Photosynthesis, Productivity, and Yield of Maize Are Not Affected by Open-Air Elevation of CO_2 Concentration in the Absence of Drought[1][OA]

Andrew D.B. Leakey*, Martin Uribelarrea, Elizabeth A. Ainsworth, Shawna L. Naidu, Alistair Rogers, Donald R. Ort, and Stephen P. Long

Institute for Genomic Biology (A.D.B.L., E.A.A., D.R.O., S.P.L.), Department of Plant Biology (A.D.B.L., E.A.A., D.R.O., S.P.L.), and Department of Crop Sciences (M.U., S.L.N., A.R., S.P.L.), University of Illinois at Urbana-Champaign, Urbana, Illinois 61801; Photosynthesis Research Unit, United States Department of Agriculture Agricultural Research Service, Urbana, Illinois 61801 (E.A.A., D.R.O.); and Environmental Sciences Department, Brookhaven National Laboratory, Upton, New York 11973 (A.R.)

While increasing temperatures and altered soil moisture arising from climate change in the next 50 years are projected to decrease yield of food crops, elevated CO_2 concentration ($[CO_2]$) is predicted to enhance yield and offset these detrimental factors. However, C_4 photosynthesis is usually saturated at current $[CO_2]$ and theoretically should not be stimulated under elevated $[CO_2]$. Nevertheless, some controlled environment studies have reported direct stimulation of C_4 photosynthesis and productivity, as well as physiological acclimation, under elevated $[CO_2]$. To test if these effects occur in the open air and within the Corn Belt, maize (Zea mays) was grown in ambient $[CO_2]$ (376 μmol mol^{-1}) and elevated $[CO_2]$ (550 μmol mol^{-1}) using Free-Air Concentration Enrichment technology. The 2004 season had ideal growing conditions in which the crop did not experience water stress. In the absence of water stress, growth at elevated $[CO_2]$ did not stimulate photosynthesis, biomass, or yield. Nor was there any CO_2 effect on the activity of key photosynthetic enzymes, or metabolic markers of carbon and nitrogen status. Stomatal conductance was lower (-34%) and soil moisture was higher (up to 31%), consistent with reduced crop water use. The results provide unique field evidence that photosynthesis and production of maize may be unaffected by rising $[CO_2]$ in the absence of drought. This suggests that rising $[CO_2]$ may not provide the full dividend to North American maize production anticipated in projections of future global food supply.

Global climate change, in the form of rising temperature and altered soil moisture, is projected to decrease the yield of food crops over the next 50 years (Thomson et al., 2005). Meanwhile, the simultaneous increase in CO_2 concentration ($[CO_2]$) is predicted to stimulate crop production and offset these detrimental components of climate change (Thomson et al., 2005). This encouraging projection results from species-specific "$[CO_2]$ fertilization" factors in yield models (Phillips et al., 1996; Brown and Rosenberg, 1999; Parry et al., 2004; Thomson et al., 2005). These simulate the enhancements of net CO_2 assimilation rate (A) and yield observed, for both C_3 (17%–29%) and C_4 crops (6%–10%), under elevated $[CO_2]$ in controlled environment studies (Kimball, 1983; Allen et al., 1987).

While early projections of "$[CO_2]$ fertilization" were based on studies in glasshouses and other protected environments, Free-Air Concentration Enrichment (FACE) experiments are fully open-air trials of crop performance. They provide realistic simulations of future growing conditions and provide perhaps the best opportunity to requantify CO_2 fertilization effects and elucidate the mechanism of crop response. FACE experiments on the C_3 crops rice (Oryza sativa), wheat (Triticum aestivum), and soybean (Glycine max) have observed smaller increases in yield than were predicted from the early chamber studies (Ainsworth and Long, 2005; Long et al., 2005; Morgan et al., 2005). Yet the primary response mechanisms of C_3 crops have not been controversial (Ainsworth and Long, 2005). First, elevated $[CO_2]$ directly stimulates A, growth, and yield by decreasing photorespiration and accelerating carboxylation by Rubisco. Second, it decreases stomatal aperture, which can reduce plant water use and indirectly enhance performance by ameliorating water stress. In contrast, the response of C_4 crops to future elevated $[CO_2]$ is uncertain. In C_4 plants, Rubisco is

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Andrew D.B. Leakey (leakey@life.uiuc.edu).

