



Biochemical acclimation, stomatal limitation and precipitation patterns underlie decreases in photosynthetic stimulation of soybean (*Glycine max*) at elevated [CO₂] and temperatures under fully open air field conditions



David M. Rosenthal^a, Ursula M. Ruiz-Vera^b, Matthew H. Siebers^b, Sharon B. Gray^{b,1}, Carl J. Bernacchi^{b,c,d}, Donald R. Ort^{b,c,d,*}

^a Department of Environmental and Plant Biology, Ohio University, Athens, OH 45701, USA

^b Department of Plant Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^c Global Change and Photosynthesis Research Unit, USDA Agricultural Research Service, Urbana, IL 61801, USA

^d Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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ABSTRACT

The net effect of elevated [CO₂] and temperature on photosynthetic acclimation and plant productivity is poorly resolved. We assessed the effects of canopy warming and fully open air [CO₂] enrichment on (1) the acclimation of two biochemical parameters that frequently limit photosynthesis (A), the maximum carboxylation capacity of Rubisco ($V_{c,max}$) and the maximum potential linear electron flux through photosystem II (J_{max}), (2) the associated responses of leaf structural and chemical properties related to A , as well as (3) the stomatal limitation (l) imposed on A , for soybean over two growing seasons in a conventionally managed agricultural field in Illinois, USA. Acclimation to elevated [CO₂] was consistent over two growing seasons with respect to $V_{c,max}$ and J_{max} . However, elevated temperature significantly decreased J_{max} contributing to lower photosynthetic stimulation by elevated CO₂. Large seasonal differences in precipitation altered soil moisture availability modulating the complex effects of elevated temperature and CO₂ on biochemical and structural properties related to A . Elevated temperature also reduced the benefit of elevated [CO₂] by eliminating decreases in stomatal limitation at elevated [CO₂]. These results highlight the critical importance of considering multiple environmental factors (i.e. temperature, moisture, [CO₂]) when trying to predict plant productivity in the context of climate change.

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1. Introduction

It is well known that increases in atmospheric CO₂ concentration ([CO₂]) will have direct effects on photosynthesis of C₃ plants, particularly in conventional agricultural systems managed to maximize productivity [1]. Rising [CO₂] and emissions of other more potent greenhouse gases due to anthropogenic activities are likely to increase global mean air temperatures by ≥ 3 °C before the end of this century [2]. The coupled impact of these changes on carbon assimilation and photosynthetic acclimation in the field is highly

uncertain because experiments examining the combined effects of elevated [CO₂] and temperature have been largely restricted to enclosed chambers [3,4], open top chambers [5] and gradient tunnels [6]. Moreover, the realized benefit of the combined effect of elevated [CO₂] and temperature on carbon assimilation differs depending on the type of enclosure, as well as the species and the functional types examined as they acclimate in different ways [7]. Taken together, these observations underscore the importance of evaluating and analyzing plant responses to future [CO₂] and elevated temperature under field conditions.

The rate of photosynthesis of C₃ plants is most frequently limited or co-limited by two biochemical processes: the maximum in vivo rate of the carboxylation of ribulose biphosphate (RuBP) by the enzyme RuBP carboxylase/oxygenase (Rubisco; $V_{c,max}$) and/or the maximum potential linear electron flux through photosystem II (J_{max}) which is directly linked to the regeneration of RuBP [8]. Both of these processes respond, and may acclimate, to increases in [CO₂]

* Corresponding author at: Department of Plant Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA. Tel.: +1 217 333 2093.

E-mail addresses: rosentha@ohio.edu (D.M. Rosenthal), d-ort@illinois.edu (D.R. Ort).

¹ Current address: University of California, Davis, Davis, CA 95616, USA.

and/or temperature [9]. At atmospheric $[\text{CO}_2]$ of 395 ppm, net leaf level photosynthetic carbon assimilation (A) in herbaceous plants is mainly Rubisco-limited [10]. As atmospheric $[\text{CO}_2]$ continues to increase [11], so too will the $[\text{CO}_2]$ at the Rubisco catalytic site thereby stimulating A . Rubisco can also catalyze the oxygenation of RuBP leading to the energetically expensive photorespiration process [12,13]. Therefore, elevating $[\text{CO}_2]$ will stimulate A both by increasing the velocity of the carboxylation reaction and through competitive suppression of the energetically expensive oxygenation reaction. In contrast, as temperature increases, the kinetics of Rubisco increasingly favor oxygenation of RuBP leading to more photorespiration [12,13]. Thus, while elevated $[\text{CO}_2]$ almost always increases rates of photosynthesis [1,14–16], higher temperatures may lead to an increase, decrease, or no change in photosynthesis depending on whether photosynthesis is operating below, at, or above the thermal optimum [17].

Acclimation of $V_{c,max}$ and J_{max} to growth at elevated $[\text{CO}_2]$ and warmer temperatures is predicted to alter the response of A in these environments [13]. A relatively consistent reduction of $V_{c,max}$ in Free Air CO_2 Enrichment (FACE) experiments (ca. 13% on average) has been observed for a range of species [1,10,18], however, since increasing $[\text{CO}_2]$ is also predicted to shift the control of A away from predominately Rubisco-limited and toward predominantly RuBP regeneration-limited control [13,19,20], this down-regulation response of $V_{c,max}$ has little or no impact on A at the growth $[\text{CO}_2]$ [19]. While J_{max} has also been shown to down-regulate in plants grown at elevated $[\text{CO}_2]$, it does so to a much lesser extent and not as consistently [21]. Yet, as plants will increasingly be limited by RuBP regeneration as atmospheric $[\text{CO}_2]$ continues to increase small decreases of J_{max} are likely to represent a proportionally greater limitation on A . Should J_{max} acclimate more than $V_{c,max}$ to the combined effects of elevated temperature and $[\text{CO}_2]$, then photosynthetic acclimation to elevated temperatures will reduce photosynthetic stimulation by elevated CO_2 . Because of roles that $V_{c,max}$ and J_{max} play in determining photosynthetic rates, it is critical to assess how $V_{c,max}$ and J_{max} will acclimate to increases in $[\text{CO}_2]$ and temperature to accurately predict C_3 crop productivity in the future [22].

In addition to the biochemical limitations, photosynthetic rate is physically limited by stomatal conductance (g_s) [23], which sets the upper limit on the flux of CO_2 into the leaf. Elevated CO_2 is well known to decrease g_s [14], yet A remains higher at elevated CO_2 because of the non-linear relationship between A and the substomatal CO_2 concentration (C_i) [23]. Thus stomatal limitation of photosynthesis (l) is lower at elevated CO_2 [19]. However, the combined effect of elevated CO_2 and temperature on l is uncertain and has never been assessed under field conditions.

In addition to being a model C_3 species and the world's most important seed legume, soybean, grown in rotation with maize, covers an estimated planting area of 68 million hectares in the United States making it the country's largest single land-use [24]. For these reasons, the effects of combined increases in temperature and $[\text{CO}_2]$ on soybean productivity will have important regional and global implications and, elucidating the responses of key photosynthetic processes to these treatments will carry implications for other C_3 species. Using Free Air CO_2 Enrichment (FACE) coupled with infrared heating arrays [25,26], we recently showed that soybean yield gains from photosynthetic stimulation by elevated CO_2 may be negated by global warming [27]. To elucidate potential ecophysiological mechanisms leading to this lower than expected stimulation, here we (1) assess the degree to which soybean photosynthetic parameters acclimate to elevated temperature and elevated $[\text{CO}_2]$, alone and in combination, under field conditions, (2) determine how photosynthetic parameter acclimation alters A and (3) elucidate the roles stomatal limitation and leaf nitrogen allocation may play in mediating photosynthetic acclimation to

elevated $[\text{CO}_2]$ and elevated temperature. Specifically, we hypothesized that (1) J_{max} would acclimate more to elevated temperature at ambient and elevated CO_2 than $V_{c,max}$ and (2) acclimation to the combined effect of elevated temperature and elevated CO_2 would lead to lower photosynthetic stimulation than elevated $[\text{CO}_2]$ alone. The experiment was conducted in 2009 and 2011. These two years differed substantially with respect to ambient temperature and precipitation, the former was cooler and wetter and the latter hotter and drier than the 30 year average [27].

