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Impairment of C_4 photosynthesis by drought is exacerbated by limiting nitrogen and ameliorated by elevated [CO_2] in maize

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Abstract

Predictions of future ecosystem function and food supply from staple C_4 crops, such as maize, depend on elucidation of the mechanisms by which environmental change and growing conditions interact to determine future plant performance. To test the interactive effects of elevated [CO_2], drought, and nitrogen (N) supply on net photosynthetic CO_2 uptake (*A*) in the world's most important C_4 crop, maize (*Zea mays*) was grown at ambient [CO_2] ($\sim$385 ppm) and elevated [CO_2] (550 ppm) with either high N supply (168 kg N ha^{-1} fertilizer) or limiting N (no fertilizer) at a site in the US Corn Belt. A mid-season drought was not sufficiently severe to reduce yields, but caused significant physiological stress, with reductions in stomatal conductance (up to 57%), *A* (up to 44%), and the *in vivo* capacity of phosphoenolpyruvate carboxylase (up to 58%). There was no stimulation of *A* by elevated [CO_2] when water availability was high, irrespective of N availability. Elevated [CO_2] delayed and relieved both stomatal and non-stomatal limitations to *A* during the drought. Limiting N supply exacerbated stomatal and non-stomatal limitation to *A* during drought. However, the effects of limiting N and elevated [CO_2] were additive, so amelioration of stress by elevated [CO_2] did not differ in magnitude between high N and limiting N supply. These findings provide new understanding of the limitations to C_4 photosynthesis that will occur under future field conditions of the primary region of maize production in the world.

Key words: Climate change, stomata, stress, Zea mays.

Introduction

The C_4 plant functional type contributes $\sim$25–30% of global terrestrial productivity, includes many of the world's worst weeds, and contributes $\sim$40% of the world's grain harvest (Patterson, 1995; Gillon and Yakir, 2001; USDA, 2005). The most globally important C_4 grain crop is maize (*Zea mays*), which is grown in over 160 countries and contributed $\sim$712 of the 800 million metric tonnes of total C_4 grain harvest in 2006 (http://faostat.fao.org/). Maize production has been dramatically increasing since 1960 and it is predicted to outpace wheat and rice as the number one cereal crop by the year 2020 (Pingali, 2001). In addition to C_4 species being ecologically and nutritionally important, many of the current and candidate biofuel crops possess C_4 photosynthesis, including sugarcane (*Saccharum officinarum*), maize (*Zea mays*), switchgrass (*Panicum virgatum*), and Miscanthus (*Miscanthus×giganteus*; Somerville *et al.*, 2010). Therefore, it is increasingly important to understand C_4 responses to global environmental change in order to predict future ecosystem function, food availability, and energy security. However, current predictions are limited by inadequate understanding of how interactions with other environmental variables enhance or exacerbate C_4 photosynthetic responses to rising atmospheric CO_2 concentration ([CO_2]) (Ghannoum *et al.*, 2000; Sage and Kubien, 2003; Leakey, 2009).

Theoretically, net CO_2 assimilation rates (*A*) of C_4 species should not be directly stimulated by elevated [CO_2] under

optimal growth conditions of temperature, water availability and nutrient supply (Ghannoum *et al.*, 2000). This is because at current atmospheric [CO_2] the CO_2 concentrating mechanism of C_4 plants results in saturating [CO_2] for the Rubisco enzyme in the bundle sheath cells (von Caemmerer and Furbank, 2003). This theoretical expectation is supported by experimental data from free-air CO_2 enrichment (FACE) studies of maize in the mid-west US, irrigated sorghum in the south-west US, and *Paspalum dilatatum* in a New Zealand pasture (von Caemmerer *et al.*, 2001; Wall *et al.*, 2001; Leakey *et al.*, 2006). This lack of a consistent, direct enhancement of photosynthesis and yield in C_4 species across a broad range of growing conditions diminishes the extent that elevated [CO_2] will offset global yield loss resulting from other aspects of environmental change, even if elevated [CO_2] acts locally to ameliorate stress associated with greater drought and temperature (Leakey, 2009).

C_4 plants in natural and agricultural ecosystems frequently grow in conditions of limiting water availability and/or limiting N supply. Globally, water availability is a key factor limiting plant productivity and crop yield (Boyer, 1982; Churkina and Running, 1998; Nemani *et al.*, 2003; Gerten *et al.*, 2004; Mu *et al.*, 2007). Summer precipitation events in mid-continental areas are projected to decrease in volume and/or frequency (Giorgi *et al.*, 2001; Kling *et al.*, 2003; Weltzin *et al.*, 2003), and greater temperatures across the world will increase crop water use and deplete soil moisture, thereby resulting in a greater risk of droughts this century (Meehl *et al.*, 2007). Although fertilizer use is rising to address the N limitation of many crops, there is economic and ecological pressure to limit fertilizer use in all regions (Wallace and Knausenberger, 1997; Smil, 1999; Galloway *et al.*, 2008). Water and N limitation will be particularly acute in many developing countries of Africa and the Americas, which are characterized by (i) heavy reliance on C_4 crops for food (Leakey, 2009), (ii) the strongest links between local agricultural productivity and human well-being (Millennium Ecosystem Assessment, 2005), and (iii) the most severe predicted impacts of global environmental change (Lobell *et al.*, 2008).

The mechanisms determining photosynthetic performance can be evaluated in terms of non-stomatal and stomatal limitations through analysis of the response of A to intercellular [CO_2] (c_i), or A/c_i curves (Farquhar and Sharkey, 1982; Lawlor and Cornic, 2002; Long and Bernacchi, 2003; Ghannoum, 2009). Non-stomatal limitations to A include numerous biochemical and structural properties of leaves that are commonly quantified and modelled in terms of their effects on the capacities for (i) carboxylation by PEPC (V_{pmax}), which determines the initial slope of the C_4 A/c_i curve, and (ii) carboxylation by Rubisco as well as regeneration of PEP by PPDK, each of which can limit the asymptote of the C_4 A/c_i curve (V_{max}; von Caemmerer, 2000). Stomatal limitation to A determines the c_i at which A is operating on the A/c_i curve. It is quantified from A/c_i curves by comparing observed A with the value that would be achieved if there was no resistance to the diffusion of CO_2 through the stomata from the atmosphere to the intercellular leaf space (Farquhar and Sharkey, 1982).

