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Interactive Effects of Elevated CO_2 and Ozone on Leaf Thermotolerance in Field-grown *Glycine max*

Sasmita Mishra[1*], Scott A. Heckathorn[1], Deepak Barua[1], Dan Wang[1], Puneet Joshi[1], E. William Hamilton III[2] and Jonathan Frantz[3]

([1]*Department of Environmental Sciences, University of Toledo*, Toledo, Ohio, USA;
[2]*Department of Biology, Washington and Lee University*, Lexington, Virginia, USA;
[3]*United States Department of Agriculture-Agricultural Research Service, University of Toledo*, Toledo, Ohio 43606, USA)

Abstract

Humans are increasing atmospheric CO_2, ground-level ozone (O_3), and mean and acute high temperatures. Laboratory studies show that elevated CO_2 can increase thermotolerance of photosynthesis in C_3 plants. O_3-related oxidative stress may offset benefits of elevated CO_2 during heat-waves. We determined effects of elevated CO_2 and O_3 on leaf thermotolerance of field-grown *Glycine max* (soybean, C_3). Photosynthetic electron transport (Φ_{et}) was measured in attached leaves heated *in situ* and detached leaves heated under ambient CO_2 and O_3. Heating decreased Φ_{et}, which O_3 exacerbated. Elevated CO_2 prevented O_3-related decreases during heating, but only increased Φ_{et} under ambient O_3 in the field. Heating decreased chlorophyll and carotenoids, especially under elevated CO_2. Neither CO_2 nor O_3 affected heat-shock proteins. Heating increased catalase (except in high O_3) and Cu/Zn-superoxide dismutase (SOD), but not Mn-SOD; CO_2 and O_3 decreased catalase but neither SOD. Soluble carbohydrates were unaffected by heating, but increased in elevated CO_2. Thus, protection of photosynthesis during heat stress by elevated CO_2 occurs in field-grown soybean under ambient O_3, as in the lab, and high CO_2 limits heat damage under elevated O_3, but this protection is likely from decreased photorespiration and stomatal conductance rather than production of heat-stress adaptations.

Key words: anti-oxidants; global change; heat-shock proteins; photosynthesis.

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Anthropogenic increases in atmospheric carbon dioxide (CO_2) are a major contributor to increased global mean surface temperature, which rose by 0.74 °C from 1906 to 2005 and is predicted to increase by 1.9–4.6 °C by 2100 (IPCC 2007). Mean temperature increases will be accompanied by increases in the frequency, duration, and severity of periods with unusually high temperatures (i.e., heat waves or acute heat stress). Increases in heat waves are likely to have significant ecological impact, including decreasing primary production (Ciais et al. 2005) and biodiversity (Thomas et al. 2004) and altering community composition and function (White et al. 2001; Van Peer et al. 2004; Marchand et al. 2005, 2006; Wang et al. 2008).

Elevated CO_2, relative to current CO_2 levels, has been demonstrated to affect plant tolerance to acute heat stress (most studies have focused on photosynthetic responses, as photosynthesis is among the most heat-sensitive of plant processes; Weis and Berry 1988), and such high-CO_2 effects have been positive (Faria et al. 1996; Ferris et al. 1998; Huxman et al. 1998; Faria et al. 1999; Taub et al. 2000), negative (Bassow et al. 1994; Roden and Ball 1996), or neutral (Coleman et al. 1991). However, these studies varied in the methods used to measure heat-stress effects on photosynthesis. Also, in those that compared elevated-CO_2 effects on tolerance to acute heat stress in relatively heat-sensitive *vs*. tolerant species, or in species with different photosynthetic pathways (Coleman et al. 1991; Bassow et al. 1994; Roden and Ball 1996; Huxman et al. 1998; Taub et al. 2000), all species were grown under identical thermal regimes, which were likely closer to optimal for some of the species examined, but supra- or sub-optimal for others. More recently, Wang et al. (2008) grew cool-season

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*Author for correspondence.
Tel: +1 419 530 2925;
Fax: +1 419 530 4421;
E-mail: <sasmita.mishra@utoledo.edu>.

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C_3, warm-season C_3, C_4, and crassulacean acid metabolism (CAM) species at normal and elevated CO_2, and at species-specific optimal growth temperatures and at a common growth temperature of 30 °C (if optimal different than 30 °C); the CAM species were grown at three temperatures (25, 30, and 35 °C). The results indicated that elevated CO_2 benefited basal heat tolerance of photosynthesis in the C_3 species, though less so at 30 °C *vs.* 25 °C, and decreased photosynthesis in the C_4 species. Further, in the CAM species, elevated CO_2 was beneficial to, or had no effect on, heat tolerance of photosynthesis at the two lower growth temperatures, but at the highest growth temperature, elevated CO_2 decreased photosynthesis during heat stress. One of the species examined in this study was *Glycine max* (soybean), a warm-season C_3 species that benefited photosynthetically from elevated CO_2 during heat stress. However, the effects of elevated CO_2 on plant tolerance to acute heat stress has not been examined in field-grown plants, so the extent to which elevated CO_2 may help or hurt photosynthesis during heat stress in naturally-occurring plants is unknown.