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localized in the bundle sheath cell chloroplasts, where $[CO_2]$ is 3 to 6 times higher than in the atmosphere (He and Edwards, 1996; Kiirats et al., 2002; von Caemmerer and Furbank, 2003). Thus, C_4 crops avoid photorespiration, are CO_2 saturated at the current atmospheric $[CO_2]$, and should not theoretically display greater A at elevated $[CO_2]$. Yield improvements could still be achieved under elevated $[CO_2]$ if reduced stomatal conductance (g_s) lowers crop water use and ameliorates short-term drought stress by conserving soil moisture (Ghannoum et al., 2000). At elevated $[CO_2]$, g_s of C_4 plants is typically reduced (Ainsworth and Long, 2005). However, lower g_s does not guarantee lower water use in the field, where canopy size, structure, and microclimate also regulate water use (Collatz et al., 1991; Meinzer et al., 1997). Also, lower water use will not typically benefit a crop when sufficient soil moisture is available. Yet current projections of grain production assume a stimulation of maize (*Zea mays*) production by elevated $[CO_2]$ in all situations (Phillips et al., 1996; Brown and Rosenberg, 1999; Parry et al., 2004; Thomson et al., 2005). The need for accuracy in these projections is significant because global demand for the C_4 crop maize is expected to exceed that for wheat and rice by 2020, making it the world's most important crop (Pingali, 2001).

Some controlled environment studies of well-watered plants suggest that growth at elevated $[CO_2]$ can directly impact C_4 photosynthesis by a number of mechanisms (for review, see Ghannoum et al., 2000). As examples, intercellular $[CO_2]$ (c_i) below the saturation point of the photosynthetic intercellular CO_2 response (A/c_i) curve has been reported under ambient $[CO_2]$, allowing direct stimulation of photosynthesis under elevated $[CO_2]$ (Wong, 1979; Watling and Press, 1997; Ziska and Bunce, 1997). Bundle sheath leakiness increased under elevated $[CO_2]$, reducing the initial slope and CO_2-saturated photosynthetic rate of the A/c_i curve in sorghum (*Sorghum bicolor*; Watling et al., 2000). In developing *Flaveria trinervia* leaves, 10% of CO_2 fixation occurred directly in the bundle sheath, without involvement of the C_4 concentrating mechanism, allowing the possibility that elevated $[CO_2]$ could directly stimulate photosynthesis (Moore et al., 1986). Some immature C_4 leaves have C_3-like photosynthesis and are therefore more sensitive to enhanced photosynthesis under elevated $[CO_2]$ (Dai et al., 1995; Ziska et al., 1999). Enzymes of both the C_4 cycle and Calvin cycle in maize were consistently lower under elevated $[CO_2]$, with malate dehydrogenase (-37%) and glyceraldehyde-3-phosphate dehydrogenase activities (-29%) declining to the greatest extent in young leaves (Maroco et al., 1999). Such acclimation was interpreted to potentially benefit maize growth by improving nitrogen (N) use efficiency while maintaining rates of photosynthesis. Of these studies, those investigating crop species at elevated $[CO_2]$ predicted for 2050 to 2100 reported that the light-saturated rate of photosynthesis (A_{sat}) was stimulated by an average of 23%. Such direct effects of CO_2 fertilization on photosynthesis

suggest a more optimistic future for food production from C_4 crops in the face of increasing temperatures and water stress. Conversely, theoretical treatment of C_4 photosynthesis suggested that differences in either leakiness or direct CO_2 fixation are unlikely to play a significant role in the responsiveness of C_4 photosynthesis to high CO_2 (Ghannoum et al., 2000). Also, young C_4 leaves in *Panicum antidotale* and *Panicum coloratum* are not C_3-like (Ghannoum et al., 1998). Unfortunately, growth in chambers and the confinement of the rooting system to pots could generate substantial and surprising artifacts, and responses might not therefore reflect the response of crops in open-air field situations (Arp, 1991; Thomas and Strain, 1991; McLeod and Long, 1999; Ainsworth et al., 2002). Sorghum grown under FACE in Arizona displayed some sensitivity to $[CO_2]$ in young, C_3-like leaves (Cousins et al., 2001). However, the primary effect of elevated $[CO_2]$ on sorghum performance was reported to be improved water relations and amelioration of drought stress (Wall et al., 2001). At SoyFACE in 2002, A of maize under field conditions was episodically stimulated under elevated $[CO_2]$ (Leakey et al., 2004). While CO_2 effects on photosynthesis were limited to periods of low rainfall, the mechanistic basis for the episodic response was not demonstrated.