Elevated [CO_2] has the potential to play an important role in future C_4 plant performance if it relieves limitations to A that result from inadequate supplies of water and N. Elevated [CO_2] consistently ameliorates reductions in A caused by drought stress in C_4 species (Samarakoon and Gifford, 1996; Ghannoum *et al.*, 2000; Wall *et al.*, 2001; Leakey, 2009), but how stomatal and non-stomatal factors contribute to the response is still uncertain. For example, in some cases, the initial slope of the A/c_i curve is lower in plants grown at elevated [CO_2] (Maroco *et al.*, 1999; Watling *et al.*, 2000; Driscoll *et al.*, 2006), which would counteract amelioration of drought stress resulting from reduced stomatal conductance and water use. Yet, in other situations, the shape of the A/c_i curve does not change (von Caemmerer *et al.*, 2001, Leakey *et al.*, 2006). Historical improvements in yields of maize in the US Corn Belt have been attributed to greater root growth supporting greater water capture (Hammer *et al.*, 2009). Despite generally greater root:shoot ratios, limiting N supply can reduce root growth (Hocking and Meyer, 1991) and thereby has the potential to prevent maize roots from accessing water deep in the soil during periods of low rainfall. Limiting N can increase leakiness of the C_4 cycle, and also reduce the capacity of key enzymes involved in the C_4 carbon concentrating mechanism and CO_2 fixation (Ranjith *et al.*, 1995; Ghannoum and Conroy, 1998; von Caemmerer, 2000; Ghannoum *et al.*, 2005), which could alter whether A remains CO_2-saturated at ambient [CO_2]. The effect of limiting N supply on C_4 photosynthetic and productivity responses to elevated [CO_2] have been studied under well-watered conditions (Hocking and Meyer, 1991; Ghannoum and Conroy, 1998), but the results were inconsistent.

The FACE facility at the University of Illinois at Urbana-Champaign in the mid-west US allowed treatments of ambient [CO_2] and high N (ACHN), ambient [CO_2] and limiting N (ACLN), elevated [CO_2] and high N (ECHN), and elevated [CO_2] and limiting N (ECLN) to be imposed on maize growing under rain-fed, open-air field conditions with an undisturbed soil–plant–atmosphere continuum; thereby avoiding the unintended artefacts on plant microclimate caused by experimental enclosure (Long *et al.*, 2006; Ainsworth *et al.*, 2008). In conjunction with a significant drought event in August 2008, this provided a rare opportunity to test the response of the model C_4 plant, maize, to the interactive effects of elevated [CO_2], drought, and limiting N supply under field conditions. The low environmental and genetic variability of the study system also maximized the power of the experimental design to detect subtle treatment effects while testing the following hypotheses: (i) limiting N supply will reduce the capacities of the CO_2 concentrating mechanism and CO_2 fixation, causing a higher [CO_2] saturation point for A, and thus greater sensitivity of A to elevated growth [CO_2]; (ii) growth at elevated [CO_2] will relieve both stomatal and non-stomatal limitations to A during periods of drought; and (iii) limiting N supply will exacerbate stomatal and non-stomatal limitation to A during drought, thereby enhancing the beneficial effects of elevated [CO_2].

Materials and methods

Experimental design, cultivation, FACE system, and crop growing conditions

During the 2008 growing season the SoyFACE experimental facility (www.soyface.illinois.edu) in Champaign, IL was used to test the effects of growth [CO_2] and N supply on *Zea mays* cv. 34b43 (Pioneer Hi-Bred International). The crop was planted on 29 May, emerged on 5 June, and was harvested on 1 October. The experiment was laid out as a fully factorial, split-plot design in four experimental blocks ($n=4$ for all statistical tests) with CO_2 treatment as the between-plot factor and N treatment as the split-plot factor. Each block contained one plot at current ambient [CO_2] (~385 ppm) and one plot at elevated [CO_2] (550 ppm). Half of each plot received standard N fertilization (168 kg N ha^{-1}, HN) while the other half received no N fertilization (LN). Both sub-plots had an estimated soil N credit of 45 kg N ha^{-1} from the soybean crop of the previous year. Soil N was measured on DOY (day of year) 198 to ensure continued lower N availability in the limiting N treatments. Fumigation operated from planting until harvest to a target [CO_2] of 550 ppm, which was chosen to simulate growing conditions projected to occur in 2050 (Prentice *et al.*, 2001). In all other regards, the agronomic techniques, site management, and fumigation technology used were the same as in previous experiments (Leakey *et al.*, 2004, 2006). Air temperature (T_{air}), relative humidity (RH%), rainfall, and incident photosynthetic photon flux density (PPFD) were measured by an on-site weather station as previously described (Leakey *et al.*, 2004). At four locations in each subplot, volumetric soil moisture content (H_2O%) was measured in increments of 10 cm between depths of 5–105 cm every 3–5 d across the season using a capacitance probe (Diviner 2000, Sentek Sensor Technologies).

In situ leaf photosynthetic gas exchange

Diurnal courses of *in situ* photosynthetic gas exchange were measured on the youngest most fully expanded leaves of two plants in each subplot on four dates that corresponded to four developmental stages (Table 1). On each date, measurements began once dew had evaporated from the leaf surfaces and continued at 2 h intervals until just before sunset. Four open gas-exchange systems (Li-6400 and Li-6400-40; Li-Cor, Lincoln, NE, USA) were used simultaneously and rotated among treatments and blocks to avoid sampling bias as described by Leakey *et al.* (2006). Immediately before each time point, T_{air}, and incident PPFD were determined above the canopy. These conditions and growth [CO_2] were reproduced in the leaf chamber of the gas exchange systems for all measurements during the timepoint. Leaf assimilation rate (A), stomatal conductance (g_s), and c_i were calculated following von Caemmerer and Farquhar (1981).

A/c_i curves

The youngest most fully expanded leaves of two plants per subplot (eight plants total per treatment, from four replicate ambient or elevated [CO_2] plots) were harvested pre-dawn, re-cut under water, and the cut surface kept immersed until measurements were completed. This was repeated on four dates corresponding to four different developmental stages (Table 1). A/c_i curves of the excised leaves were assessed in the laboratory using the gas exchange apparatus described in the previous section and the protocol of Bernacchi *et al.* (2005), with the following modifications. Measurements were performed at 27 °C, 1750 µmol m^{-2} s^{-1} PPFD, and [CO_2] of 25, 50, 75, 100, 150, 200, 300, 400, 575, 800, and 1000. The A to c_i relationship at a c_i <50 ppm was used to solve for V_{pmax} following von Caemmerer (2000). The horizontal asymptote of a four-parameter non-rectangular hyperbola was used as an estimate for V_{max}. Stomatal limitation to A was estimated as described by Long and Bernacchi (2003), using mean values of *in situ* c_i in combination with A/c_i curves drawn using V_{pmax} and V_{max} parameter values that corresponded to statistically significant treatment effects on dates representing non-drought (DOY 197 and 204) and drought-stressed conditions (DOY 228 and 232).

Development, leaf area index, biomass, yield

Plant ontological development was monitored every 3–5 d throughout the life cycle of the crop and developmental stages were determined based on classifications given in Ritchie *et al.* (1993). Leaf area index (LAI) was measured at the developmental stage corresponding to maximum vegetative canopy leaf area (Ritchie *et al.*, 1993) using a plant canopy analyser (LAI-2000, Li-Cor, Lincoln, NE, USA). At the end of the growing season six plants were harvested from each plot to assess above-ground biomass accumulation. Material was aggregated into three fractions (ears, leaves, and stalks) that were oven-dried at 70 °C before weighing to determine dry mass. Dried grain was shelled from the ears and weighed to determine seed yield. From this sample, three hundred random maize kernels were weighed to determine individual grain size.