As with CO_2 and temperature, human activity is also increasing ground-level ozone (O_3) (Chameides et al. 1994); a pollutant that causes oxidative damage to plants, especially to the photosynthetic apparatus (e.g. Pell et al. 1992; Heath 1994; Christ et al. 2006). Several studies have shown that growth of plants in elevated CO_2 ameliorates damage from elevated O_3 (McKee et al. 1995; Rao et al. 1995; Kellomäki and Wang 1997; Reid and Fiscus 1998), in part because elevated CO_2 induces a decrease in stomatal conductance, and this limits diffusion of O_3 into leaves. Thus, it is expected that elevated CO_2 may also limit O_3-related damage during heat stress, though this has not been examined.

As growth of plants in elevated CO_2 typically decreases leaf N concentration and increases C concentration and C:N (e.g., Ehleringer et al. 2002), elevated CO_2 will likely affect the production of cellular adaptations to heat and O_3 (oxidative) stress, as most such adaptations are affected by plant C or N status. For example, among the most important cellular adaptations to acute heat stress in plants are: (i) heat-shock proteins (HSPs), of which there are five major families (HSP 100, HSP 90, HSP 70 HSP 60, and small-molecular-weight HSPs); (ii) anti-oxidants; (iii) protective solutes; and (iv) redox-state regulators (Wang et al. 2004). For O_3 stress, as predicted, anti-oxidants are major adaptations (including key anti-oxidant enzymes, such as super-oxide dismutase (SOD) and catalase, and non-enzymatic anti-oxidants like carotenoids), but so too are HSPs (Mittler 2002; Wang et al. 2004). Past studies on HSPs observed that production of HSPs is dependent on plant N status (increasing with N%; Heckathorn et al. 1996). Similarly, anti-oxidants are also affected by both CO_2 and N (Polle et al. 1993, 1997; Logan et al. 1999; Schwanz and Polle 2001), and soluble carbohydrates are increased by elevated CO_2 (Ehleringer et al. 2002).

In this study, we investigated the effects of elevated CO_2 on plant tolerance to acute heat stress in field-grown plants, and also examine the effects of O_3 and its interaction with CO_2 on heat tolerance. We conducted the study on *G. max* (soybean) grown under Free Air Concentration (CO_2 and O_3) Enrichment (FACE) (SoyFACE, University of Illinois, IL, USA). Attached leaves of plants were heat-stressed in the field, but in the lab, heat stress was imposed on detached leaves that were harvested from plants in the field. In both field and lab, we monitored the effects of heat stress, CO_2, and O_3 on photosynthetic electron transport (Φ_{et}). Also, leaves were harvested after heat treatments for determination of the content of pigments (chlorophyll, carotenoids, anthocyanin), HSPs (HSP 70, HSP 60, and small HSPs), anti-oxidant enzymes (catalase, mitochondrial Mn-SOD, and cytosolic/chloroplastic Cu/Zn-SOD), and total soluble carbohydrates. A priori, we expected that growth of plants in elevated CO_2 would increase heat tolerance of leaves, monitored by Φ_{et} and chlorophyll, which are both heat sensitive (Weis and Berry 1988; Heckathorn et al. 2002), as observed previously in the lab (Wang et al. 2008). We also expected elevated O_3 to exacerbate heat-related damage and for elevated CO_2 to minimize O_3-related damage during heat stress. Measurements of HSP, anti-oxidant, and soluble carbohydrate content would provide insight as to whether CO_2 and O_3 effects on heat tolerance were associated with changes in cellular adaptations to heat and O_3 stress.

Results

In field-grown plants experiencing ambient or elevated CO_2 and/or O_3, heat treatment of attached leaves decreased the quantum yield of photosynthetic electron transport (Φ_{et}) (Figure 1, Table 1). Elevated CO_2 increased Φ_{et}, primarily on 29 June and in heat-stressed plants (CO_2 and heat $\times$ CO_2 effects); a marginal CO_2 effect was observed on 10 September. Elevated O_3 decreased Φ_{et}, primarily on 10 September (comparing three-way ANOVA results for individual days in Table 1 to results from four-way ANOVA, where $P = 0.0161$ for O_3 effect for both days combined), and there was a heat-stress $\times$ O_3 interaction on 29 June. On both days, elevated CO_2 prevented the elevated-O_3-related decrease in Φ_{et} in heat-stressed plants. Relative to un-heated controls for each treatment, on 29 June, heat stress decreased Φ_{et} by 51.8%, 39.6%, 64.9%, and 49.0% in control, $+CO_2$, $+O_3$, and $+CO_2+O_3$ treatments, respectively. On 10 September, heat stress decreased Φ_{et} by 34.8%, 20.8%, 51.3%, and 31.4% in control, $+CO_2$, $+O_3$, and $+CO_2+O_3$ treatments, respectively.