2. Methods

2.1. Site and plots description

The SoyFACE facility is located in the Experimental Research Station of the University of Illinois at Urbana-Champaign [28]. In 2009 and 2011 eight plots 20 m in diameter, four that were enriched with pure CO_2 from dawn until dusk to a target concentration of the current global mean $[\text{CO}_2]$ + 200 $\mu\text{mol mol}^{-1}$ (585 ppm in 2009 and 590 ppm in 2011) and four that served as controls, were nested at 100 m spacing within a soybean (*Glycine max*, Pioneer 93B15) field of ca. 16 ha. Agronomic management practices were typical of corn soybean rotations in this region, so no N was applied the year of these experiments. However, N was applied prior to corn plantings in 2008 (160 kg ha⁻¹) and 2010 (180 kg ha⁻¹). Ambient atmospheric $[\text{CO}_2]$ for these two growing seasons at SoyFACE were ca. 385 ppm (2009) and 390 ppm (2011). The CO_2 enrichment of the four elevated $[\text{CO}_2]$ plots was performed using a modification of the method of Miglietta et al. (2001) described in detail previously [29]. For simplicity, the ambient and elevated $[\text{CO}_2]$ plots are hereafter referred to as 385 and 585.

The canopy temperature of a 7 m² circular sub-plot within the larger 385 and 585 $[\text{CO}_2]$ plots was raised by 3.5 °C using an infrared heating array [27]. Briefly, a ceramic infrared heating array was mounted 1.2 m above the canopy following the design of Kimball et al. [26] except that the number of heating elements per heater was increased from one to four with a total of six heaters per plot. A proportional-integrative-derivative (PID) feedback control system similar to that assembled by Kimball [25] was designed to maintain the day and night canopy temperature of the warmed plots to 3.5 °C above that of the corresponding reference plots for the duration of growing season from crop emergence to harvest. The ambient and elevated temperature plots are hereafter referred to as CON and HOT. The experimental treatments were established in a full factorial design with a total of four treatments replicated four times: ambient $[\text{CO}_2]$ at ambient temperature (385-CON); ambient $[\text{CO}_2]$ at +3.5 °C (385-HOT); elevated $[\text{CO}_2]$ at ambient temperature (585-CON); elevated $[\text{CO}_2]$ at +3.5 °C (585-HOT).

2.2. Measurement and analysis of photosynthetic sub-stomatal $[\text{CO}_2]$ response (A vs. C_i) curves

In vivo values of maximum carboxylation capacity ($V_{c,max}$) and maximum linear electron transport through photosystem II (J_{max}) were determined from photosynthesis (A) vs. sub-stomatal $[\text{CO}_2]$ (C_i) measurements using an open gas exchange system (LI-6400, LI-COR, Lincoln, Nebraska) on six days in 2009 and five days in 2011. Before dawn, the petioles of uppermost fully expanded leaves were cut and immediately submerged in water. Leaves were returned to the lab within 30 min, petioles were recut and kept under water and low light (ca. <50 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Photosynthesis and conductance of detached soybean leaves collected in this manner are similar to those measured in the field [19,27,30]. Fifteen minutes prior to measurements leaves were pretreated to high light (ca. 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Leaves were then placed in the measurement

cuvette and allowed to reach steady state photosynthesis at their growth $[\text{CO}_2]$ (i.e. 385 ppm or 585 ppm $[\text{CO}_2]$) at a saturating light level of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$. The petioles of all leaves remained submerged in water throughout the measurements. The mean leaf temperature (T_l , °C) and vapor pressure deficit determined based on T_l (VpdL; kPa) for the A vs. C_i curves was $25 \pm 0.9^\circ\text{C}$ and $1.39 \pm 0.25 \text{ kPa}$ (s.d.) for $n=212$ A vs. C_i curves in 2009. Similarly, mean VpdL was $1.17 \pm 0.18 \text{ kPa}$ and leaf temperature was $25.4 \pm 0.7^\circ\text{C}$ for $n=219$ A vs. C_i curves in 2011. Once steady state A was reached, measurements of photosynthesis, C_i , and operating efficiency of photosystem II (Φ_{PSII} ; Supplemental Figure 1) were recorded at the growth $[\text{CO}_2]$; $[\text{CO}_2]$ was then decreased stepwise to $50 \mu\text{mol mol}^{-1}$, increased again to the growth $[\text{CO}_2]$, and then increased stepwise to 1500 ppm $[\text{CO}_2]$. A minimum of 11 data points were collected for each leaf and the A vs. C_i data were fitted to the biochemical model of photosynthesis [31] and solved for $V_{c,\text{max}}$, J_{max} and R_d , following the methods outlined by Long and Bernacchi [32].

2.3. Leaf N and structural traits

Two leaf disks were collected from upper canopy leaves of 3–5 plants in each plot on 6 days in 2009 and 6 days in 2011. Leaf disks were dried at 60°C for 48 h and weighed to determine leaf mass per area (LMA , g m^{-2}), Leaf N per area (N_{area} ; g m^{-2}) and N percentage per unit mass (N_{mass} ; %). Disks were ground to a fine powder and nitrogen (N) content was determined by total combustion (Costech ECS 4010, Valencia, CA, USA). Leaf disks were collected at the same time as field measurement of gas exchange reported in [27], therefore photosynthetic nitrogen use efficiency (PNUE; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) was estimated using these leaf N estimates and measurements of A reported in [27].

2.4. Stomatal limitation

Stomatal limitation (l) of A is an estimate of the proportion by which actual stomatal conductance (g_s) decreases A relative to what A would be assuming infinite conductance between the atmosphere and the leaf sub-stomatal airspace (A^0) and is usually estimated from A vs. C_i curves using the following relationship [23]:

$$l = 1 - \left(\frac{A}{A^0} \right) \quad (1)$$

A is the rate of carbon assimilation at the measurement $[\text{CO}_2]$ and based on the actual g_s and A^0 is photosynthesis assuming infinite g_s (i.e. when $C_i = \text{atmospheric } [\text{CO}_2]$).

Stomatal limitation for this study is referred to as the “field” stomatal limitation (l_{field}) and was calculated using A and C_i measured in the field reported in Ruiz-Vera et al. [27] coupled with modeled estimates of A^0 . Modeled values of A^0 were calculated with the Farquhar et al. [33] photosynthesis model by using the laboratory determinations of $V_{c,\text{max}@25}$ and $J_{\text{max}@25}$ corrected for field light (Q) and temperature (T_l) conditions (i.e. from Ruiz-Vera et al. [27]). Stomatal limitation was calculated in this way to resolve treatment effects on stomatal limitation from diurnal changes in biochemical limitations to photosynthesis.

2.5. Temperature response curves

The response of leaf photosynthesis to temperature was measured on leaves collected predawn at three different phenological stages during the 2009 growth season (July 6–7, vegetative; August 3–4, beginning pod; August 17–18, beginning seed). Temperature response curves were not measured in 2011. Photosynthesis was measured using a gas exchange system (LI-6400; LI-COR Inc., Lincoln, Nebraska) at a saturating light level of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and

at $[\text{CO}_2]$ of 385.8 ± 7 (s.d.) $\mu\text{mol mol}^{-1}$ for ambient or 583.1 ± 8.5 (s.d.) $\mu\text{mol mol}^{-1}$ for elevated $[\text{CO}_2]$ grown plants. Leaf vapor pressure deficit (VpdL; kPa) ranged from 0.77 at 20°C to 3.12 at 38°C with a mean VpdL for all measurements of 1.7 ± 0.45 (s.d.). At each temperature, leaves were monitored until the target temperature and steady state A was reached based on the measured coefficient of variation of the gas exchange system falling below 1%; this took approximately 8–10 min for each stepwise change in temperature. Each temperature response curve took slightly longer than 60 min to complete after the leaf reached steady-state and the gas exchange system stabilized. Following the measurement at 38°C , leaves were allowed to cool and were measured again at 29°C to ensure that any decrease in A above the optimum was reversible and not due to permanent damage to the photosynthetic apparatus.