The North American Corn Belt is the largest single area of global maize production and is characterized by high growing season rainfall and deep fertile soils capable of substantial water storage. This region accounted for more than 40% of the world's total maize grain production in 2004 (U.S. Department of Agriculture, 2005). The extent of any direct or indirect stimulation of A, growth, and yield of maize in this region by elevated $[CO_2]$ has major economic and social implications. The 32-ha FACE facility at the University of Illinois, and an absence of even intermittent water stress in 2004, provided a unique opportunity to test the following three predictions concerning the effects of elevated $[CO_2]$ on C_4 plants, and maize specifically, under field conditions. In the absence of water stress there is (1) no direct effect of $[CO_2]$ on photosynthetic rate, growth, or yield; (2) no $[CO_2]$ effect on the development of photosynthetic capacity, as reflected by in vivo and in vitro activities of the key enzymes; and (3) a decrease in g_s and water use under elevated $[CO_2]$. This builds upon previous studies to provide a novel mechanistic understanding of the responses to elevated $[CO_2]$ of a major food crop, in the major region of production. Most importantly, changes in crop water use under elevated $[CO_2]$ are quantified while also testing for direct effects of elevated $[CO_2]$ on C_4 photosynthesis in the absence of water stress.

RESULTS

Palmer Crop Moisture Index and Microclimate

Total rainfall in June, July, and August of 2004 was 347 mm, 11% above the average for the past 50 years of

312 mm. Palmer Crop Moisture Index (PCMI) is a dynamic, meteorological estimate of short-term moisture conditions, based on temperature, precipitation, and modeled soil water content (Palmer, 1968). As PCMI decreases below zero, it indicates progressively greater drought stress conditions. Throughout the 2004 growing season, the PCMI for East Central Illinois was greater than zero (Fig. 1; National Oceanic and Atmospheric Administration [NOAA]/U.S. Department of Agriculture [USDA]; http://www.usda.gov/oce/waob/jawf/). Conditions were rated as favorable for normal growth and field work; moisture adequate for present crop needs for 13 out of the 19 weeks in the growing season, with the remaining weeks rated as some fields too wet; prospects above normal. In other words, 2004 was an ideal growing season in which the crop did not experience drought stress at any time. For comparison, PCMI was often less than zero during a previous experiment at the same site in 2002 (Fig. 1), which indicates that the crop experienced drought stress even though growing season rainfall was also close to average at 321 mm. It is rare in East Central Illinois to have a growing season without any drought stress, in other words, when PCMI is always greater than or equal to zero (Fig. 2). Conditions that were that favorable have only occurred three times since 1973, including 2004 (Fig. 2). However, moderate, episodic drought stress such as in 2002 occurs frequently, with PCMI ≤ -1, occurring roughly one in every three growing seasons.

In situ physiological performance was assessed on five dates, corresponding to five discrete and key stages of crop development (Table I). Conditions were predominantly clear and dry on each day except day of year (DOY) 173, when heavy cloud cover and rain affected measurements (Fig. 3). Daily peak values of photosynthetic photon flux density (PPFD; approximately 1,250–2,000 μmol mol^{-1}) covered the range
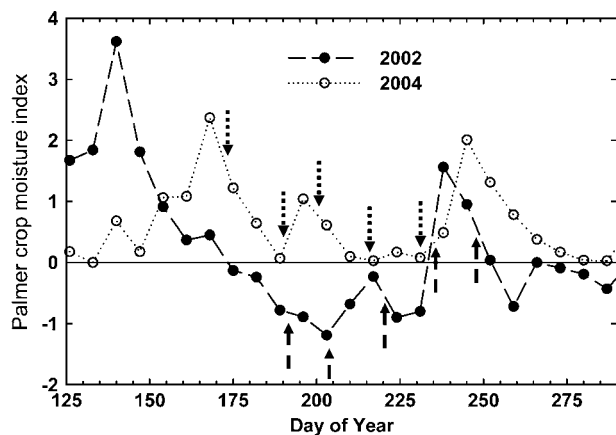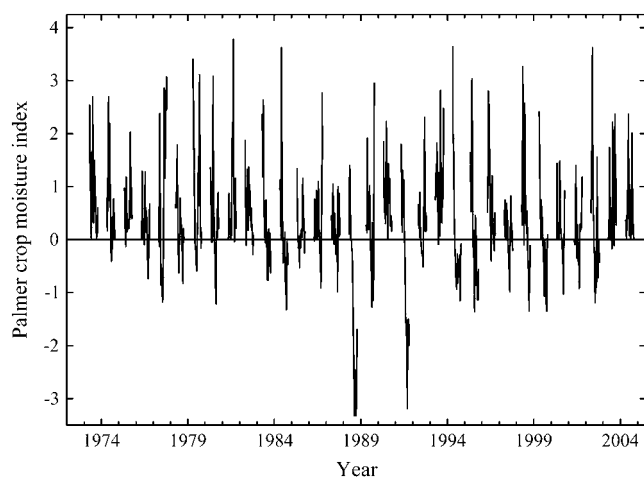