When leaves were removed from plants in the field, and then heat stressed in the lab at 40 °C under ambient CO_2 and O_3, heat stress decreased Φ_{et} within 30 min (Figure 2, Table 1). As in the field, elevated-O_3 treatment exacerbated the heat-related decrease in Φ_{et}, and elevated-CO_2 treatment largely

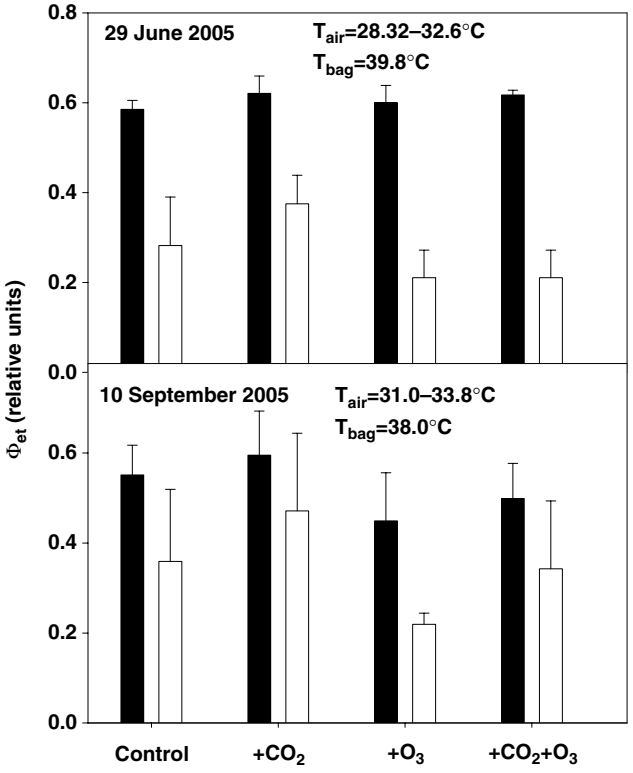

Figure 1. Effect of elevated (+) CO_2 and O_3 on *in situ* thermotolerance of photosynthetic electron transport (Φ_{et}) in field-grown soybean plants.

Attached leaves were heat stressed by enclosing recently-expanded leaves in clear vented plastic bags and orienting the bags towards the sun (29 June: bags on at 09:30 hours; measurements made at 13:30–15:00 hours; 10 September: bags on at 10:30 hours; measurements made at 12:30–14:00 hours). Ambient air temperatures during the heat treatment and temperatures of bags/leaves at time of measurements indicated in panels. Results are means (± *SD*) of four plots within each treatment combination, and plot means were based on three replicates within each plot. (■) Control; (□) heat stressed.

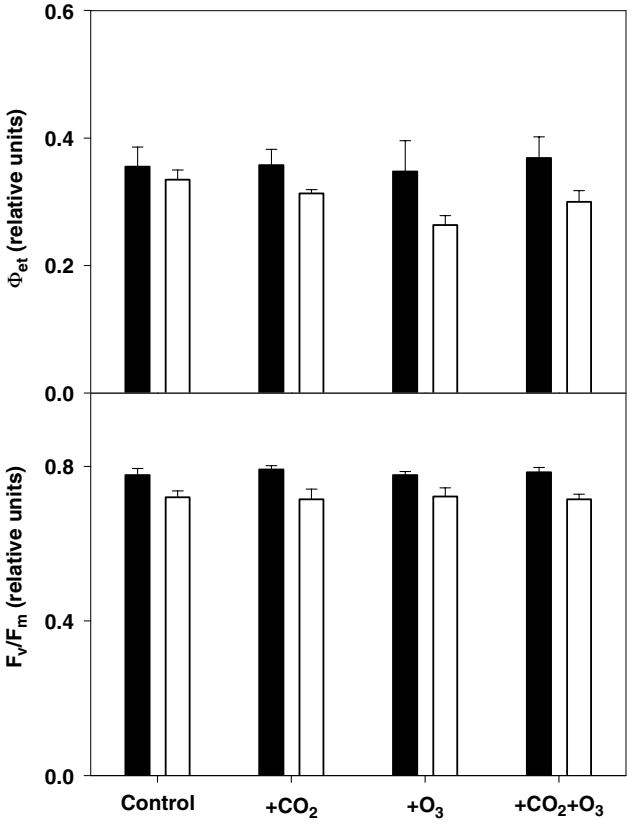

Figure 2. Effect of elevated (+) CO_2 and O_3 on photosynthetic thermotolerance of detached leaves of field-grown soybean plants assayed in the laboratory.

Recently-expanded leaves were harvested from plants in the field, transported to the laboratory, and then assayed for either electron transport of leaves adapted to moderate light (600 μmol/m^2per s PAR) or photochemical efficiency of dark-adapted leaves (F_v/F_m). Leaves were incubated at either 27 or 40 °C ([■] control; (□) heat stressed, respectively) and in normal air. Results are means (± *SD*) of three to four replicates.

prevented the O_3-related decrease in Φ_{et} during heat stress (similar results were obtained in an independent replicated experiment; not shown). However, in contrast to the field, in the lab, leaves from elevated-CO_2 plants did not exhibit higher Φ_{et} in either heat-stressed or unstressed leaves relative to controls. Relative to un-heated controls for each treatment, in the lab, heat stress decreased Φ_{et} by 5.6%, 12.4%, 24.2%, and 18.6% in control, +CO_2, +O_3, and +CO_2+O_3 treatments, respectively. Heat treatment of detached leaves also decreased dark F_v/F_m, indicating damage to photosystem II (PSII), but neither CO_2 nor O_3 had an effect on F_v/F_m (similar CO_2 and O_3 effects were observed at 43 and 46 °C, even though heat effects were larger; not shown).