2.6. Statistical analysis

The seasonal responses of parameters to growth at elevated temperature and elevated $[\text{CO}_2]$ were analyzed separately for each year using a mixed model repeated measures analysis of variance (PROC mixed, SAS). Temperature (Temp), $[\text{CO}_2]$, day of year (DOY) and year (2009, 2011) were treated as fixed effects and block was a random effect. Seasonal or pre-senescence means are averages of all the plots for a given treatment for the specified time period. Significant differences between seasonal least square means were detected using post hoc tests adjusted for multiple comparisons. As there were only four blocks, significant probability was set a priori at $p < 0.1$ to reduce the likelihood of type II errors [34,35].

3. Results

3.1. Soil moisture depletion profiles differed by treatment and year

At planting, soil volumetric water content (VWC; % v) for the upper most soil layer (upper; 0–20 cm) was similar in 2009 (DOY 160) and 2011 (DOY 159) with upper soil at field capacity in 2009 (ca. 41%) and slightly lower than field capacity in 2011 (ca. 35%) (Fig. 1). However, seasonally averaged VWC was higher in 2009 than 2011 because precipitation consistently replenished soil moisture and returned VWC to near field capacity several times during the 2009 growing season.

The response of VWC to treatments differed between years. In 2009, VWC was lower in HOT plots when compared to CON plots and lower in 385 plots compared to 585 plots for the upper and middle (30–50 cm) soil layers. By contrast in 2011, VWC was only detectably lower in the upper soil layer in HOT plots and there were no detectable differences of VWC in 385 vs. 585 plots. However, in both years there was a significant $\text{CO}_2 \times \text{temperature}$ interaction effect for soil moisture in the middle soil layer because soil moisture was more depleted in 385-HOT than in 585-HOT when compared to the 385-CON and 585-CON plots, respectively (Table 1).

3.2. The responses of $V_{c,\text{max}}$ to elevated CO_2 and J_{max} to elevated temperature were consistent between years

Both $V_{c,\text{max}@25}$ and $J_{\text{max}@25}$ responded to temperature and $[\text{CO}_2]$ dynamically within years but the overall trends were similar between years (Fig. 2). There was no significant interactive effects of elevated temperature and elevated $[\text{CO}_2]$ for either parameter in either year (Table 2). There was, however, a statistically significant main effect for $V_{c,\text{max}@25}$, which was consistently lower at elevated $[\text{CO}_2]$ (i.e. $-13.6 \pm 1.4\%$ in 585-CON and $-11.8 \pm 1.4\%$ in 585-HOT), and for $J_{\text{max}@25}$, which was consistently lower at

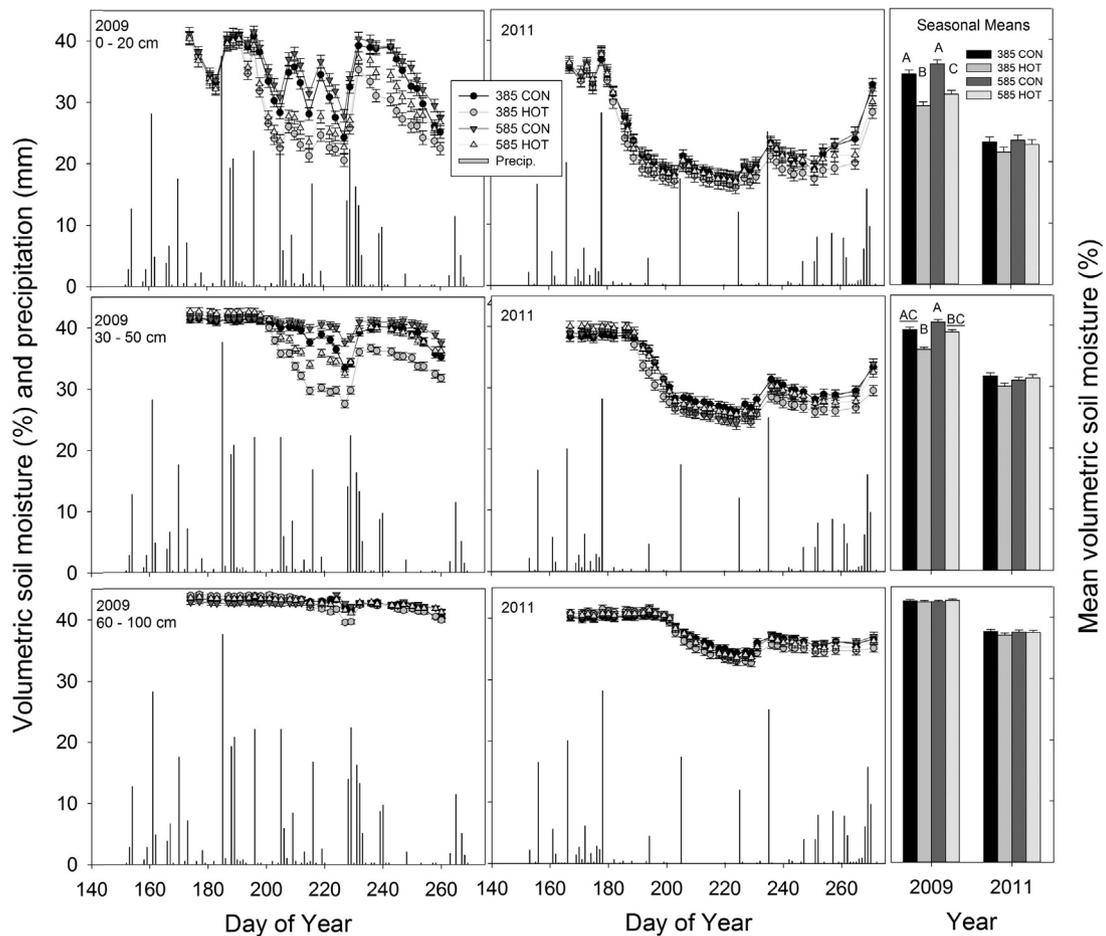


Fig. 1. Volumetric soil moisture content (VWC, % v) for top (0–20 cm), middle (30–50 cm), and bottom (60–100 cm) soil layers, measured on 33 and 39 days in 2009 and 2011, respectively. Data are for control (385-CON; black circle), elevated temperature (385-HOT; gray circle), elevated CO₂ (585-CON; inverted triangle), and the combined treatment (585-HOT; upright triangle). For line graphs symbols are daily means of 4 plots (\pm se). The thin bars within line graph panels are daily precipitation data (mm) which were reported previously [27] and are shown to illustrate that the variance in VWC is related to precipitation as well as the experimental treatments. The plot means averaged over the entire season for each year at each depth are presented on the far left (Seasonal Means) bar graphs and are in the same shade as the symbols. Within panels bars with different letters are significantly different.

elevated temperature (i.e. $-7.8 \pm 0.4\%$ in 385-HOT and $-10.7 \pm 1.8\%$ in 585-HOT) (Table 2). As a result of the contrasting responses of $V_{c,max@25}$ and $J_{max@25}$ to temperature and CO₂, the ratio of $V_{c,max@25}$ to $J_{max@25}$ ($V_{c,max@25}/J_{max@25}$) was increased by temperature (i.e. $9.6 \pm 0.2\%$ in 385-HOT and $9.0 \pm 2.7\%$ in 585-HOT) and decreased by elevated [CO₂] in both years. Because the 585-HOT treatment response is similar to the 585-CON treatment with regards to $V_{c,max@25}$ and similar to the 385-HOT treatment with regards to $J_{max@25}$ the $V_{c,max@25}/J_{max@25}$ ratio in 585-HOT was indistinguishable from 385-CON.