Figure 2. Weekly reported PCMI between mid-March and October each year from 1973 to 2004 for Illinois Climate Division 5 (including SoyFACE) by the Climate Operation Branch of the NOAA (http://www.usda.gov/oce/waob/jawf/).

typically experienced in the Midwest United States. The daily mean temperatures (17°C–23°C) were at or slightly below the 40-year average for summer months of 23°C (http://www.sws.uiuc.edu/data/climatedb/).

Diurnal Courses of Leaf Gas Exchange and Chlorophyll Fluorescence

There was no significant effect of CO_2 treatment on A at any time on any day (Fig. 3). This lack of difference applied to all photosynthetic parameters measured, including A, quantum yield of photosynthesis (Φ_{CO2}), quantum yield of PSII (Φ_{PSII}), proportion of open PSII reaction centers (qP), intrinsic efficiency of PSII (F_v'/F_m'), and nonphotochemical quenching (NPQ; Figs. 3 and 4). On all dates of measurement and for all photosynthetic parameters investigated, the probability of supporting the null hypothesis was high. As this coincided with relatively small standard errors, the absence of significance is not likely to be the result of high variability leading to a Type II error, but rather because there was no difference. A power test indicated that, with this data set, there was an 88% probability of detecting a 10% stimulation of A by elevated [CO_2], even with the Type I error rate of $P = 0.05$. Under elevated [CO_2], g_s was 29% lower across the growing season (Fig. 3) and significant for all or part of each day.

Leaf Midday Gas Exchange; Leaf Photosynthetic Enzyme Activities; and Leaf Carbohydrate, Protein, Amino Acid, Chlorophyll, Specific Leaf Area, Nitrogen, and Water Status

To investigate the basis for any photosynthetic enhancement or acclimation under elevated [CO_2], photosynthetic enzyme activities, leaf metabolite pools, and water status were measured at midday alongside

Figure 1. PCMI reported weekly during 2002 (●) and 2004 (○) for Illinois Climate Division 5 by the Climate Operation Branch of NOAA (http://www.usda.gov/oce/waob/jawf/). Dates on which diurnal courses of gas exchange were measured are indicated by dashed arrows for 2002 and dotted arrows for 2004.

Table I. *Calendar and DOY of experimental measurements with corresponding crop growth stage described as days after emergence (DAE), developmental stage (defined in Ritchie et al., 1993), and height for maize grown under ambient (370 $\mu mol\ mol^{-1}$) and elevated [CO_2] (550 $\mu mol\ mol^{-1}$) during 2004 at SoyFACE, Urbana, IL*

Date	DOY	DAE	Developmental Stage	
			Ambient [CO_2]	Elevated [CO_2]
June 21	173	43	Tenth leaf	Tenth leaf
July 8	190	60	Silking	Silking
July 19	201	71	Blister kernel	Blister kernel
August 2	215	85	Milky kernel	Milky kernel
August 16	229	89	Dented kernel	Dented kernel

gas exchange (Table II). There was no significant [CO_2] effect on *A* at midday across the growing season. Nor was there any significant effect of growth at elevated [CO_2] on the activity of the key photosynthetic enzymes phospho*enol*pyruvate (PEP) carboxylase (PEPc), pyruvate orthophosphate dikinase (PPDK), or Rubisco, measured at 25°C. In contrast, g_s at midday was significantly lower at elevated [CO_2], by 34% on average across the season. Therefore, leaf-level transpiration (*E*) at midday was also significantly lower at elevated [CO_2]. Midday c_i at elevated [CO_2] was significantly greater, by 34% on average across the season. Consequently, there was no significant effect of growth at elevated [CO_2] on the ratio of intercellular [CO_2] to atmospheric [CO_2] (c_i/c_a) at midday. There was no significant effect of growth at elevated [CO_2] on the

midday leaf content of total nonstructural carbohydrates (TNC; Table II) or its component pools of starch, Suc, Fru, and Glc (data not shown). There was also no significant effect of growth at elevated [CO_2] on the midday leaf content of total protein, total free-amino acids, leaf N, or specific leaf area (SLA). Nor was there a significant [CO_2] effect on leaf water status at midday, measured as relative water content (RWC) and total leaf water potential (ψ_{leaf}).