As with Φ_{et}, heat stress decreased total chlorophyll content of leaves, but in contrast to Φ_{et}, elevated CO_2 also decreased chlorophyll content, especially during heat stress (Figure 3, Table 1). Similar heat and CO_2 effects were observed for carotenoids, though these effects were only marginally significant. No significant treatment effects were observed for anthocyanin content or chlorophyll a-to-b ratio.

Heat treatment induced the *de novo* production of HSP 70 and small HSP (these proteins were not detected constitutively, as expected; Wang et al. 2004), and heat stress induced an increase in HSP 60 content (Figure 4, Table 1). However, neither CO_2 nor O_3 had an effect on HSP content.

Table 1. Results from ANOVA (*P* values) on effects of heat stress, elevated CO_2, and elevated ozone (O_3) on leaf response variables

	Treatments						
Variables	Heat stress	CO_2	O_3	HS × CO_2	HS × O_3	CO_2 × O_3	HS × CO_2 × O_3
Φ_{et} field 29 June	<.0001*	0.0033*	0.1282	0.0629	0.0659	0.9505	0.7087
Φ_{et} field 10 September	0.0005*	0.0703	0.0131*	0.4237	0.6855	0.9218	0.9750
Φ_{et} lab	<.0001*	0.3912	0.0810	0.8451	0.0584	0.0966	0.3812
Dark F_v/F_m lab	<.0001*	0.6946	0.8442	0.1771	0.6946	0.6946	0.8442
Anthocyanin	0.4167	0.6709	0.8821	0.4625	0.7233	0.8199	0.6959
Carotenoids	0.0763	0.0599	0.5759	0.3077	0.4902	0.5390	0.3181
Total chlorophyll	0.0012*	0.0376*	0.1598	0.0953	0.4389	0.9034	0.1810
Chlorophyll a:b	0.6110	0.8115	0.3039	0.2122	0.9890	0.9546	0.8435
Catalase	0.0020*	0.0920	0.0536	0.1506	0.0717	0.0392*	0.6144
Mn-SOD	0.6565	0.7227	0.6985	0.4325	0.3382	0.9493	0.6128
Cu/Zn-SOD	<.0001*	0.8136	0.5490	0.9077	0.4767	0.5953	0.8933
HSP 60	0.0137*	0.9304	0.9741	0.6666	0.9584	0.4592	0.6521
HSP 70		0.7344	0.8953			0.5968	
Small HSP		0.5467	0.9863			0.1813	
Soluble sugars	0.8571	≤.0001*	0.0537	0.0903	0.6506	0.3352	0.9298

*Asterisk indicates significant difference at $P < 0.05$

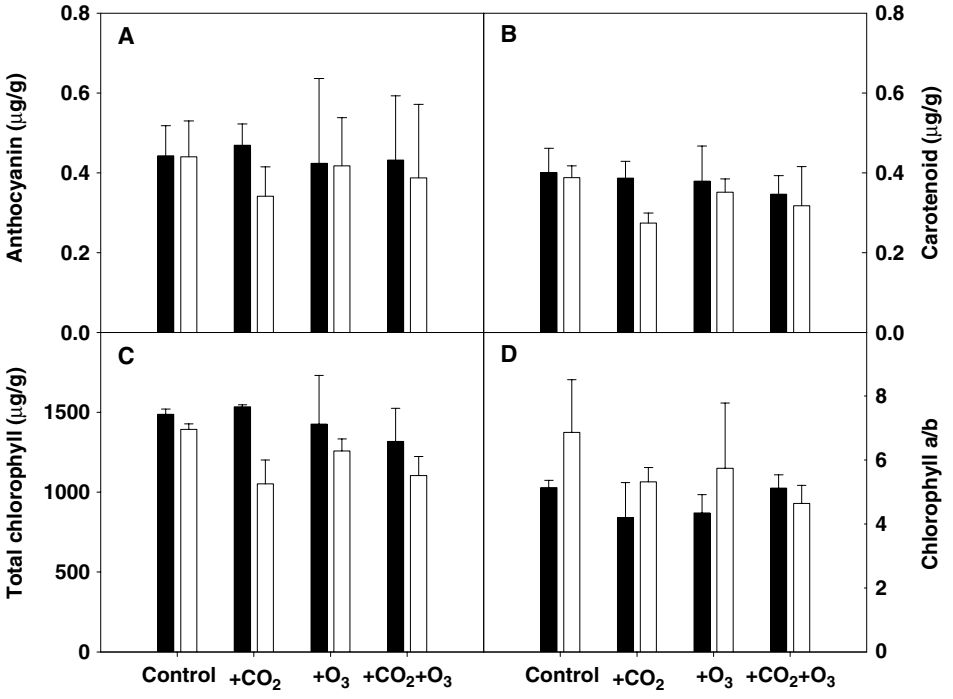

Figure 3. Effect of elevated (+) CO_2 and O_3 on pigment content (per fresh mass) of control and heat-stressed soybean leaves.