To resolve the differences in biochemical acclimation due to treatments from those that included monocarpic senescence caused by translocation of resources from leaves to pods [36] we removed the last days in 2009 and 2011 from the analysis (see Fig. 2, vertical dashed line) and compared the mean response. The latter analyses revealed that prior to senescence in 2009, $V_{c,max@25}$ was actually significantly higher in HOT compared to CON plots ($F_{1,12.6} = 5.24, p < 0.05$) and $J_{max@25}$ was only detectably lower in 585-HOT when compared to 385-CON. In 2011, the acclimation patterns were similar pre and post senescence.

Table 1

Repeated measures ANCOVA of the effect of CO₂ elevation (CO₂), temperature elevation (Temp), day of year (DOY) on volumetric soil moisture content (VWC, % v) for top (0–20 cm), middle (30–50 cm), and bottom (60–100 cm) soil layers, measured on 33 and 39 days in 2009 and 2011, respectively. The soil moisture at planting (Initial Soil Moisture) is included as covariate to account for soil moisture differences across plots at planting.

	Main effects and interactions							Initial Soil Moisture
	CO ₂	Temp	Temp × CO ₂	DOY	DOY × CO ₂	DOY × Temp	DOY × CO ₂ × Temp	
2009								
Top (0–20)	0.0008	<0.0001	ns	<0.0001	ns	<0.0001	ns	0.037
Middle (30–50)	0.0001	<0.0001	0.0466	<0.0001	<0.0001	<0.0001	ns	ns
Bottom (60–100)	ns	ns	ns	<0.0001	0.0052	<0.0001	ns	0.0069
2011								
Top (0–20)	ns	0.0513	ns	<0.0001	ns	0.0089	0.0442	0.008
Middle (30–50)	ns	ns	0.0179	<0.0001	ns	0.0002	ns	ns
Bottom (60–100)	ns	ns	ns	<0.0001	ns	ns	ns	ns

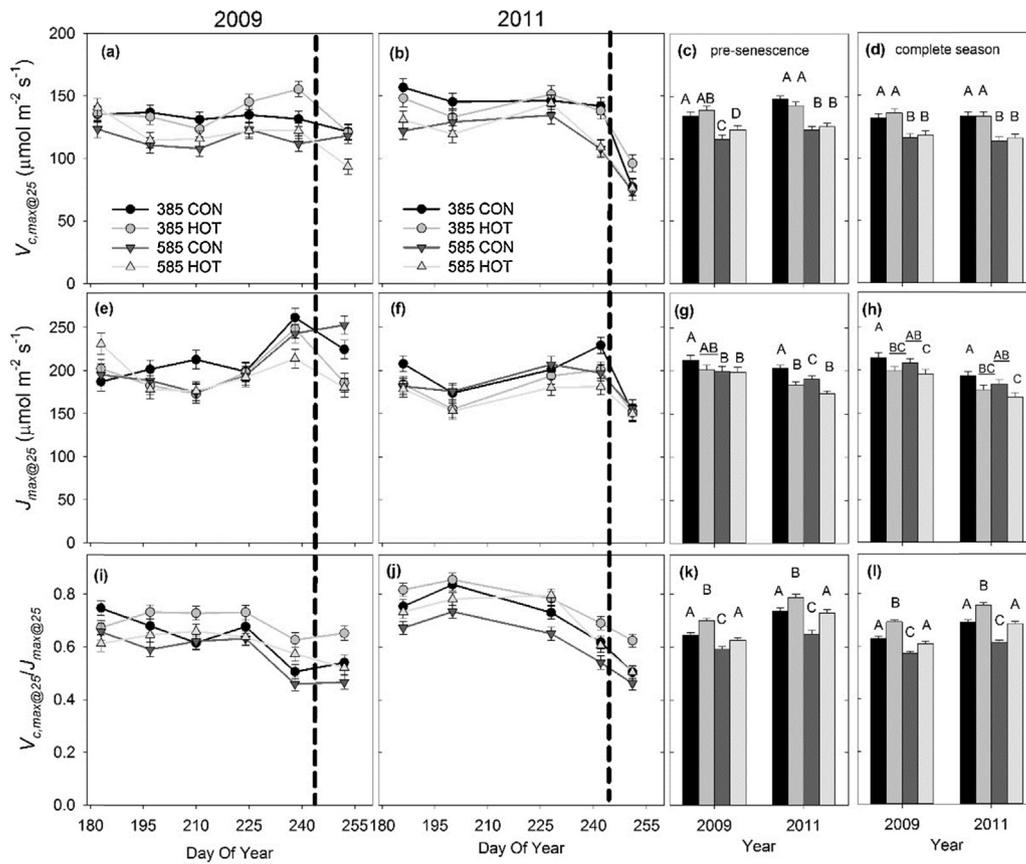


Fig. 2. Seasonal variation and annual means in 2009 and 2011 of $V_{c,max@25}$ (a–d), $J_{max@25}$ (e–h), $V_{c,max@25}/J_{max@25}$ (i–l) in control (385-CON), elevated CO_2 (585-CON), elevated temperature (385-HOT) and the combined treatment (585-HOT). Symbols and shading are identical to Fig. 1 and daily means are for 4 plots (\pm SE). Pre-senescence means (c, g and k) are estimated including days prior to the vertical dashed line whereas complete season means (d, h and l) include all 6 days in 2009 and 5 days in 2011. Within panels bars with different letters are significantly different.

Table 2
Full season repeated measures ANOVA of the effect of elevated CO_2 (CO_2), elevated temperature (Temp) and day of year (DOY) on photosynthetic parameters ($V_{c,max@25}$, $J_{max@25}$, $V_{c,max@25}/J_{max@25}$), light saturated photosynthesis measured growth [CO_2] and at a common ambient [CO_2] of 385 ppm ($A_{@growth}$, $A_{@385ppm}$), stomatal limitation in the field (l_{field}), leaf nitrogen percent by mass (N_{mass}), leaf nitrogen per unit area (N_{area}), leaf mass per unit area (LMA) and photosynthetic nitrogen use efficiency (PNUE) in 2009 and 2011.

	Main effects and interactions						
	CO_2	Temp	Temp \times CO_2	DOY	DOY \times CO_2	DOY \times Temp	DOY \times CO_2 \times Temp
2009							
$V_{c,max@25}$	<0.0001	ns	ns	<0.0001	ns	<0.0001	0.0027
$J_{max@25}$	0.0395	<0.0001	ns	<0.0001	0.0605	<0.0001	ns
$V_{c,max@25}/J_{max@25}$	<0.0001	<0.0001	ns	<0.0001	ns	0.0008	ns
$A_{@growth}$	0.0025	0.0216	ns	<0.0001	ns	<0.0001	ns
$A_{@385ppm}$	<0.0001	ns	0.0683	<0.0001	ns	<0.0001	ns
l_{field}	0.0015	<0.0001	ns	<0.0001	ns	<0.0001	ns
N_{mass}	<0.0001	ns	0.0967	<0.0001	0.0083	<0.0001	0.0366
N_{area}	0.0156	0.0799	ns	<0.0001	ns	<0.0001	ns
LMA	<0.0001	0.016	ns	<0.0001	0.0071	0.0253	ns
PNUE ^a	0.0002	ns	ns	<0.0001	ns	0.0098	ns
2011							
$V_{c,max@25}$	<0.0001	ns	ns	<0.0001	0.0913	ns	ns
$J_{max@25}$	ns	0.0074	ns	<0.0001	ns	ns	ns
$V_{c,max@25}/J_{max@25}$	<0.0001	<0.0001	ns	<0.0001	ns	ns	ns
$A_{@growth}$	0.0014	0.0134	ns	<0.0001	ns	ns	ns
$A_{@385ppm}$	0.0148	0.0275	ns	<0.0001	ns	ns	ns
l_{field}	0.0067	<0.0001	0.035	<0.0001	0.0304	0.0093	0.0222
N_{mass}	<0.0001	ns	0.0653	<0.0001	0.0007	ns	0.0771
N_{area}	0.0452	0.0278	ns	0.0002	0.0493	0.0317	ns
LMA	<0.0001	0.0015	0.0705	<0.0001	ns	ns	ns
PNUE ^a	<0.0001	0.0082	ns	<0.0001	ns	0.0023	ns

^a PNUE was estimated with using N_{area} reported here and measurements of A reported by [27]. See methods for details.