A/c_i and Light Response Curves

Leaves cut predawn, maintained hydrated and measured at 30°C in the laboratory, had rates of *A* equal or higher to fluxes measured in situ, suggesting that the photosynthetic capacity of these leaves was unaffected by this short-term detachment. The A/c_i and light response (*A/Q*) curves (Fig. 5) showed the classical C$_4$ patterns, and the parameter values were close to theoretical expectations (von Caemmerer, 2000). There was no significant effect of growth at elevated [CO_2] on maximum apparent rate of PEPc (V_{pmax}), CO_2-saturated rate of photosynthesis (V_{pr}), A_{sat}, or maximum apparent quantum yield as determined from these response curves.

Crop Biomass, Development, and Yield

There was no significant effect of growth at elevated [CO_2] on stover biomass, grain biomass, kernel number, individual kernel weight, total leaf area, anthesis

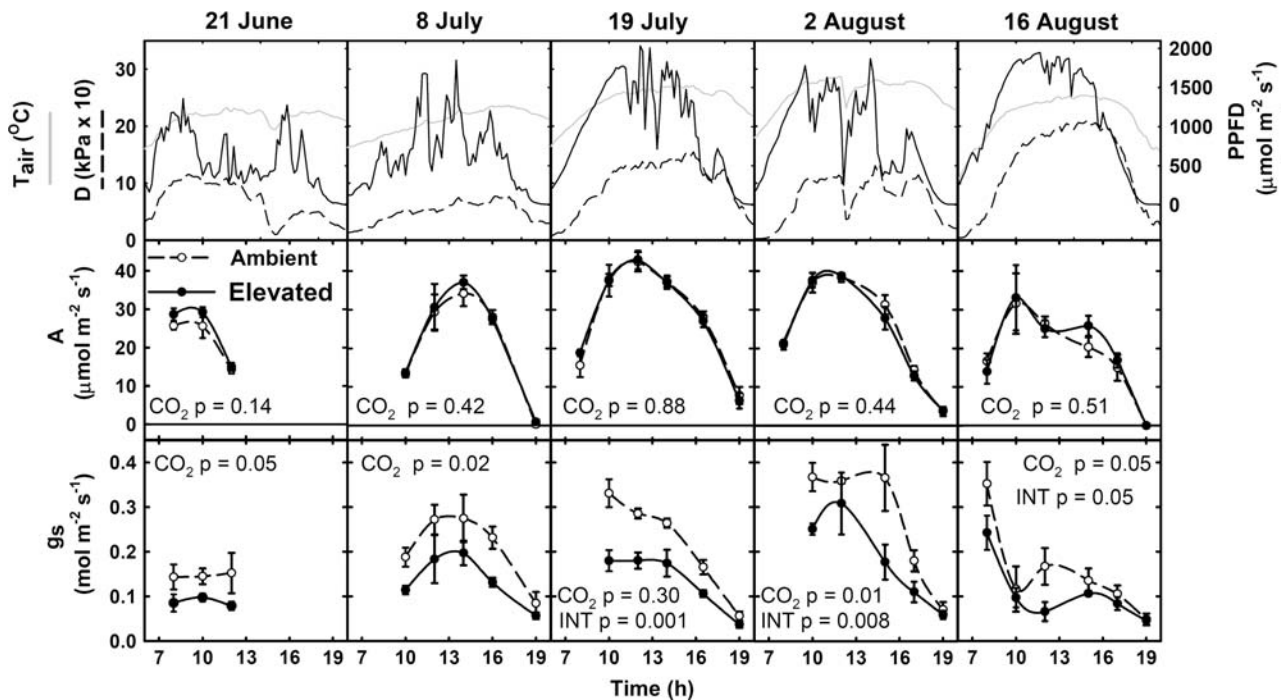**Figure 3.** Diurnal courses of PPFD, T_{air}, vapor pressure deficit (*D*), *A*, and g_s of the youngest and uppermost fully expanded leaf of maize grown under ambient (○) and elevated CO_2 (●) on five dates during 2004 at SoyFACE. Each point is the mean (±SE) of the replicate plots measured at that time (*n* = 4). *P* values indicate statistical significance of CO_2 and CO_2 × time interaction effects.