Plants were heat stressed as in Figure 2, and then detached leaves were harvested, frozen, and assayed for content of: (**A**) anthocyanin, (**B**) carotenoids, (**C**) total chlorophyll, and (**D**) chlorophyll a-to-b ratio. Results are means (± *SD*) of three to four replicates. (■) Control; (□) heat stressed.

Of the three anti-oxidant enzymes assayed, heat treatment increased content of catalase and Cu/Zn-SOD, but had no effect on Mn-SOD (Figure 5, Table 1). Neither CO_2 nor O_3 affected Mn-SOD or Cu/Zn-SOD, but both elevated CO_2 and O_3 had marginally significant effects on catalase. Both elevated CO_2 and O_3 tended to decrease catalase relative to the ambient control, and elevated O_3 (but not elevated $O_3 + CO_2$) prevented the heat-related increase in catalase.

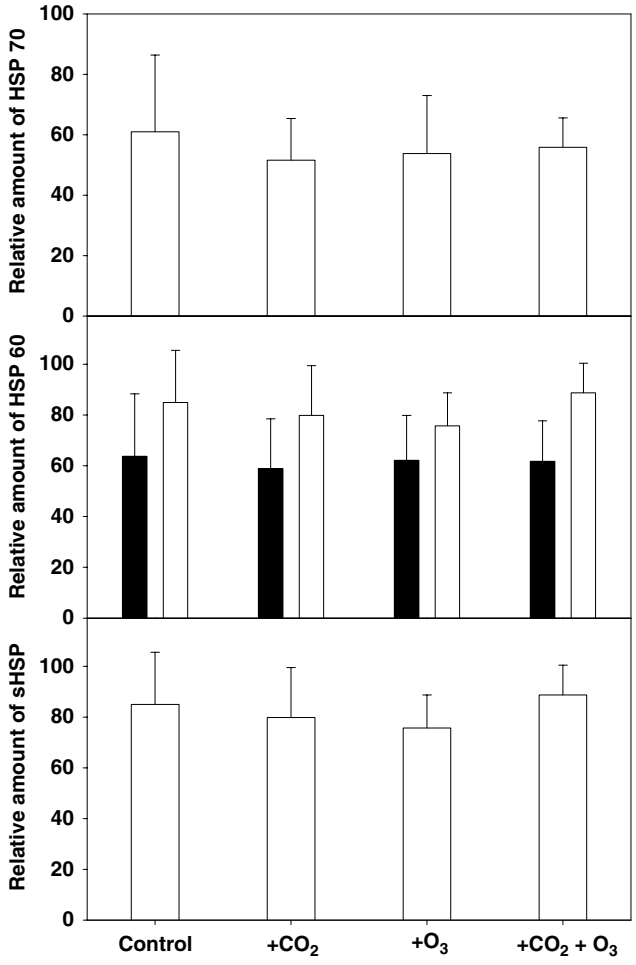

Figure 4. Effect of elevated (+) CO_2 and O_3 on heat-shock protein (HSP) content of control and heat-stressed soybean leaves.

Plants were heat stressed as in Figure 2, and then detached leaves were harvested, frozen, and assayed for content of: HSP 70, HSP 60, and small sHSP. Results are means (± *SD*) of three replicates. Protein content is expressed as a percent of a standard (heat-shocked) leaf extract. (■) Control; (□) heat stressed.

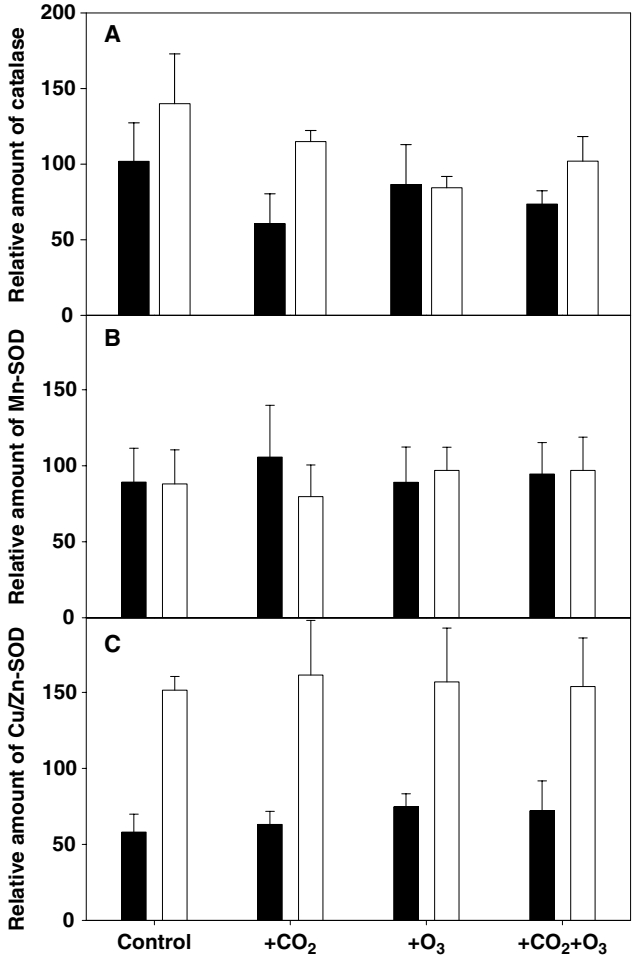

Figure 5. Effect of elevated (+) CO_2 and O_3 on anti-oxidant enzyme content of control and heat-stressed soybean leaves.