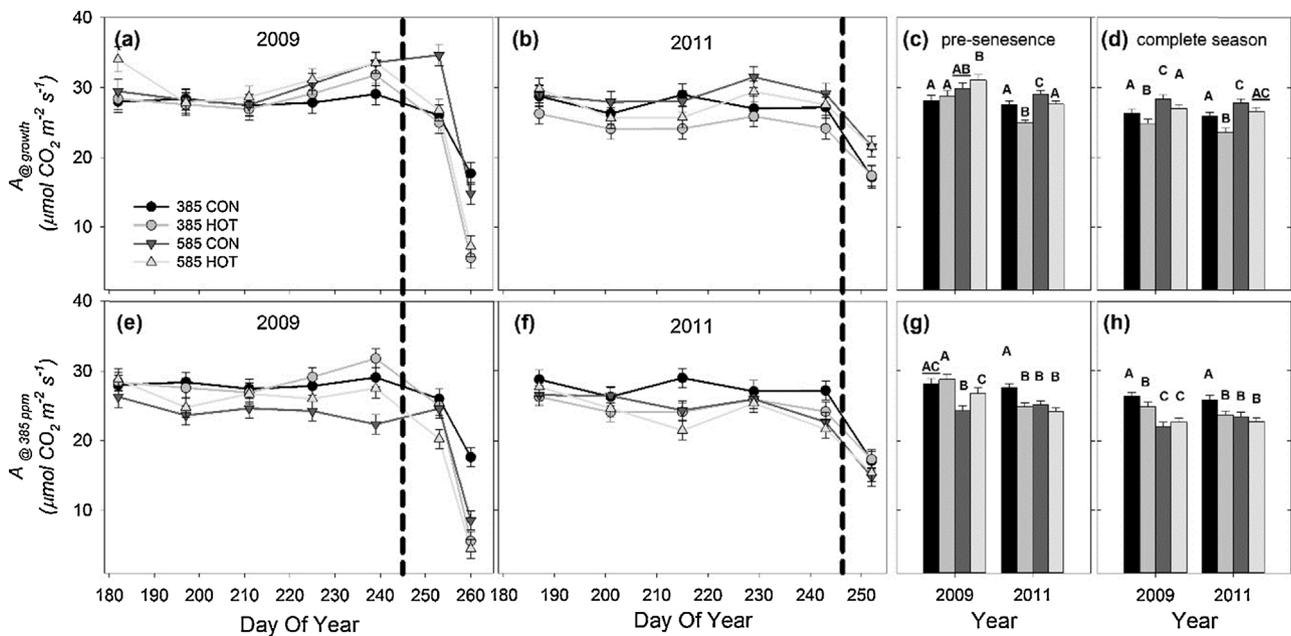


Fig. 3. Steady state light saturated photosynthesis, measured at growth $[CO_2]$ ($A_{@growth}$; panel a–d), and at a common ambient $[CO_2]$ of 385 ppm ($A_{@385ppm}$; panels e–h) for control (385-CON), elevated CO_2 (585-CON), elevated temperature (385-HOT) and the combined treatment (585-HOT) in 2009 and 2011. Symbols and shading are the same as Fig. 1 and are daily means of 4 plots (\pm se). Pre-senesence means (c and g) are estimated including only days prior to the vertical dashed line whereas complete season means (d and h) are for 7 days in 2009 and 6 days in 2011. Within years and panels bars with different letters are significantly different.

3.3. Acclimation to temperature ameliorated biochemical acclimation of A to elevated CO_2 in 2009 but not 2011

Light saturated steady state photosynthesis rates at growth $[CO_2]$ ($A_{@growth}$) and at a common ambient CO_2 of 385 ppm ($A_{@385ppm}$) were collected during A vs. C_i measurements. $A_{@growth}$ was greater in 585-CON and 585-HOT when compared to ambient CO_2 controls (Fig. 3d) but $A_{@growth}$ was significantly lower in 385-HOT and 585-HOT when compared to 385-CON and 585-CON, respectively (Fig. 3d). When measured at a common ambient CO_2 of 385 ppm ($A_{@385ppm}$, Fig. 3e–h), photosynthetic rates of 585-CON and 585-HOT plots were significantly lower than 385-CON and 385-HOT plots, respectively, in 2009 and 2011 (Table 2), consistent with photosynthetic acclimation to growth at elevated CO_2 (Fig. 3h).

As above, we resolved the differences in A due to treatments from those potentially related to senescence by removing two days in 2009 and 1 day in 2011 from the analysis of the response of $A_{@growth}$ to CO_2 and temperature (Fig. 3c and g). This latter analysis showed that $A_{@growth}$ remained significantly lower in HOT plots in 2011 ($F_{1,11.5} = 16.81, p < 0.01$). In contrast, in 2009 $A_{@385ppm}$ was significantly higher in 585-HOT compared to 585-CON, following post hoc tests (Fig. 3g). Measured values of $A_{@growth}$ (above) and $g_{s@growth}$ (Supplemental Figure 2) were consistent with A and g_s rates measured on this cultivar in the field [19,28,37] indicating that predawn excision did not detectably alter leaf photosynthesis.

3.4. Elevated temperature eliminated the benefit of elevated CO_2 for stomatal limitation in both years and stomatal limitation was greater in 2011 than in 2009

While elevating CO_2 decreased stomatal limitation in the field (l_{field}) by similar amounts ($-13.7 \pm 0.6\%$) in both years (Table 2 and Fig. 4) elevating temperature increased l_{field} by $40.7 \pm 2.5\%$ in 2009 and by $55.4 \pm 6.5\%$ in 2011 in the HOT treatments plots relative to the controls. Stomatal limitation was always greatest in 385-HOT plots and the $CO_2 \times$ temperature \times time interaction was statistically significant (Table 2) in 2011 because stomatal limitation increased more rapidly over time in 385-HOT than in any other

plot. Consequently, l_{field} was nearly two-fold greater in 385-HOT plots when compared with other plots at the middle and end of August in 2011 (i.e. DOY 228 and 243) (Fig. 4).

3.5. Elevating temperature had the opposite effect of elevated $[CO_2]$ on leaf structure, nitrogen allocation and photosynthetic nitrogen use efficiency

Season mean total leaf nitrogen content (N_{mass} ; %) for both years was $19 \pm 0.2\%$ lower in 585-CON and $16.8 \pm 1.6\%$ lower in 585-HOT when compared to 385-CON plots (Table 2 and Fig. 5). A significant $CO_2 \times$ temperature interaction with respect to N_{mass} in both years indicates that elevated temperature consistently lowered N_{mass} in 385 plots but increased N_{mass} in 585 plots. The significant three way interaction of $DOY \times [CO_2] \times$ Temp in both years is the result of differences in the timing of peak levels of N_{mass} . The three way interaction was particularly evident in 2009 where, for instance, N_{mass} peaks are at different times for 385-CON and 385-HOT whereas in 2011 the peaks differ for 585-CON and 585-HOT (Fig. 5a and d).

In 2009, when averaged over the whole season, elevated CO_2 decreased nitrogen allocation per unit area (N_{area} , $g\ m^{-2}$) by 6.5% and elevated temperature decreased N_{area} by 4.8% and 2.5% in 385-HOT and 585-HOT plots, respectively (Table 2 and Fig. 5e and h). The combined effects of elevated $[CO_2]$ and temperature were additive in 2009, as N_{area} was 8.9% lower in 585-HOT when compared to ambient unheated plots. Increasing temperature also caused N_{area} to increase faster early in the season and decrease faster late in the season when compared to unheated plots in the 2009 season. In 2011, elevated $[CO_2]$ decreased N_{area} by 4.8% (Fig. 5f and h); elevated temperature decreased N_{area} by similar amounts in 385-HOT and 585-HOT plots (ca. 5%) and N_{area} was 9.6% lower in 585-HOT when compared to control. Thus the main effects of elevated CO_2 and temperature were also additive with respect to N_{area} in 2011.