Figure 4. Diurnal courses of Φ_{CO2}, Φ_{PSII}, F_v'/F_m', qP, and NPQ of the youngest fully expanded leaf of maize grown under ambient (O) and elevated CO_2 (●) on five dates during 2004 at SoyFACE, Urbana, IL. Each point is the mean (±SE) of the replicate plots measured at that time (n = 4). P values indicate statistical significance of CO_2 and CO_2 × time interaction effects.
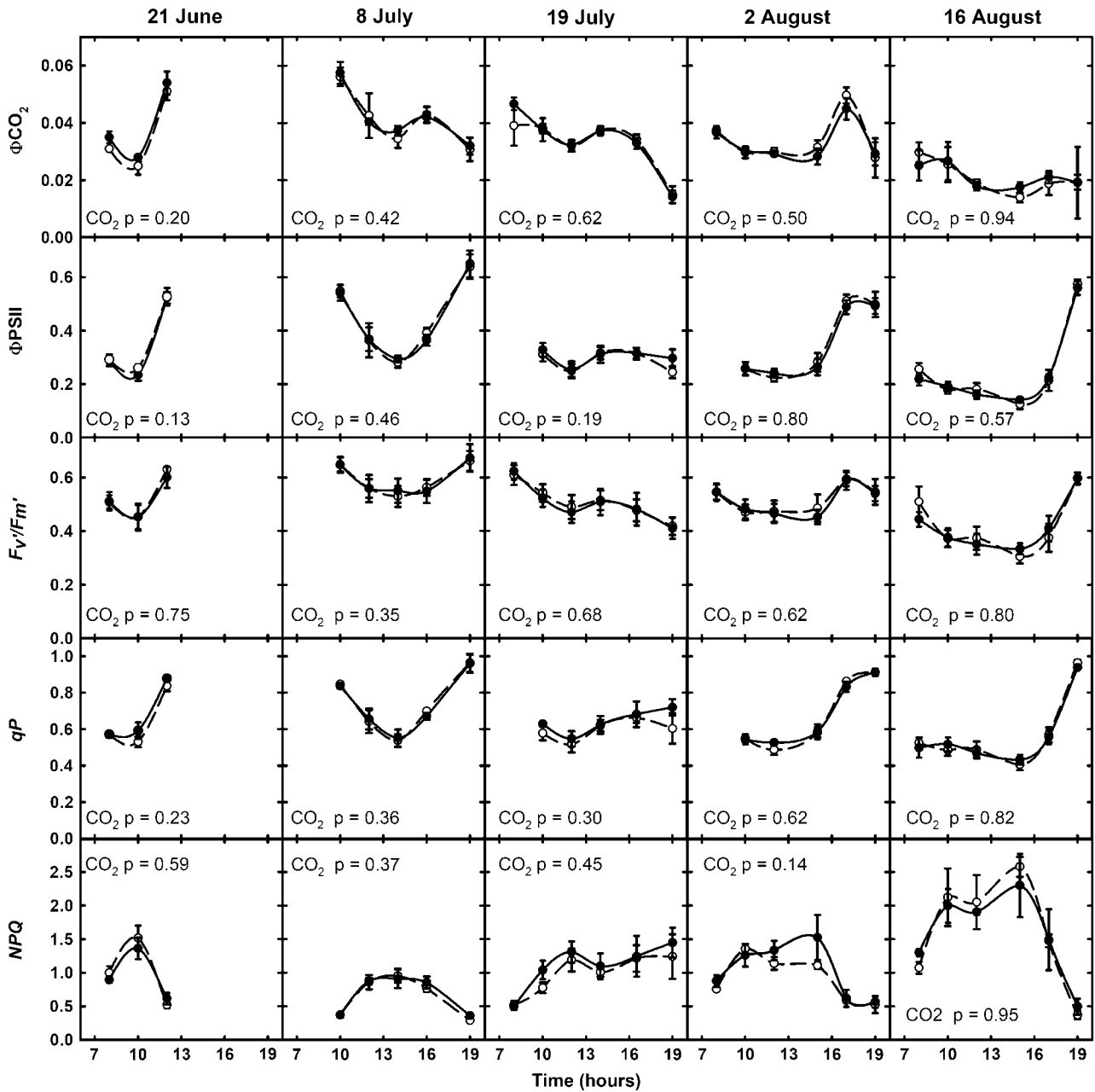
date, or silking date (Table III). The yields of approximately 10.5 t (seed) ha^{-1} and approximately 20.3 t (total biomass) ha^{-1} are among the higher yields for the Corn Belt, showing that the crop was representative of current agriculture.