Plants were heat stressed as in Figure 2, and then detached leaves were harvested, frozen, and assayed for content of: (**A**) Catalase, (**B**) Mn-superoxide dismutase (SOD), and (**C**) Cu/Zn-SOD. Results are the means (± *SD*) of three replicates. Protein content is expressed as a percent of a standard leaf extract. (■) Control; (□) heat stressed.

Finally, neither heat treatment nor O_3 affected the content of soluble carbohydrates of leaves, but elevated CO_2 increased the content of soluble carbohydrates (Figure 6, Table 1).

Discussion

Human activities are increasing the concentrations of atmospheric CO_2 and ground-level ozone, and increases in these and other greenhouse gases are increasing both mean and extreme high temperatures (IPCC 2007; Stich et al. 2007; USEPA 2007; http://www.epa.gov/ozone/2007). Our recent studies indicate that elevated CO_2 will benefit photosynthesis during heat stress in C_3 and CAM plants grown at near-optimal temperatures, but that high CO_2 will often decrease photosynthesis during heat stress in C_4 plants at both near-optimal and supra-optimal growth temperatures and CAM plants at supra-optimal growth temperatures (Hamilton et al. 2008; Wang et al. 2008). However, it remains to be determined if effects of elevated CO_2 on heat tolerance observed in the lab hold true for field-grown plants. For example, it is possible that nutrient or water limitations interact with elevated CO_2 to decrease or increase the effect of CO_2 on thermotolerance.

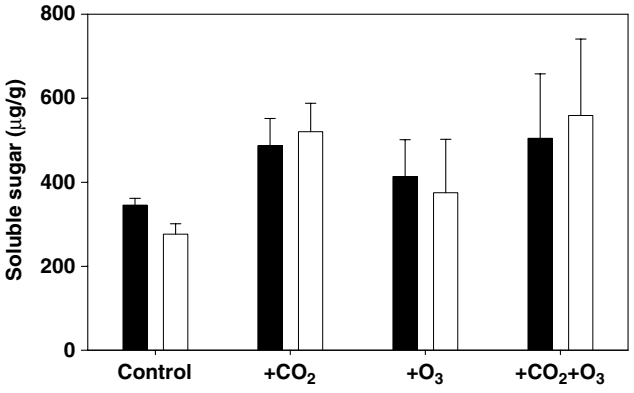

Figure 6. Effect of elevated (+) CO_2 and O_3 on total soluble-sugar content (per fresh mass) of control and heat-stressed soybean leaves.

Plants were heat stressed as in Figure 2, and then detached leaves were harvested, frozen, and assayed. Results are the means (± *SD*) of three replicates. (■) Control; (□) heat stressed.

Results from this study indicate that growth of plants in the field under elevated CO_2 does benefit the heat tolerance of photosynthetic electron transport in the C_3 crop plant, *G. max*, as in the lab (Wang et al. 2008). Further, elevated O_3 exacerbated heat-related decreases in electron transport, and elevated CO_2 minimized or prevented light-dependent O_3-related decreases in electron transport (and thus photoinhibition) during heat stress. The benefits of elevated CO_2 to electron transport during heat stress in the field in this study were comparable in magnitude to those observed in the lab in Wang et al. (2008) for both electron transport and net photosynthesis (i.e., CO_2 assimilation). Benefits of elevated CO_2 to electron transport under ambient O_3 were evident in both control and heat-stressed field-grown plants measured *in situ* under elevated CO_2. Specifically, electron transport was greater in elevated, as compared to ambient, CO_2 for both control and heat-stressed plants, and under both elevated CO_2 and O_3, electron transport was approximately equal to that of ambient CO_2 and O_3 in heat-stressed plants on 10 September. However, in the lab where measurements were made under ambient CO_2, benefits of growth under elevated CO_2 were evident only in heat-stressed leaves of plants grown under elevated O_3. Further, elevated CO_2 and O_3 had no effect on HSP production in leaves during heat stress, and affected only one of the anti-oxidant enzymes examined (i.e., decreasing catalase, but not affecting Mn- or Cu/Zn-SOD). Elevated CO_2 did increase soluble carbohydrate content, but neither O_3 nor heat stress had an effect on soluble carbohydrates. Together, these observations indicate that the benefits of elevated CO_2 to tolerance of electron transport during heat stress are likely related in large part to decreased photorespiration and/or stomatal conductance, which would increase net CO_2 fixation and decrease O_3 diffusion into leaves, respectively (McKee et al. 1995; Fiscus et al. 1997; Sage and Monson 1999). However, the benefits of elevated CO_2 to thermotolerance of electron transport in this study would be offset by the heat-related decreases in total chlorophyll and carotenoid content of leaves, which was exacerbated by elevated CO_2. Heat stress tended to increase chlorophyll a-to-b, indicating that decreases in total chlorophyll were driven mostly by decreases in chlorophyll bound to light-harvesting complexes relatively rich in chl b and carotenoids, rather than chlorophyll in reaction centers rich in chl a. In general, decreases in chlorophyll should decrease light capture and thus net photosynthesis.