Removing two days in 2009 from the analysis of N_{area} and N_{mass} to resolve the differences due to treatments from those potentially related to senescence, we see that temperature had the opposite effect to that of the full season trend. That is, this analysis revealed that in the HOT plots there were significant increases in

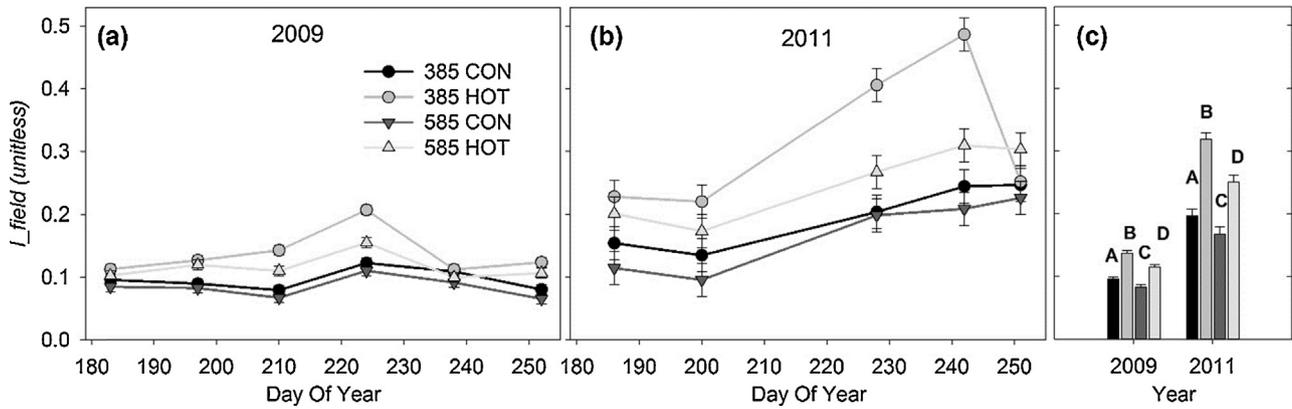


Fig. 4. Stomatal limitation (l_{field}) in 2009 (a), 2011 (b), and overall annual means (c) for control (385-CON), elevated $[CO_2]$ (585-CON), elevated temperature (385-HOT) and the combined treatment (585-HOT). Symbols and shading in line graphs are the same as Fig. 1 and are daily means of 4 plots (\pm se). In bar graphs, overall means (\pm se) are for 6 days in 2009 and 5 days in 2011. Within years bars with different letters are significantly different.

N_{mass} ($F_{1,12} = 28.87$, $p < 0.001$) and N_{area} ($F_{1,12} = 4.04$, $p < 0.07$) prior to senescence in 2009. Unfortunately, such a comparison is not possible for 2011 as there were no late season N_{area} data.

Elevated CO_2 increased leaf mass per unit area (LMA , $g\ m^{-2}$) by 15% in 2009 and by 18.9% in 2011. Averaged over all treatments, elevated temperature decreased LMA by about 5.7% when compared to 385-CON; post hoc test show that LMA was significantly lower in 585-HOT plots when compared to 585-CON in both years (Table 1 and Fig. 5i) with LMA decreasing twice as much due to temperature in 2011 (i.e. -9.9%) than in 2009 (-5%). Removing two days

in 2009 from the analysis of LMA revealed a similar response to elevated CO_2 but the effect of temperature was not detectable.

Photosynthetic nitrogen use efficiency (PNUE) was significantly higher in elevated CO_2 increasing by 16% in 2009 and 26% in 2011 when compared to 385-CON (Table 2; Supplemental Figure 3). Temperature elevation affected PNUE differently between years. In 2009 only 385-HOT had significantly lower (-10%) PNUE than 385-CON whereas in 2011 PNUE was lower in 385-HOT (-11%) and 585-HOT (-8%) when compared to 385-CON and 585-CON, respectively. The effects of elevated temperature and $[CO_2]$ were similar

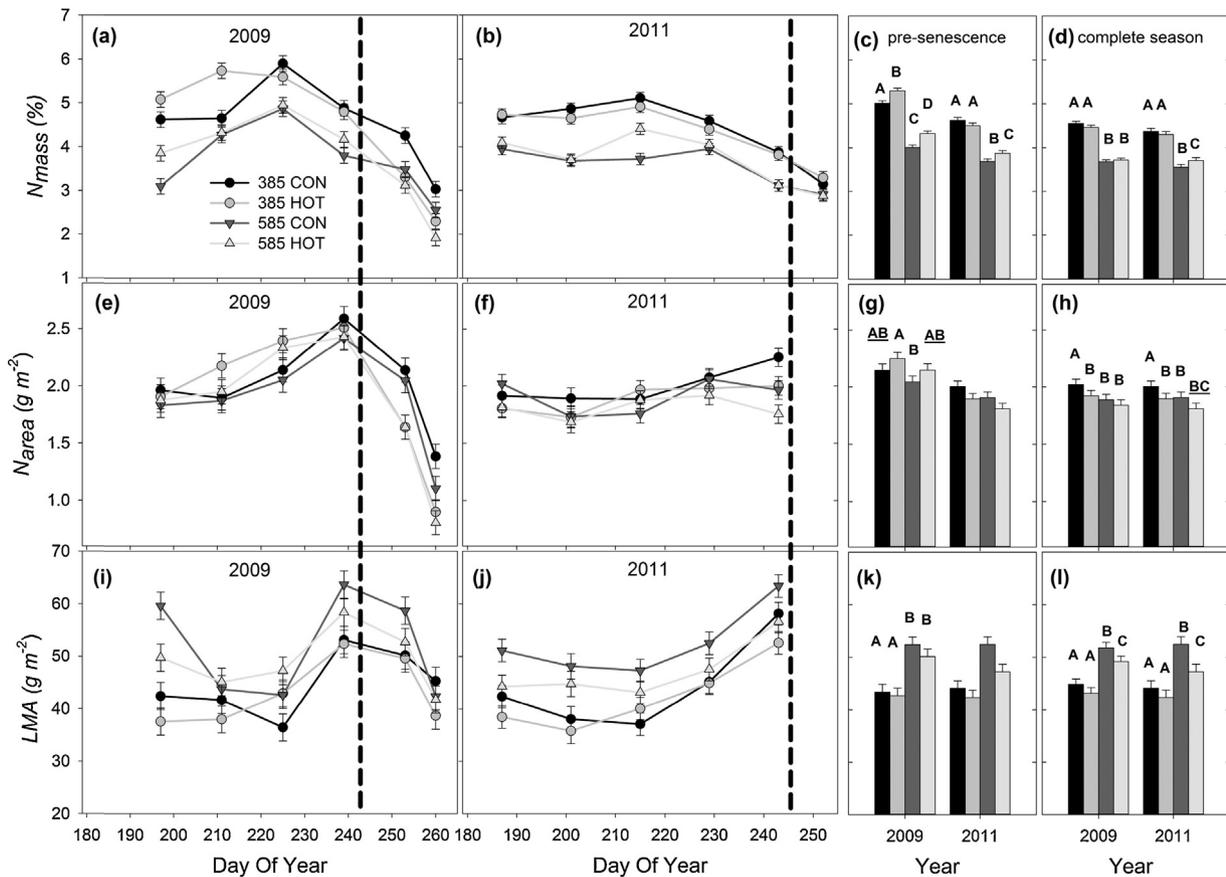


Fig. 5. Leaf nitrogen content by weight (N_{mass}), by area (N_{area}), and leaf mass per area (LMA) for control (385-CON), elevated CO_2 (585-CON), elevated temperature (385-HOT) and the combined treatment (585-HOT). Symbols and shading are the same as Fig. 1 and are daily means of 4 plots (\pm se). Pre-senescence means (c, g and k) are estimated excluding days after the vertical dashed line whereas complete season means (d, h and l) are for 6 days in 2009 and 6 days in 2011, except N_{area} and LMA in 2011, which were analyzed for 5 days. Within years bars with different letters are significantly different.

both prior to senescence and for the whole season (Supplemental Figure 3).