Soil Water Content

There was no difference in volumetric soil water content ($H_2O\%$) between treatments at the beginning of the season (Fig. 6, A and B). Over the growing season, $H_2O\%$ decreased due to crop water use but was regularly replenished by rain. The ratio of $H_2O\%$ in elevated [CO_2] compared to ambient [CO_2] plots gradually increased to reach 1.31 between 5 and 25 cm depth on DOY 215, and 1.11 between 25 and 55 cm depth on DOY 223 (Fig. 6C). This greater $H_2O\%$ under elevated [CO_2] reflected a significant interaction between the [CO_2] and time in both the upper and lower soil layers.

Table II. A ($\mu mol\ m^{-2}\ s^{-1}$), PEPc activity ($\mu mol\ m^{-2}\ s^{-1}$), PPDK activity ($\mu mol\ m^{-2}\ s^{-1}$), Rubisco activity ($\mu mol\ m^{-2}\ s^{-1}$), g_s ($mol\ m^{-2}\ s^{-1}$), transpiration (T; $mmol\ m^{-2}\ s^{-1}$), c_i ($\mu mol\ mol^{-1}$), ratio of intercellular to atmospheric CO_2 (c_i/c_a), TNC ($mmol\ m^{-2}$), total protein content ($g\ m^{-2}$), RWC (%), total water potential (ψ; MPa), N (%) content, and SLA ($mm^2\ mg^{-1}$) of the youngest fully expanded leaf of maize grown under ambient (370 $\mu mol\ mol^{-1}$) and elevated $[CO_2]$ (550 $\mu mol\ mol^{-1}$) at noon on five dates in 2004 at SoyFACE, Urbana, IL

There was no effect of CO_2 on A ($P = 0.81$), PEPc activity ($P = 0.26$), PPDK activity ($P = 0.76$), Rubisco activity ($P = 0.59$), c_i/c_a ($P = 0.52$), TNC ($P = 0.25$), protein ($P = 0.80$), RWC ($P = 0.60$), total ψ ($P = 0.86$), N ($P = 0.21$), and SLA ($P = 0.48$). Asterisk (*), There was a significant CO_2 effect on g_s ($P = 0.037$), T ($P = 0.049$), and c_i ($P = 0.05$).

Parameter	DOY 173 $[CO_2]$ 370	DOY 173 $[CO_2]$ 550	DOY 190 $[CO_2]$ 370	DOY 190 $[CO_2]$ 550	DOY 201 $[CO_2]$ 370	DOY 201 $[CO_2]$ 550	DOY 215 $[CO_2]$ 370	DOY 215 $[CO_2]$ 550	DOY 229 $[CO_2]$ 370	DOY 229 $[CO_2]$ 550
A	14.3 ± 2.8	15.0 ± 2.8	29.4 ± 2.8	30.6 ± 2.8	42.4 ± 2.8	42.9 ± 2.8	38.4 ± 2.8	38.8 ± 2.8	26.4 ± 2.8	25.1 ± 2.8
PEPc	222 ± 39	213 ± 18	259 ± 32	248 ± 13	283 ± 19	243 ± 27	230 ± 12	222 ± 18	152 ± 19	174 ± 19
PPDK	32.5 ± 1.7	32.4 ± 5.0	33.0 ± 2.0	34.5 ± 3.3	39.1 ± 2.7	37.9 ± 1.1	25.0 ± 3.0	27.6 ± 2.2	37.6 ± 1.9	32.8 ± 2.6
Rubisco	21.1 ± 3.1	17.9 ± 1.8	17.7 ± 3.1	19.2 ± 2.9	22.4 ± 2.3	18.9 ± 1.2	18.0 ± 2.2	19.6 ± 1.8	13.2 ± 1.0	12.9 ± 1.0
g_s*	0.15 ± 0.04	0.08 ± 0.04	0.27 ± 0.04	0.18 ± 0.04	0.29 ± 0.04	0.18 ± 0.04	0.36 ± 0.04	0.31 ± 0.04	0.17 ± 0.04	0.07 ± 0.04
T*	1.6 ± 0.6	0.9 ± 0.6	2.9 ± 0.6	2.2 ± 0.6	5.7 ± 0.6	3.9 ± 0.6	4.8 ± 0.6	3.6 ± 0.6	4.7 ± 0.6	1.9 ± 0.6
c_i*	167 ± 31	226 ± 31	187 ± 31	241 ± 31	118 ± 31	141 ± 31	187 ± 31	292 ± 31	126 ± 31	154 ± 31
c_i/c_a	0.45 ± 0.13	0.41 ± 0.13	0.49 ± 0.13	0.43 ± 0.13	0.31 ± 0.13	0.25 ± 0.13	0.50 ± 0.13	0.52 ± 0.13	0.33 ± 0.13	0.19 ± 0.13
TNC	14.2 ± 1.1	13.0 ± 0.9	11.4 ± 1.1	11.9 ± 0.9	18.3 ± 0.9	18.1 ± 0.9	20.1 ± 0.9	17.7 ± 0.9	20.4 ± 0.9	18.4 ± 1.1
Protein	10 ± 1	10 ± 1	11 ± 1	12 ± 2	12 ± 1	12 ± 1	13 ± 1	12 ± 1	11 ± 1	11 ± 1
RWC	93.5 ± 0.7	90.8 ± 2.0	93.7 ± 0.5	92.6 ± 1.4	94.1 ± 0.8	94.6 ± 1.0	90.2 ± 2.4	91.2 ± 0.9	94.0 ± 1.5	96.4 ± 1.2
Total ψ	−1.0 ± 0.1	−1.0 ± 0.1	−1.5 ± 0.1	−1.4 ± 0.1	−1.5 ± 0.1	−1.7 ± 0.1	−2.1 ± 0.1	−1.9 ± 0.1	−2.1 ± 0.1	−2.1 ± 0.2
N	4.0 ± 0.1	4.0 ± 0.1	3.2 ± 0.2	3.2 ± 0.1	3.5 ± 0.1	3.7 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	2.6 ± 0.1	2.8 ± 0.1
SLA	27 ± 2	30 ± 2	23 ± 1	23 ± 1	18 ± 1	21 ± 1	17 ± 1	19 ± 2	17 ± 1	18 ± 1