Finally, a number of past studies have shown that, in the absence of heat stress, elevated CO_2 can suppress the toxic effects of ozone to growth or photosynthesis (Barnes and Pfirrmann 1992; Mulchi et al. 1992; Balaguer et al. 1995; Rao et al. 1995; McKee et al. 1995; Booker et al. 1997; Reid and Fiscus 1998; Fuhrer 2003), although other reports have found no such protective effects (Tausz et al. 2004; Paoletti et al. 2007). Results from this study demonstrate that photoprotection from O_3 by elevated CO_2 also occurs during acute heat stress. Cellular anti-oxidants play a key role in protecting plants from O_3-related oxidative damage (Morre et al. 1990; Willekens et al. 1994), and CO_2 can have interactive effects with O_3 on anti-oxidant production (Polle et al. 1993; Azevedo et al. 1998; Tausz et al. 2004; Erice et al. 2007). Our limited examination of anti-oxidants in this study (i.e., catalase, Mn-SOD, Cu/Zn-SOD, carotenoids) suggests that the protective effects of elevated CO_2 from elevated O_3 during heat stress are not related to CO_2-related increases in anti-oxidants.

Materials and Methods

Plant material and treatments

The study was conducted on *Glycine max* (soybean) plants grown at ambient (approximately 370 parts per million [ppm]) or elevated (550 ppm) CO_2 and/or ambient or elevated (1.2 × ambient) O_3 at the University of Illinois Soybean Free-Air Concentration Enrichment (SoyFACE) site (Urbana-Champaign, IL, USA; http://www.soyface.uiuc.edu/). At this facility, plants are grown in the field from seed under the four different CO_2 and O_3 treatments (ambient CO_2 + O_3, elevated CO_2 + ambient O_3, ambient CO_2 + elevated O_3, elevated CO_2 + O_3), and there are four replicate 350 m^2 plots for each treatment combination (16 plots total). Field heat-treatments and *in situ* measurements of intact plants were made on 29 June and 10 September 2005, laboratory measurements on detached heat-treated leaves were made during the week of 29 June, and biochemical assays of leaves collected from plants that were heat-treated in the lab were done so on plants receiving heat treatment during the week of 29 June 2005. Climate data for the field site (i.e., air temperatures = T_{air} and photosynthetically-active radia-

tion = PAR) were obtained from the SoyFACE website. The two field sampling days, 29 June and 10 September, were mostly-sunny warm days with no rain (29 June: T_{air} = 28.3–32.6 °C from 09:30 to 15:00 hours, mean PAR = 1684 μmol/m^{2}per s; 10 September: T_{air} = 29.8–33.8 °C from 09:30 to 15:00 hours, mean PAR = 1430 μmol/m^2 per s).

Heat treatment of attached leaves of plants in the field was imposed by enclosing individual recently-expanded trifoliate leaves in clear flat plastic 16 × 20 cm ziplock bags, and then holding the bags and leaves mostly-perpendicular to the sun with wooden stakes and clips. The bags, which are sealed on three sides and sealable of the fourth side, were left unsealed around the leaf petiole to allow for air exchange. Three leaves from three different plants were heat-treated per plot, and data were collected from three different untreated (unbagged) control plants per plot; measurements from the three plants in each plot were averaged to obtain plot means used in statistical analyses. Leaves were heat treated on 29 June from 09.30 to 16:00 hours, with electron transport results collected from 13:30 to 15:00 hours (based on preliminary experiments), and with harvesting of leaves for biochemical assays occurring from 15:00 to 16:00 hours. On 10 September, leaves were heat treated from 10.30 to 16.00 hours, with electron transport results collected from 12.30 to 14.00 hours, and with harvesting of leaves occurring from 14.30 to 16.00 hours. Leaf and internal bag temperatures were monitored through the course of the heat treatment, using data-loggers and fine-wire thermocouples or hand-held IR thermometers, and leaf temperatures measured just prior to electron transport measurements are reported here. From separate plants than those that were heat treated in the field (i.e., from untreated unmeasured controls), we collected (detached) leaves (n = 3–4 per plot per assay), which were placed in a chilled insulated cooler in the dark. These detached leaves were transported to a nearby laboratory (within 30 min), and then used in electron transport assays (± heat stress applied in the lab).