Statistics

All analyses were performed on plot means ($n=4$) in SAS (SAS 9.1, SAS Institute, Cary, NC) using the MIXED procedure with the Kenward–Rogers option. A threshold of P <0.1 was used to determine statistical significance for this field study. In all cases, block was a random effect, while [CO_2] and N treatments were fixed effects. The [CO_2] treatment was tested as the between-plot factor and N was tested as the within-plot factor. Averages of H_2O% in three layers of the soil profile (5–25 cm, 25–55 cm, 55–105 cm) were independently analysed with DOY as a repeated measure and early season saturated soil $H_2O_{v/v\%}$ as a covariate. For all gas exchange parameters, data from different DOY were

Table 1. Calendar date, Julian day of year (DOY), and days after emergence (DAE) when measurements were made of diurnal courses of leaf gas exchange and A/c_i curves along with the corresponding developmental stages as defined by Ritchie *et al.* (1993) for field-grown maize under either ambient (385 µmol mol^{-1}; AC) or elevated [CO_2] (550 µmol mol^{-1}; EC) and either high nitrogen supply (HN) or limiting nitrogen supply (LN) during 2008 at SoyFACE

Date	DOY	DAE	Type	Developmental stage			
				ACHN	ACLN	ECHN	ECLN
3 July	185	29	A/c_i	Leaf 7	Leaf 6	Leaf 7	Leaf 6
11 July	193	37	Diurnal	Leaf 10	Leaf 9	Leaf 10	Leaf 9
15 July	197	41	Diurnal	Leaf 12	Leaf 10	Leaf 12	Leaf 11
22 July	204	48	A/c_i	Leaf 16	Leaf 14	Leaf 17	Leaf 15
7 August	220	64	Diurnal	Blister kernel	Blister kernel	Blister kernel	Blister kernel
14 August	227	71	A/c_i	Milky kernel	Milky kernel	Milky kernel	Milky kernel
19 August	232	76	Diurnal	Milky kernel	Milky kernel	Milky kernel	Milky kernel
27 August	240	84	A/c_i	Dough kernel	Dough kernel	Dough kernel	Dough kernel

tested independently. For *in situ* photosynthetic gas exchange, time of day (TOD) was treated as a repeated measure of time.

Results

Rainfall and soil moisture

January to July total rainfall was the second greatest in 119 years and August rainfall was the sixth lowest on record (Illinois State Water Survey, http://www.isws.illinois.edu/ atmos/statecli/cuweather/2008/aug2008.pdf). As a consequence, the seasonal course of soil volumetric moisture content ($H_2O\%_{v/v}$) was dominated by wet early-season conditions, a single extended drying event in the mid-season, and late-season rewetting of the soil (Fig. 1A–C). On day of year (DOY) 190, soil $H_2O\%_{v/v}$ at all depths was near field capacity. Significant soil drying occurred from DOY 190–240, first in shallow depths, and then also in progressively deeper soil layers. This period of soil drying corresponded with the vegetative growth of the crop and the early stages of reproductive development (see Supplementary Fig. S1 at *JXB* online). Significant rainfall between DOY 242 and 260 then returned soil $H_2O\%_{v/v}$ to field capacity during the later stages of reproductive development (Fig. 1A–C; see Supplementary Fig. S1 at *JXB* online).

At the beginning of the season there was no difference in soil $H_2O\%_{v/v}$ between any of the treatments (Fig. 1A–C). However, both CO_2 and N treatments affected the rate at which soil moisture was depleted by crop water use. Consequently, for a significant fraction of the growing season both elevated [CO_2] and LN treatments resulted in greater soil $H_2O\%_{v/v}$, when considering the soil profile as a whole (Fig. 1A–C). The nature of these treatment effects varied between soil layers. At depths of 5–25 cm, soil $H_2O\%_{v/v}$ was significantly greater in ECLN than the other three treatments (Fig. 1A). Despite this, at the peak of the drought (DOY 235–239) no further drying of the soil at depths of 5–25 cm was achieved in any treatment, suggesting that all of the accessible soil moisture had been exhausted by the plants in every treatment. In other words, a significant fraction of the root system in every treatment experienced soil water potentials near or at the permanent wilting point. At depths of 25–55 cm, the CO_2 and N treatment effects were additive. Consequently, the rank order of soil $H_2O\%_{v/v}$ in the four treatments was: ECLN>ECHN=ACLN>ACHN (Fig. 1B). At the peak of the drought (DOY 239), significant soil moisture extraction was still occurring at depths of 25–55 cm in all four treatments. At depths of 55–105 cm, only plants grown at ambient [CO_2] extracted significant soil moisture (Fig. 1C).

In situ *diurnal courses of leaf gas exchange*

Early in the growing season when soil $H_2O\%_{v/v}$ was close to field capacity (DOY 193 and 197), there were no significant differences in the diurnal course of *A* between any of the treatments (Fig. 2). However, significant effects of CO_2 and N treatments emerged over time, coincident with the
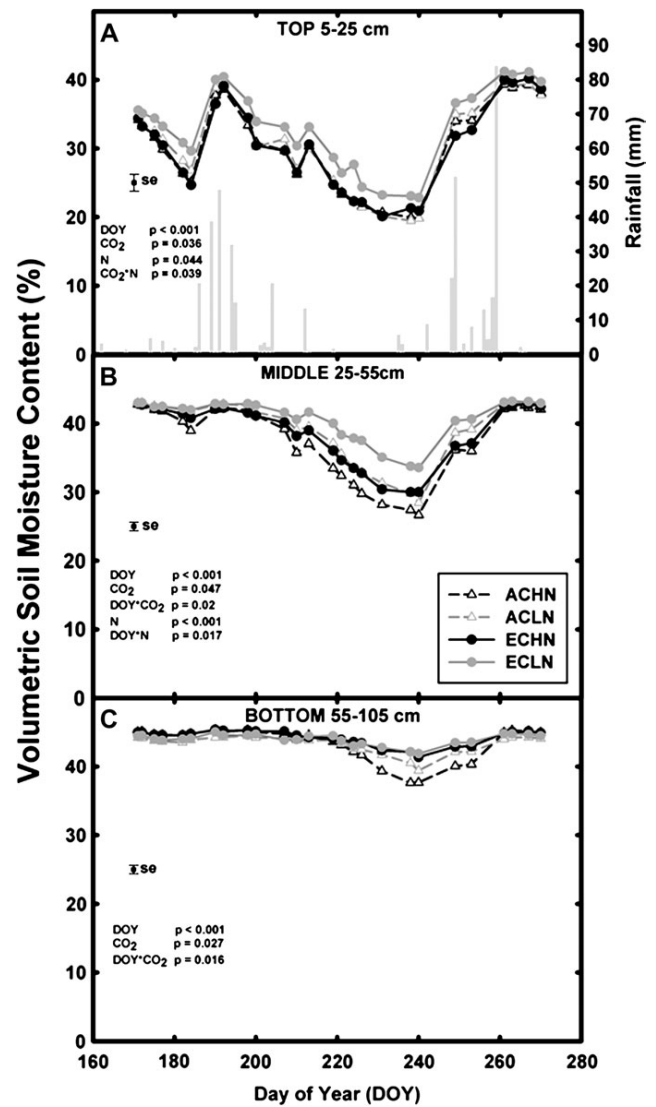