3.6. Synergistic effect of elevated CO₂ and temperature on the thermal response of A is transient

The response of $A_{@growth}$ to leaf temperature (T_l) was measured in the laboratory on three days in 2009 for all four treatments (Fig. 6). The synergistic effect of elevated [CO₂] and elevated temperature on the thermal response of A (i.e. A is significantly more stimulated in 585-HOT than 585-CON) was only evident during vegetative growth at 30 and 35 °C (Fig. 6a).

4. Discussion

The goals of this experiment were to quantify photosynthetic acclimation in soybean subjected to growth at elevated [CO₂] and elevated temperature, singly and in combination, under field conditions. We build on the recently published results of Ruiz-Vera et al. [27], by elucidating some of the key ecophysiological and associated biochemical mechanisms underlying the negation of CO₂ stimulation of assimilation by elevated temperature reported earlier. Because precipitation substantially altered field conditions between the two years, we assessed how changes in soil moisture may have modulated acclimation responses to elevated temperature and CO₂. The data fully supported our first hypothesis that $J_{max@25}$ would acclimate more to elevated temperature, regardless of [CO₂], than would $V_{c,max@25}$. Moreover, the substantial acclimation of $J_{max@25}$ likely reduced some of the purported synergistic effects of increases in temperature at elevated [CO₂] [27,38,39]. The data only partially supported our second hypothesis; that is, the combined effect of elevated temperature and elevated [CO₂] did not always lower photosynthetic stimulation when compared to the effect of elevated [CO₂] alone. While biochemical acclimation to elevated CO₂ was similar between years, acclimation to temperature, and its effect on assimilation, differed substantially between years.

Higher soil moisture in 2009 ameliorated the detrimental effects of temperature whereas warmer ambient temperatures and low soil moisture likely interacted to exacerbate the effect of chronic temperature elevation in 2011. In spite of plentiful precipitation in 2009, VWC profiles revealed that moisture depletion was greater at depth in HOT plots compared to CON plots. Plants in HOT plots apparently accessed moisture deeper in the soil than plants in CON plots, probably because soybeans in HOT plots were rooted more deeply. Nevertheless, soil VWC was lower in the HOT plots throughout the soil profile in 2009 (Fig. 1), consistent with greater moisture demand and lower midday water potential in HOT plots [27].

By contrast, in 2011 there was little or no soil moisture replenishment after planting, and elevating temperature exacerbated this effect in the upper soil, which was at or near the wilting point (ca. 21% VWC) for most July and August (Fig. 1). Also unlike 2009, VWC profiles were similar between treatments in 2011 suggesting that plants in HOT plots may not have had access to additional moisture compared to CON plots in 2011, which is consistent with a greater temperature effect on plant water potential in HOT plots in 2011 compared to 2009 [27]. Thus, the low soil moisture in 2011, when combined with the detrimental effect of higher ambient temperatures and greater vapor pressure deficits [27], exacerbated the effects of chronic temperature elevation and likely contributed to the substantial decreases in yield reported for HOT plots in 2011.

As seen in numerous previous field and enclosure studies [10,21], soybean acclimated to growth at elevated [CO₂] by lowering in vivo carboxylation capacity ($V_{c,max@25}$) and elevating [CO₂] had a smaller effect on $J_{max@25}$ (Fig. 1). Additionally, assimilation

rates of plants in 585 plots were lower than those of plants in 385 plots when compared at a common ambient [CO₂] of 385 ppm (i.e. $A_{@385ppm}$), consistent with downregulation of $V_{c,max}$ at elevated CO₂ [10]. The decrease in $V_{c,max@25}$ at elevated CO₂ is likely due to decreases in Rubisco amount or activation state [3]. However, regardless of lower $V_{c,max@25}$, N_{mass} and N_{area} , light saturated photosynthesis measured at growth [CO₂] ($A_{@growth}$) remained higher in elevated CO₂ plots (Fig. 2) [27], consequently, photosynthetic nitrogen use efficiency remained higher at elevated [CO₂] (Supplemental Figure 3) [40].

Acclimation of $V_{c,max}$ and J_{max} to combined increases in [CO₂] and temperature varies substantially across species and experiments [3,41–43]. Moderately elevated temperature alone (i.e. +4 °C) has been reported to either increase [43,44], decrease [3,6,43] or not change $V_{c,max}$ [43]. At [CO₂] of ca. 350 ppm moderate increases in temperature may have little effect on $V_{c,max}$ as long as growth temperatures remain below the photosynthetic optimum; when growth temperatures consistently exceed the temperature optimum of A, $V_{c,max}$ down-regulates significantly [3,6]. On average, J_{max} acclimates more than $V_{c,max}$ at higher growth temperatures [44–46] and less than $V_{c,max}$ at elevated CO₂. Here, $V_{c,max@25}$ did not detectably change in response to elevated temperature when averaged for the entire season in either year suggesting that plant canopy temperatures did not exceed the temperature optimum of A. Moreover, $V_{c,max@25}$ did not acclimate more to elevated [CO₂] in the dryer, hotter, 2011 season which is consistent with nodulated alfalfa where down regulation of $V_{c,max}$ at elevated [CO₂] occurred irrespective of temperature and drought [47]. In contrast, non-leguminous species including red maple [48], black spruce [49], wheat [6] and spinach [50] report decreases in $V_{c,max@25}$ in plants grown at moderately elevated daytime temperatures (i.e. +3.5 °C to +6 °C).

Seasonal increases and subsequent declines in leaf N, which were more clearly seen in 2009, are typical for soybean [51], but could alter $V_{c,max@25}$. Leaf N remains relatively low in young soybeans, because of a lag between nodule formation just after emergence and active N fixation, after which leaf N increases rapidly to a peak level [52]. Subsequent declines in leaf N following pod formation in soybeans are due to senescence as leaf N is retranslocated to developing pods [36,52]. Because of the dependence of leaf assimilation and biochemistry on leaf N we also assessed trait responses to treatments prior to senescence. In the latter analysis, we see that $V_{c,max@25}$ was greater in HOT compared to CON plots prior to senescence consistent with higher N_{mass} and N_{area} during the same period of 2009. Increased access to moisture in 2009 and warmer temperature in the HOT plots would likely increase the nitrogen nutrition of plants whereas nitrogen nutrition in soybeans was inhibited in drought [53] potentially explaining why N_{area} was higher in HOT plots compared to control plots in 2009 and why in 2011 N levels were generally lower than at comparable developmental stages in 2009. Thus, when leaf N was higher in HOT plots and in the absence of water stress, elevated temperature and [CO₂] increased carboxylation capacity relative to elevated [CO₂] alone which is consistent with a recent meta-analysis of plant responses to elevated [CO₂] and temperature [7] and mirrors nodulating *Medicago truncatula* grown in temperature gradient tunnels (+4 °C) at ambient and elevated (700 ppm) CO₂ [54].