PS II electron transport

To examine the effects of heat stress, CO_2, and O_3 on photosynthetic metabolism, we monitored the quantum yield of electron transport (Φ_{et}) and the photochemical efficiency of PSII (F_v/F_m) (Genty et al. 1989). In attached leaves in the field, electron transport was measured in leaves adapted to ambient sunlight using a portable pulse-amplitude-modulated (PAM) fluorometer (model OS1-FL, Opti-Sciences, Hudson, NH, USA); in the lab, electron transport was measured with a bench-top PAM fluorometer (model 101/103, Heinz Walz GmbH, Effeltrich, Germany) (as in Wang et al. 2008). In both cases, 1-sec saturating flashes of >5 000 μmol/m^{2}per s PAR were used. PSII efficiency of dark-adapted leaves (F_v/F_m) was monitored after 30 min of incubation in the dark at various temperatures. In preliminary experiments, F_v/F_m was measured after 30 min at 27, 37, 40, 43, or 46 °C; leaves were placed on moistened filter paper in plastic Petri dishes and floated on water in a temperature-controlled water-bath. In subsequent experiments, electron transport was monitored in light-adapted leaves incubated at 27 or 40 °C (control or heat stressed); based on preliminary experiments wherein light level and incubation duration varied, results are reported for assays wherein leaves were incubated for 30 min at 600 μmol/m^{2}per s PAR.

Pigment and carbohydrate content

Anthocyanin, carotenoid, and chlorophyll content of leaf tissue were estimated spectrophotometrically after extraction in dimethylsulfoxide (DMSO) and using the equations of Sims and Gamon (2002). The content of total soluble carbohydrates in leaf tissue was estimated by using the phenol-sulfuric acid method of Dubois et al. (1956), with minor modification. Leaf tissue (50 mg dry mass) was ground in liquid N_2, and then mixed with 2 mL of 1M phosphate buffer (pH 7.2) and re-ground. The homogenate was centrifuged at 21 000*g*, and then 1 mL of supernatant was taken and mixed with 1 mL of aqueous phenol. Concentrated sulfuric acid (5 mL) was added, and absorbance at 470 nm was determined after 20 min. Glucose was used for generating a standard curve.

Content of heat-shock proteins and anti-oxidant enzymes

Proteins were extracted from leaf tissues (400 mg fresh weight) by grinding in liquid N_2 in a mortar and pestle, and then in an extraction buffer containing 0.15 M Tris-HCl (pH 8.0), 1% SDS (sodium dodecyl sulfate) (w/v), 1 mmol/L PMSF (phenylmethylsulfonyl fluoride), 10 mmol/L DTT (dithiothreitol), 10% sucrose (w/v), 10 μM leupeptin, 1 mmol/L benzamidine, 10 mmol/L ascorbic acid, 2% PVPP (polyvinylpolypyrrolidine) (w/v), 1 mmol/L EDTA (ethylenediaminetetraacetic acid), 10% glycerol (v/v), and 0.05% bromophenol blue. Samples were centrifuged at 21 000*g* for 10 min at 4 °C to remove insoluble debris, and the supernatant was used for further analysis. The total protein concentration of each sample was determined in triplicate by the Coomassie-dye-binding method of Ghosh et al. (1988), using bovine serum albumin (BSA) as a standard. The colorimetric density of protein in sample spots on filter-paper discs was determined using a desktop scanner and densitometry analysis, using National Institutes of Health imaging software (Scion).

Proteins were separated by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 12% acrylamide, 16 × 20-cm gels (50 μg of total protein per lane) (e.g., Heckathorn et al. 2002). Following electrophoresis, gels were either stained for total proteins using Coomassie blue R-250 or transferred to nitrocellulose membranes by electroblotting. The membranes were then probed with protein-specific antibodies and secondary antibodies conjugated to alkaline

phosphatase. Colorimetric detection of secondary antibodies was carried out by treating membranes with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP). Antiserum to Cu/Zn-SOD, Mn-SOD, HSP 70, and HSP 60 were purchased commercially (StressGen Bioreagents, Canada), as was the case for catalase (Rockland Chemicals, Gilbertsville, PA, USA), while antiserum to the small HSPs was developed by Heckathorn et al. (Preczewski et al. 2000). Antiserum to HSP 70 was for heat-inducible isoforms of HSP 70 only, and the antiserum to small HSPs detects multiple (and most) isoforms, which in most species and vegetative tissue are not constitutively expressed (Vierling 1991; Wang et al. 2004). Protein concentration on blots was determined by densitometric analysis as above, and was relativized to a standard leaf extract obtained from heat-stressed corn included on each gel.

Statistics

Most results were analyzed statistically by three-way (HS $\times$ CO_2 $\times$ O_3) ANOVA using JMP software (SAS Corp., Carey, NC, USA) (all results are shown in Table 1, excluding HSP 70 and small HSP). However, for content of HSP 70 and small HSP, as these proteins were not detected constitutively, including heat treatment (HS) as a factor in ANOVA was not relevant; hence, we analyzed these results by two-way ANOVA (CO_2 $\times$ O_3) (also shown in Table 1). In addition, for field Φ_{et}, results of both sampling days combined were analyzed by four-way ANOVA (HS $\times$ CO_2 $\times$ O_3 $\times$ day), so as to gain insight regarding treatment effects over both sampling days combined (results discussed in text).

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