Fig. 1. Volumetric soil moisture (%) measured from depths of 5–25 cm (A, TOP), 25–55 cm (B, MIDDLE), 55–105 cm (C, BOTTOM) in plots of maize grown under ambient [CO_2] and high nitrogen (ACHN, open black triangles), ambient [CO_2] and limiting nitrogen (ACLN, open grey triangles), elevated [CO_2] and high nitrogen (ECHN, closed black circles), and elevated [CO_2] and limiting nitrogen (ECLN, closed grey circles) during the 2008 growing season at SoyFACE. Each point is the mean of the replicate plots (*n*=4) measured at that time, with the corresponding standard error calculated from the repeated measures ANOVA represented by the bars around the closed black box plotted on the lower left of each panel. Statistically significant treatment effects (*P* <0.05) are listed in each panel. Precipitation per day (mm) is shown as grey bars in (A).

progressive development of soil moisture deficits and physiological indicators of drought stress. By midway through the drought period (DOY 220) *A* had decreased in all treatments compared with earlier in the growing season. This response was ameliorated by elevated [CO_2] at both levels of N, leading to ~18% greater *A* in ECHN and ECLN when

compared with ACHN and ACLN (Fig. 2). By the time soil water deficits were greatest (DOY 232), A had been reduced by up to 44% relative to the non-drought conditions at the beginning of the growing season. The decline in A associated with increasing soil moisture deficit over time was again significantly ameliorated by elevated [CO$_2$], but now also exacerbated by LN. Consequently, the rank order of A at midday on DOY 232 in the four treatments was: ECHN>ACHN>ECLN>ACLN (Fig. 2). At this time, A was 25% greater in ECHN compared to ACHN, and A was 23% greater in ECLN compared with ACLN.

Early in the season when soil H$_2$O%$_{v/v}$ was close to field capacity (DOY 193 and 197), midday stomatal conductance (g_s) was significantly lower (–33% on average) under elevated [CO$_2$] at both levels of N supply (Fig. 2). By midway through the period of low rainfall (DOY 220) the magnitude of the CO$_2$ effect on g_s was greatly diminished (–19% on average) because g_s had decreased much more over time in ACHN and ACLN than in ECHN and ECLN. By the time soil water deficits were greatest (DOY 232), g_s

had been reduced by up to 57% relative to the non-drought conditions at the beginning of the growing season, with the drought induced reduction of g_s being significantly greater in both the ambient [CO$_2$] and LN treatments.

On all four measurement dates, elevated [CO$_2$]-grown plants maintained greater c_i values when measured in the field, regardless of N treatment (Fig. 2). During the period of most severe drought (DOY 232), LN treatments also had significantly lower c_i than HN treatments, consistent with changes in g_s.

A/c$_i$ response curves

A/c_i curves (Fig. 3) were measured in order to assess the stomatal and non-stomatal factors limiting A. Both the maximum rate of PEP carboxylation (V_{pmax}) and the [CO$_2$]-saturated rate of A (V_{max}) declined in all treatments as drought progressed over time. The decline in V_{max} from DOY 204 to 228 was 13%, on average, and there were no effects of CO$_2$ or N treatments on V_{max} on any date
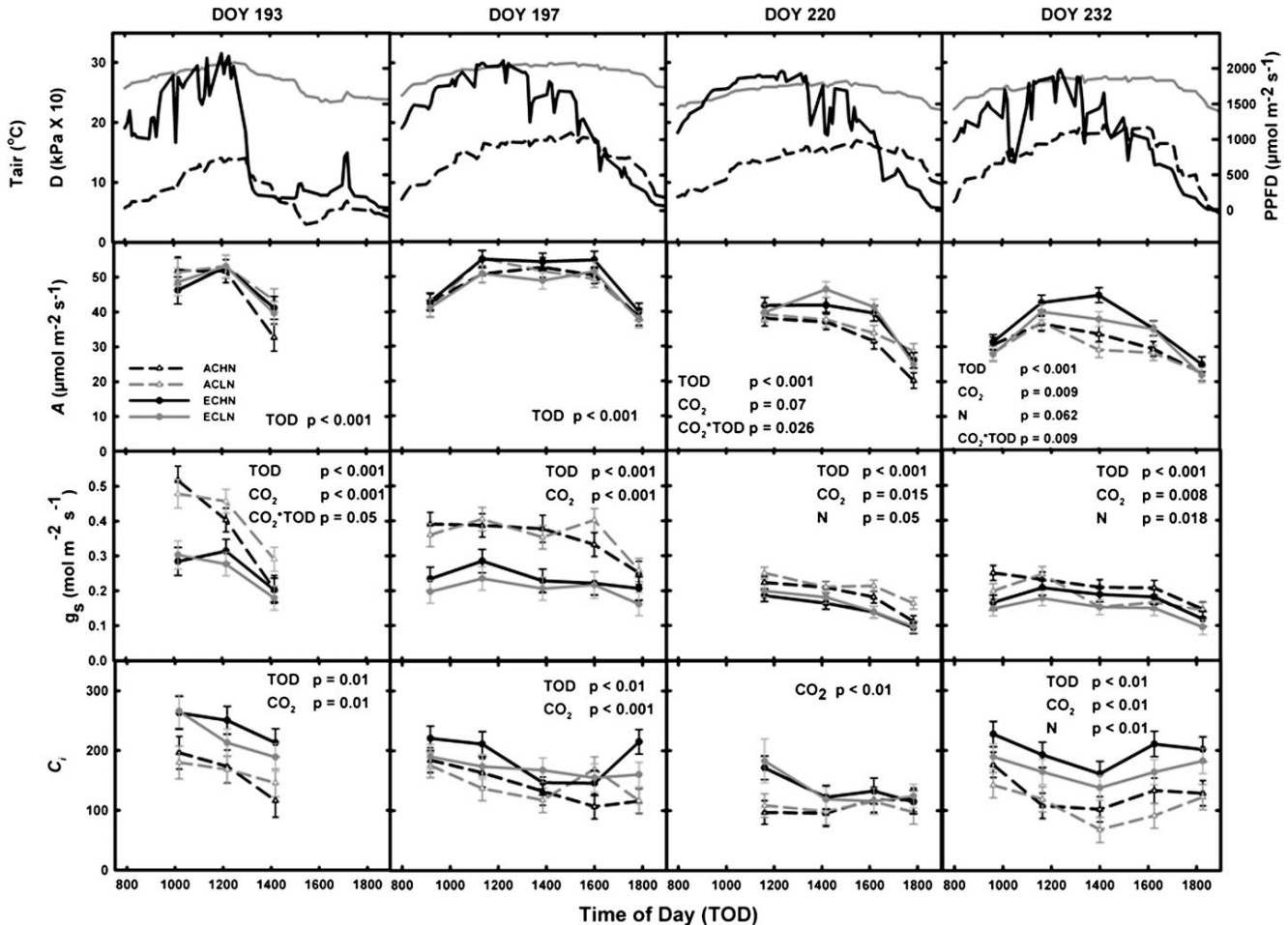

Fig. 2. *In situ* diurnal courses of T$_{air}$, vapour pressure deficit (D), PPFD, A, g_s, and c_i of the youngest fully expanded leaf of maize grown under ambient [CO$_2$] and high nitrogen (ACHN, open black triangles), ambient [CO$_2$] and limiting nitrogen (ACLN, open grey triangles), elevated [CO$_2$] and high nitrogen (ECHN, open grey triangles), and elevated [CO$_2$] and limiting nitrogen (ECLN, closed grey circles) on four dates (DOY) during the 2008 growing season at SoyFACE. Each point is the mean (±SE) of the replicate plots measured at that time point (*n*=4). Statistically significant treatment effects (*P* <0.05) are listed in each panel.