Significantly lower $J_{max@25}$ (Fig. 2) when coupled with lower operating efficiency of photosystem II (Φ_{PSII}) (Supplemental Figure 1) are indicative of a cumulative effect of acclimation to temperature and adjustments in energy partitioning in PSII [55] or possibly temperature induced damage to photosystem II because these decreases were observed in predawn collected leaves measured under similar temperatures and relative humidity. Such damage or changes in energy partitioning in photosystem II directly reduce A

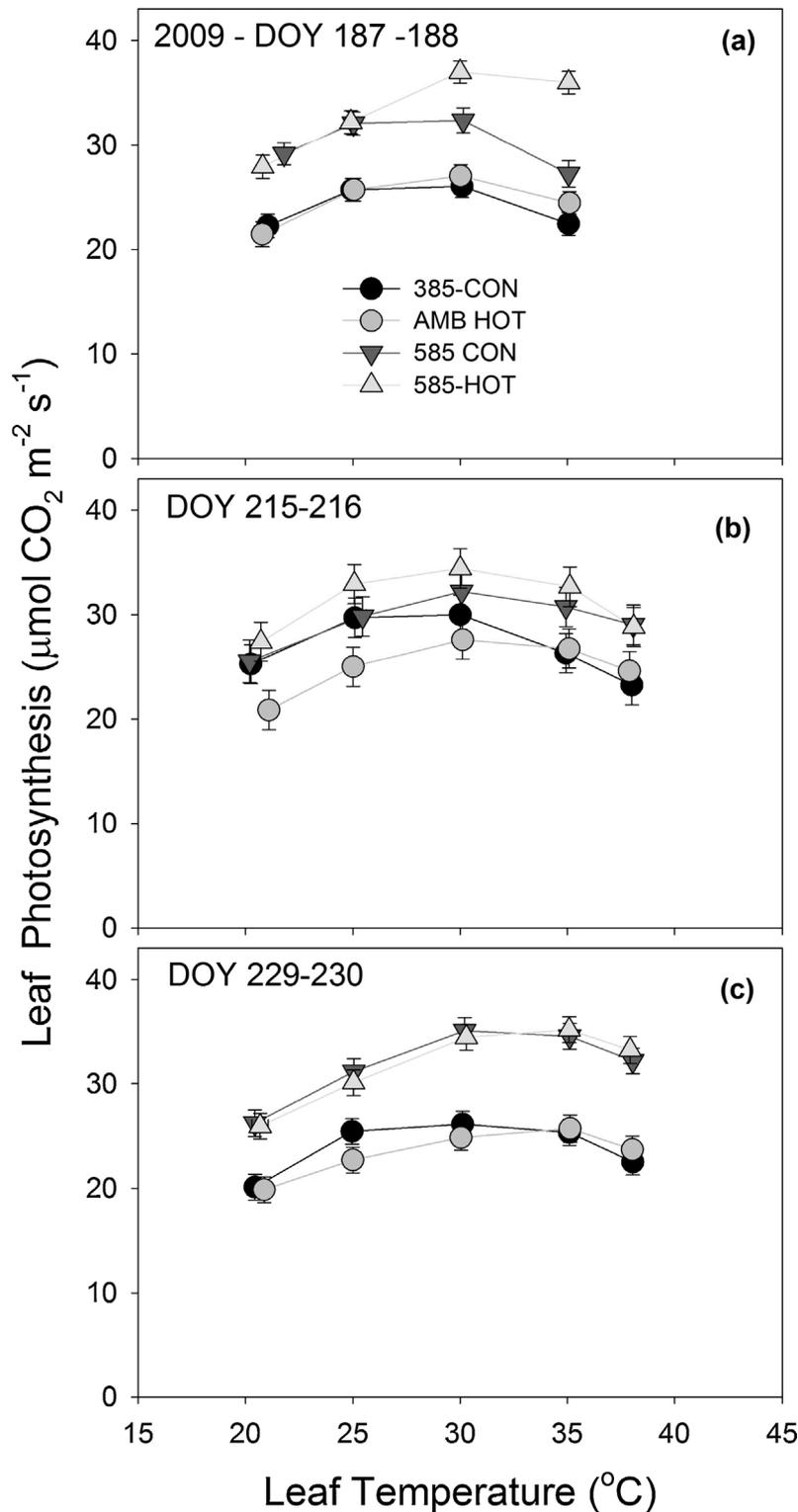


Fig. 6. The response of leaf photosynthesis to leaf temperature measured at vegetative (a), full bloom (b) and beginning seed (c) stages in the laboratory in 2009. Symbols and shading are the same as Fig. 1 and are daily means of 4 plots (\pm se). In this figure, the symbols with error bars that do not overlap at a given temperature are significantly different ($p < 0.05$).

[56] and could lead to a lower realized benefit of elevated $[\text{CO}_2]$ when coupled with increases in temperature. Indeed, A becomes increasingly RuBP limited at elevated $[\text{CO}_2]$ and the decreases in J_{max} reported here directly contributed to decreases in RuBP limited carbon assimilation and likely lower yields of soybean in the field [27]. Thus future increases in temperature will likely have a proportionally larger effect on carbon gain and future yields.

Stomatal limitation of A decreases with increasing CO_2 because of the non-linear response of A to C_i [23]; consequently in spite of a 25 and 31% decrease in g_s in 585-CON compared to 385-CON plots in 2009 and 2011, respectively [27], stomatal limitation in the field (I_{field}) was 14% less in elevated $[\text{CO}_2]$ plots when compared to controls in both years, which is within the same range previously reported for soybean grown at SoyFACE [19]. Elevated temperature,

independent of $[\text{CO}_2]$, also decreased g_s and C_i in the field [27] consistent with greater I_{field} in HOT plots (Fig. 4). However, the combination of elevated temperature and CO_2 increased I_{field} by 20–27% compared to 385-CON in 2009 and 2011, respectively. Thus, while elevated CO_2 decreased stomatal limitation of A as expected, elevated temperature eliminated the benefit of elevated $[\text{CO}_2]$ on the physical limitation of A in both years.

The measured responses of A to temperature conformed to theoretical expectations with respect to elevated CO_2 [13,17] but the synergistic effect of increasing $[\text{CO}_2]$ and temperature on the thermal response of A was absent later in the season, at least in 2009. For instance, while A was higher at all measurement temperatures in elevated $[\text{CO}_2]$ (e.g. Sage et al. [57]), A was significantly higher in the 585-HOT plots when compared to 585-CON plots, but only in July of 2009. This stimulation of the temperature response of $A_{\text{@growth}}$ occurred when leaf N levels were higher in HOT plots and when soil moisture was identical and near field capacity in all plots, following a large (37 mm) precipitation event in 2009. Thus, it appears that theoretical expectations of the thermal response of A are met in the field but possibly only under near optimal conditions.

There has been much discussion about the theoretical modification of the temperature response of leaf photosynthesis at elevated $[\text{CO}_2]$ since the seminal review by Long [13]. While the latter theoretical analysis accounted for the acclimation of $V_{c,\text{max}}$ to elevated $[\text{CO}_2]$, the study by Long [13] did not have sufficient data to support the notion of acclimation of $V_{c,\text{max}}$ or J_{max} to temperature and its effect on the modification of the temperature response of A at elevated $[\text{CO}_2]$. Here, the combined acclimation of $V_{c,\text{max}@25}$ and $J_{\text{max}@25}$ and the shift in $V_{c,\text{max}@25}/J_{\text{max}@25}$ are consistent with a shift toward RuBP regeneration limited photosynthesis at elevated $[\text{CO}_2]$ (e.g. [1,13,19]); therefore, the benefit of elevated $[\text{CO}_2]$ on the temperature response of A may also decrease because acclimation of $J_{\text{max}@25}$ to elevated temperature can lower RuBP regeneration limited A . Thus, while the temperature response of A is almost always enhanced at elevated $[\text{CO}_2]$ [8,17,19] and the realized benefit of elevated $[\text{CO}_2]$ increases with temperature [37,41] longer term growth at elevated $[\text{CO}_2]$ and elevated temperature can be less than additive with respect to A as seen here and in some other enclosure studies [6,58].

5. Concluding remarks

Improving crop productivity within the context of climate change remains a critical goal if we are to meet global food demands [39,59,60]. While photosynthetic responses to climate change differ among species and genotypes [48,50,61–64] the greater than expected acclimation of key photosynthetic processes reported here has important implications for C_3 photosynthesis beyond that of soybeans. Indeed, assessing the magnitude and direction of acclimation is crucial to our understanding of global carbon flux and food security because A modulates the largest exchange of carbon from the atmosphere into ecosystems and is an important determinant of crop yields. Finally, and perhaps most importantly, photosynthetic acclimation to temperature and CO_2 when combined under field conditions may be greater than reported in some enclosure studies (cf. Long et al. [65]) and will modify regional variation in CO_2 stimulation reported in [66].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.plantsci.2014.06.013>.

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