(Fig. 3A). From DOY 204 to 228, increasing drought stress resulted in V_{pmax} declining by up to 58%, with considerable variation among treatments. The non-stomatal limitation to *A* resulting from these reductions in V_{pmax} was ameliorated by elevated $[CO_2]$ and exacerbated by LN. As a result of significant, additive CO_2 and N effects on V_{pmax}, the rank order of V_{pmax} in the four treatments on DOY 228 was ECHN>ACHN=ECLN>ACLN (Fig. 3B). The first precipitation events greater than 2 mm in over 20 d occurred on

DOY 235 and 236 (Fig. 1A). Following this there were no longer any treatment effects on V_{pmax} (DOY 240).

Output from statistical analysis of *in situ* c_i and A/c_i curves for each treatment on dates representing non-drought (DOY 197 and 204) and drought conditions (DOY 228 and 232) were combined in order to estimate stomatal limitation to *A* (Fig. 4). Under non-drought conditions, there was almost no stomatal limitation to *A* (0.02–0.03) because mean c_i was at or above the inflexion point of the
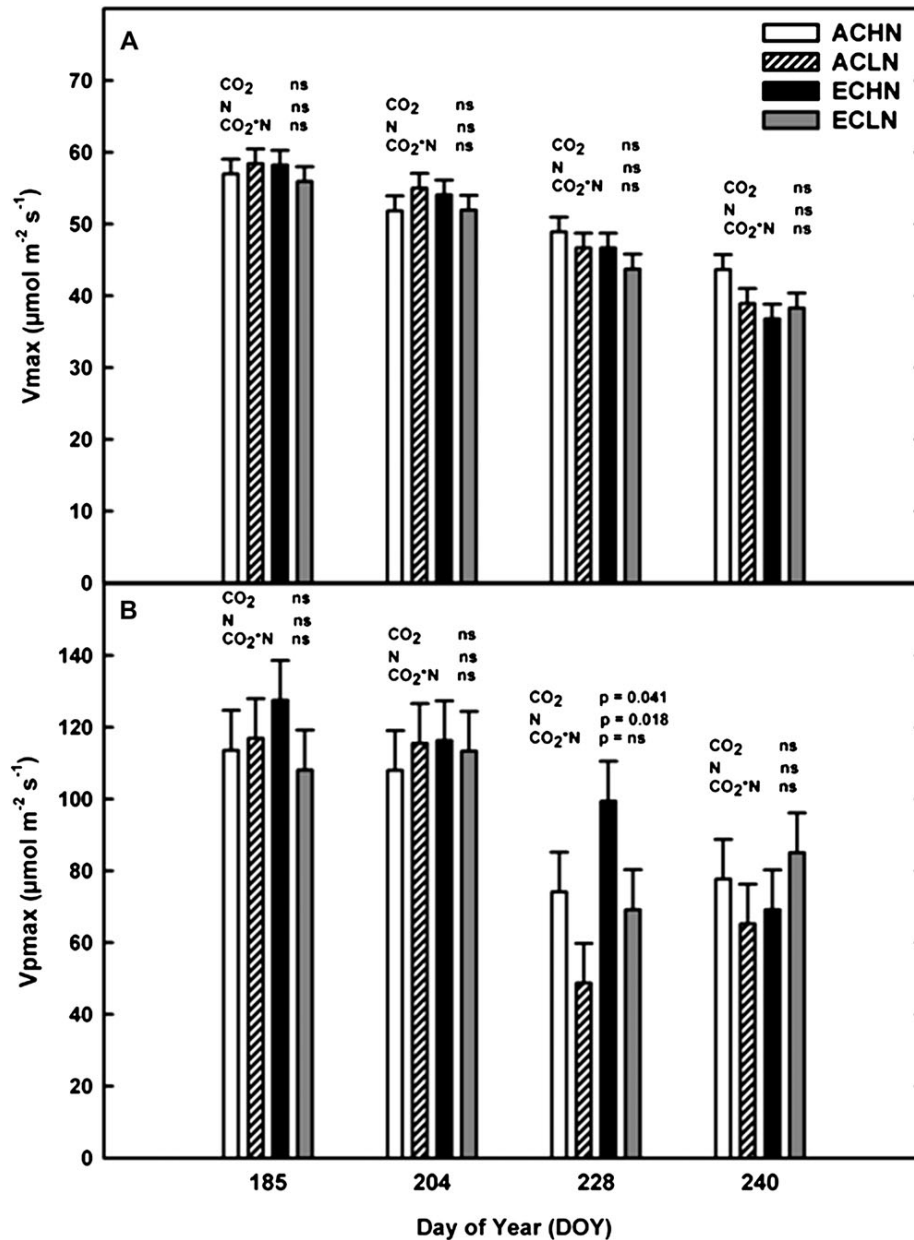

Fig. 3. (A) Asymptote of A/c_i curve (V_{max}) and (B) maximum carboxylation capacity of PEPC (V_{pmax}) of the youngest fully expanded leaf of maize grown under ambient $[CO_2]$ and high nitrogen (ACHN, white bars), ambient $[CO_2]$ and limiting nitrogen (ACLN, hatched bars), elevated $[CO_2]$ and high nitrogen (ECHN, black bars), and elevated $[CO_2]$ and limiting nitrogen (ECLN, grey bars) on four dates during the 2008 growing season at SoyFACE. Each point is the mean ($\pm$SE) of the replicate plots measured at that time point (*n*=4). The statistical significance of CO_2, N, and $CO_2\times N$ effects within each DOY are indicated (ns=not significant).

A/c_i curve. The development of significant water deficits caused stomatal limitation to A to increase many-fold in all treatments except ECHN, reaching a maximum of 0.49 in ACLN. Greater stomatal limitation under drought was ameliorated by elevated $[CO_2]$, but exacerbated by LN. As a result, the rank order of stomatal limitation to A in the four treatments under drought stress was ACLN>ACHN>ECLN>ECHN (Fig. 4).

Leaf area index, development, biomass, and yield

There were no significant effects of elevated $[CO_2]$ on leaf area index (LAI), biomass, yield, or development (Table 2; see Supplementary Fig. S1 at *JXB* online). However, peak LAI was significantly lower in LN treatments compared with HN treatments, regardless of growth $[CO_2]$ (Table 2). This was associated with the development of fewer leaves per plant under LN (see Supplementary Fig. S1 at *JXB* online). As a consequence, total biomass, stover biomass (i.e. the remaining plant biomass after the ear is removed), and kernel number at reproductive stage 6 were significantly reduced in the LN treatments, with again no significant

effect of $[CO_2]$ on growth (Table 2). Individual kernel size did not vary with either growth $[CO_2]$ or N treatment (Table 2).

Discussion

The 2008 growing season in Central Illinois featured a very wet spring followed by the sixth driest August on record. Reductions in g_s (up to 57%), A (up to 44%), and V_{pmax} (up to 58%) between DOY 193 and 232 coincided with a substantial decline in soil $H_2O\%_{v/v}$ and are consistent with the crop suffering significant physiological drought stress. Consequently, comparison of crop performance early and late in the growing season provided a rare opportunity to assess the mechanistic basis for C_4 photosynthetic responses to interactions between drought stress, N supply, and growth $[CO_2]$ under fully open-air field conditions. Consistent with previous experiments on maize at this site, there was no effect of elevated $[CO_2]$ on A under conditions of high N supply in the absence of drought (Leakey *et al.*, 2004, 2006). Contrary to our first hypothesis, limiting N
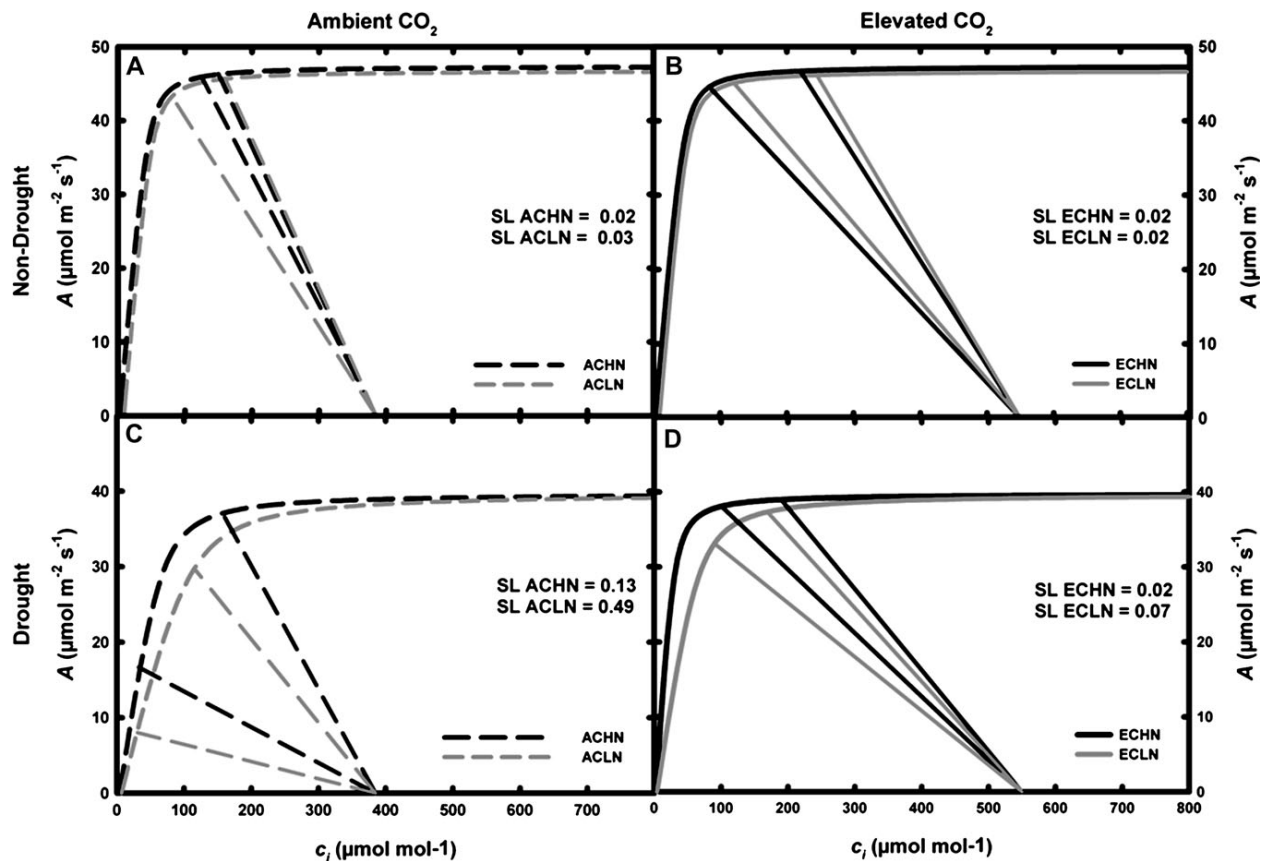
Fig. 4. Summary of A/c_i response curves and CO_2 supply functions for maize grown at ambient $[CO_2]$ (A, C, dashed lines) and elevated $[CO_2]$ (B, D, solid lines) as well as high N (black lines) and limiting N (grey lines) during non-drought conditions (A, B) or drought conditions (C, D). Where distinct, A/c_i response curves represent statistically significant treatment effects for values of V_{pmax} and V_{max} ($n=4$; see Fig. 3) under non-drought conditions (DOY 204) and drought conditions (DOY 228). Superimposed are supply functions representing the maximum and minimum c_i observed at midday in the field (see Fig. 2) under non-drought conditions (DOY 197) and drought conditions (DOY 232). Estimates of stomatal limitation (SL) using mean midday c_i in each treatment are reported in each panel.

Table 2. Final total, stover, grain biomass, kernel number, individual kernel mass, and peak leaf area index (LAI), for each of the maize plots grown under ambient [CO_2] high nitrogen (ACHN), ambient [CO_2] low nitrogen (ACLN), elevated [CO_2] high nitrogen (ECHN), and elevated [CO_2] low nitrogen (ECLN) at SoyFACE in Urbana, Illinois

Different letters indicate significant treatment differences ($P < 0.1$).

Parameter	ACHN	ACLN	ECHN	ECLN
Total biomass R6 (g plant^{-1})	256±21 a	207±21 b	268±21 a	209±21 b
Stover biomass R6 (g plant^{-1})	118±8 a	97±8 b	114±8 a	96±8 b
Grain biomass R6 (g plant^{-1})	137±11 a	110±11 b	139±14 a	114±11 b
Kernel number (plant^{-1})	515±46 a	428±46 b	574±46 a	456±46 b
Individual kernel mass (mg)	266 ±10 a	257±10 a	267±10 a	250±10 a
Peak LAI	4.3±0.2 a	3.7±0.2 b	4.5±0.21 a	3.76±0.21 b

supply did not alter leaf photosynthetic capacity and the CO_2-saturation point of *A*. Therefore, there was no stimulation of *A* by elevated [CO_2] when N was limiting and water availability was high. In accordance with our second hypothesis, elevated [CO_2] delayed and relieved both stomatal and non-stomatal limitations to *A* during periods of drought. With respect to our third hypothesis, limiting N supply exacerbated stomatal and non-stomatal limitation to *A* during drought. However, the effects of limiting N and elevated [CO_2] were additive, so the extent to which drought effects on *A* were ameliorated by elevated [CO_2] did not differ between high N and limiting N supply. These findings provide new mechanistic understanding necessary to improve model predictions of future C_4 photosynthesis, net primary productivity, and crop yield across a diverse range of growing conditions. The CO_2 effects observed during reproductive developmental stages in 2008 can be attributed to interactions with episodes of drought rather than plant developmental events because (i) no effect of elevated [CO_2] was observed at any developmental stage in the 2004 growing season that lacked any periods of drought stress (Leakey *et al.*, 2006); and (ii) in the 2002 growing season, during vegetative developmental stages, the ameliorating effects of elevated [CO_2] again coincided with periods of drought (Leakey *et al.*, 2004).

Limiting N did not make A *sensitive to elevated [CO_2] under non-drought conditions*

The interaction between N supply and elevated [CO_2] is key to the future performance of C_3 species (Stitt and Krapp, 1999; Poorter and Perez-Soba, 2001; Reich *et al.*, 2006; Rogers *et al.*, 2009), but has been largely unexplored in C_4 species. Previous experiments at SoyFACE in which unstressed maize showed no photosynthetic response to elevated [CO_2] assessed plants receiving significant fertilizer inputs (168 kg N ha^{-1}; Leakey *et al.*, 2004, 2006). Along with favourable climatic and edaphic conditions, the high rate of fertilizer application in Central Illinois results in maize yields that are amongst the greatest in the world (USDA, 2005). Using FACE technology to test the effect of elevated [CO_2]

on maize grown without fertilizer inputs resulted in an experiment with greater relevance to the limiting N supply under which C_4 crops are grown in many other regions of the world (Leakey, 2009). The photosynthetic capacity of C_4 species declines as leaf N content decreases (Ranjith *et al.*, 1995; Ghannoum and Conroy, 1998; Ghannoum *et al.*, 2005), with the potential outcome that under limiting N the CO_2-saturation point of *A* would increase. If *A* became CO_2-limited in this manner then elevated [CO_2] would stimulate carbon gain and productivity under a broader range of growing conditions, i.e. both in the presence and absence of drought stress. Diurnal courses of *in situ* leaf photosynthetic gas exchange and *A*/c_i curves measured early in the growing season (DOY 185–204) when adequate water was available to the crop revealed that, contrary to expectation, maize grown under the limiting N treatment produced leaves that were unaltered in terms of photosynthetic capacity and sensitivity to c_i. Instead of altering leaf physiological capacity, limiting N supply resulted in the production of fewer leaves, reduced LAI, biomass accumulation, and yield compared with high N treatments. This provides a mechanistic explanation for the lack of any CO_2 effect on biomass accumulation during the vegetative growth stages of maize grown in pots of sand and supplied with a range of N from 0.5 to 25 mol m^{-3} NO$_3$ (Hocking and Meyer, 1991). Together these results support the conclusion that the yield of maize, and probably other C_4 crops, will not be stimulated by rising [CO_2] this century across a wide range of soil fertility as long as they are not drought stressed.

Elevated [CO_2] delays and diminishes the stomatal and non-stomatal limitations to A *that develop during the progression into drought stress*

Drought is defined as the stage when demand for water by a plant is not matched by water supply to the plant. In this study, drought stress is considered to be any physiological impairment resulting from the plant sensing or experiencing water deficits. Over the period of soil drying where surface soil $H_2O\%_{v/v}$ declined from near field capacity (DOY 193) to near the permanent wilt point (DOY 232), there were significant reductions in g_s (up to 57%), *A* (up to 44%), and V_{pmax} (up to 58%). Many studies have reported the capacity of elevated [CO_2] to relieve drought-induced inhibition of *A*, growth, crop yield, and net primary productivity (Samarakoon and Gifford, 1996; Owensby *et al.*, 1999; Ghannoum *et al.*, 2000; Wall *et al.*, 2001; Ottman *et al.*, 2001; Leakey *et al.*, 2004; Leakey, 2009). The importance of both stomatal and non-stomatal limitations to *A* in causing reduced C_4 plant productivity under drought is also widely recognized, and has recently been comprehensively reviewed (Ghannoum, 2009). However, there is little information on the degree to which these mechanisms are engaged by drought and relieved by elevated [CO_2] under field conditions. This study provides evidence that, in the primary region of maize production, (i) lower g_s at elevated [CO_2] results in reduced water use, slower depletion of soil $H_2O\%_{v/v}$ during periods of low rainfall, and a delay in the

reduction of g_s and photosynthetic capacity by drought; and also (ii) once drought stress is experienced by the plant at elevated [CO_2], decreases in g_s and V_{pmax} do not limit A as much as at ambient [CO_2] because c_i is greater.

Early season measurements when adequate water was available to the crop (DOY 185–204) did not detect any photosynthetic response to elevated [CO_2] in plants receiving either high N or limiting N. In all treatments, *in vivo* measures of photosynthetic capacity (V_{pmax}, V_{max}) were at the upper range of those reported in the literature (von Caemmerer, 2000; Driscoll *et al.*, 2006) meaning that non-stomatal limitations to A were minimized. Likewise, g_s was high leading to c_i that were above the inflexion point of the A/c_i curve, resulting in essentially no stomatal limitation to A. During the mid-season period of low rainfall, plant water use caused soil $H_2O\%_{v/v}$ to decrease substantially, finishing near or at the permanent wilt point in shallow soil layers. The rate of soil drying was slower in elevated [CO_2] treatments, particularly in the middle (25–55 cm) and bottom (55–105 cm) soil layers. This slower soil drying at elevated [CO_2] was associated with smaller decreases in g_s, c_i, and V_{pmax} over time in elevated [CO_2] compared with ambient [CO_2] treatments. This provides evidence that elevated [CO_2] delayed drought-induced stomatal and non-stomatal limitations to A. Once drought stress was experienced by plants in elevated [CO_2] treatments, the operating c_i was maintained near or above the inflexion point of the A/c_i curve, thereby reducing the negative effects on A of drought-induced reductions in the initial slope of the A/c_i curve (V_{pmax}) and g_s. By contrast, in ambient [CO_2] treatments, drought-induced reductions in V_{pmax} and g_s resulted in c_i that was below the inflexion point of the A/c_i curve. This was the cause of the greater reductions in A under ambient [CO_2].

A comparison of the results from 2008 with data from previous growing seasons at the same field site suggests that elevated [CO_2] resulted in greater A by ameliorating episodic drought stress rather than by affecting maize physiology during specific developmental events. During the 2002 growing season, A was greater under elevated [CO_2] compared with ambient [CO_2] during drought conditions, but not when drought stress was absent (Leakey *et al.*, 2004). However, an important distinction was that, in 2002, the drought occurred during early-season, vegetative developmental phases and not during mid-season reproductive development as it did in 2008. In addition, during the drought-free growing season of 2004, there was no effect of elevated [CO_2] on A of maize at any developmental stage (Leakey *et al.*, 2006). Across all three seasons the reduction in g_s at elevated [CO_2] was greatest during non-drought periods and diminished during periods of drought, irrespective of the developmental stage at which that occurred (Leakey *et al.*, 2004, 2006). This is consistent with greater soil drying and drought sensitivity at ambient [CO_2]. Therefore, the observed changes in photosynthesis and g_s that explain the episodic treatment effects on A in 2008 are highly likely to result from progression through soil wetting and drying cycles rather than any effects on plant development or senescence.

The mechanism relieving drought stress via greater c_i is likely to be most important in situations where drought stress is prolonged. Under prolonged drought the delay in drought stress associated with lower water use and greater soil $H_2O\%_{v/v}$ at elevated [CO_2] would disappear as soil water resources in all treatments became exhausted. However, the relief of stomatal and non-stomatal limitations to A by greater c_i would remain. By contrast, the delay of drought stress will contribute more to overall amelioration of drought stress in situations featuring frequent wetting and drying cycles. Of course, at some point, drought stress will be so severe that elevated [CO_2] will not have the capacity to sustain plant performance. This threshold will likely be a key tipping point in crop responses to climate change, but remains to be determined. The slow progression into drought stress that was observed (>40 d) emphasizes the importance of soil moisture-holding capacity and a plant's capacity for proliferation of deep roots in determining the outcome of the elevated [CO_2]×drought interaction. Soils that are shallow or have a low moisture-holding capacity, as well as pot-based experimental systems, may respond very differently which is supported by modelling analysis (Weng and Luo, 2008).

Limiting N exacerbates drought inhibition of A, *but acts additively with elevated [CO_2]*

Soil $H_2O\%_{v/v}$ was greater in limiting N treatments compared with high N treatments. However, extraction of water from shallow soil layers (5–25 cm) appeared to cease in all treatments when soils were at their driest in late August. This was not because demand for water had ceased, as significant soil drying was still occurring in deeper soil layers. Rather, this suggests that limiting N supply constrained root growth and the capacity of the plants to extract all of the available water from a given volume of soil, causing greater drought stress despite smaller canopy size. This interpretation is consistent with the greater reductions in g_s, c_i, V_{pmax}, and A observed in limiting N treatments compared with high N treatments over the time that significant soil water deficits were developing. This type of interaction between N supply and drought stress may be favoured in deep, high moisture-holding soils such as the mid-west US where deeper root growth can provide access to otherwise unused water resources. However, in conditions of lower soil water storage the outcome of the interaction might be reversed and drought stress will be more prevalent in productive genotypes or higher fertility conditions where shallow water resources can be exhausted more rapidly without the possibility of finding additional water deeper in the soil. This is consistent with Ghannoum and Conroy (1998) who observed greater A and biomass accumulation of *Panicum coloratum* and *P. antidotale* in response to elevated [CO_2] when grown at high N, but not under low N. In that study, plants grown at high N and ambient [CO_2] had c_i/c_a (~0.25) that was lower than is typical for unstressed C_4 species (~0.40). The enhancement of A by elevated [CO_2] at high N may, therefore, have been driven by the amelioration of unintended drought stress caused by the high demand for

water of pot-grown plants that were more than five times larger under high N than low N.

While limiting N exacerbated the impairment of physiological functions observed over the period of increasing soil water deficits, the effects of N supply and growth $[CO_2]$ were additive. In other words, the extent to which elevated $[CO_2]$ ameliorated drought stress did not vary with N supply. In combination with the finding that limiting N did not make A sensitive to elevated $[CO_2]$ under non-drought conditions, this suggests that the nature of photosynthetic responses to elevated $[CO_2]$ in maize should be consistent across a broad range of N supply. This study also adds to the evidence that elevated $[CO_2]$ effects on A in C_4 species are strongly dependent on plant water status. In 2008, the amelioration of drought stress by elevated $[CO_2]$ resulted in up to 25% greater rates of A. By comparison, in 2002, A was up to 41% greater under elevated $[CO_2]$ than under ambient $[CO_2]$ during a period of early season drought stress (Leakey *et al.*, 2004). The greater impact of the drought on plant water status under ambient $[CO_2]$ in 2002 was apparent from observations of leaf curling (Leakey *et al.*, 2004), which did not occur in 2008. This may simply reflect a stronger drought in 2002 (minimum Palmer Crop Moisture Index= –1.19) versus 2008 (minimum Palmer Crop Moisture Index= –0.34), but could also be related to the reduced capacity of the root system early in the season to access deeper soil water.

The high water-holding capacity of the deep soils at the SoyFACE site and moderate temperatures in August 2008 meant that, although rainfall was substantially below average for >40 d, maize yield was not significantly reduced relative to favourable growing seasons (Leakey *et al.*, 2006). Consequently, while this experiment revealed the mechanisms by which elevated $[CO_2]$ ameliorated the drought-induced inhibition of A by stomatal and non-stomatal factors, the stress relief was not sufficiently sustained to result in significantly greater biomass accumulation or yield. It is important that yield at a site in the world's primary region of maize production was not enhanced by elevated $[CO_2]$ in a year with a drought episode of moderate duration and intensity. Combined with no benefit of elevated $[CO_2]$ in years lacking drought stress (Leakey *et al.*, 2006), this contrasts significantly with the assumption in current models of future food supply that maize photosynthesis and yield will be consistently enhanced by elevated $[CO_2]$ (see details in Tubiello *et al.*, 2007*a*, *b*). Nevertheless, considerable uncertainty about the impact of global environmental change impacts on ecosystem goods and services from agricultural and natural ecosystems dominated by C_4 species will remain until C_4 species responses to elevated $[CO_2]$ are examined across a much broader range of hydrological conditions than has been done to date.

Conclusion

This study revealed that elevated $[CO_2]$ primarily exerts its effects on C_4 photosynthesis of maize by modulating how drought causes stomatal and non-stomatal limitations to A. Elevated $[CO_2]$ delayed drought-induced reductions in g_s and V_{pmax} that inhibit A, while also relieving inhibition of A via greater c_i once drought stress induced reductions in g_s and V_{pmax}. Limiting N exacerbated drought stress, but the degree to which drought stress was ameliorated by elevated $[CO_2]$ did not differ between conditions of high N and limiting N supply. While elevated $[CO_2]$ ameliorated inhibition of leaf-level photosynthetic carbon gain by drought, the effect was insufficient to drive any CO_2 effect on grain yield of maize under either high N or limiting N supply. This means that even accounting for moderate variations in soil fertility and drought stress, elevated $[CO_2]$ appears not to enhance the yield of maize in its primary growing region. Further studies are needed to determine whether the CO_2-response mechanisms characterized here can relieve stress sufficiently to sustain yields of C_4 crops in times or places of severe drought.

Supplementary data

Supplemetary data can be found at *JXB* online.

Supplementary Fig. S1. The progression of vegetative and reproductive development for maize grown at either ambient or elevated $[CO_2]$ and either high or limiting N availability during the 2008 growing season at SoyFACE.

Acknowledgements

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