DISCUSSION

In 2004, the climate of Central Illinois was particularly favorable for crop growth, as reflected in the high yield in both control and elevated $[CO_2]$ plots. The absence of water stress throughout the season provided a rare opportunity to test for direct effects of elevated $[CO_2]$ on photosynthesis and water relations in a major C_4 crop, under field conditions. In accordance with our first and second predictions, growth at elevated $[CO_2]$ did not stimulate A at any time of the day and at any of the developmental stages investigated. Nor did it impact photosynthetic development by altering in vivo or in vitro activities of key enzymes. However, in accordance with our third prediction, growth at elevated $[CO_2]$ did significantly decrease g_s, corresponding to improved soil water availability by midseason. In the same genotype on the same site in 2002, A was transiently stimulated by elevated $[CO_2]$ during periods of intermittent drought stress, probably as a result of improved water relations (Leakey et al., 2004). Early chamber studies (Rudorff et al., 1996; Samarakoon and Gifford, 1996) found no increase in A and production in well-watered maize grown in elevated $[CO_2]$, but many other studies of well-watered plants have reported an increase in A and acclimation in the amounts of key photosynthetic enzymes (Wong, 1979; Moore et al., 1986; Dai et al., 1995; Watling and Press, 1997; Ziska and Bunce, 1997; Maroco et al., 1999; Ziska et al., 1999; Watling et al., 2000). It is hard to compare the results presented here with this previous work because most previous studies examined A_{sat} at only one time in the day and at one or two stages in the plant life cycle. Such data cannot be easily extrapolated to field conditions where pho-tosynthetic regulation and flux vary substantially over the diurnal course and with development of the crop. Additionally, most prior work was carried out in chambers rather than in the field. Plants in chambers do not experience normal atmospheric coupling and have limited rooting volume, both of which might impact whole-plant water relations, carbon gain, and growth. Uniquely, this study of field-grown maize, at a site very typical of the major crop production area, found no evidence of any direct effect of elevated $[CO_2]$ on photosynthetic rate, photosynthetic enzymes, development, dry matter production, or harvestable yield in the absence of water deficit. This work will enable the updating of models projecting future crop yields and food supply.

No Direct Effect of Elevated $[CO_2]$ on Photosynthesis

If any of the proposed mechanisms for direct CO_2 effects on C_4 photosynthesis (Wong, 1979; Moore et al., 1986; Dai et al., 1995; Watling and Press, 1997; Ziska and Bunce, 1997; Maroco et al., 1999; Ziska et al., 1999; Watling et al., 2000) occurred in this experiment, it was not evident in either net photosynthetic CO_2 assimilation or any chlorophyll fluorescence parameter at any time of day or at any of the five developmental stages assessed. If responses occurred at a developmental stage not assessed by the physiological analyses performed (e.g. C_3-like photosynthesis in very young leaves), then they were not of sufficient significance to alter biomass accumulation, development, or yield. In addition, there was no effect of growth under FACE on midday photosynthesis or final yield of two additional cultivars (FR1064 × LH185 and FR1064 × IHP;
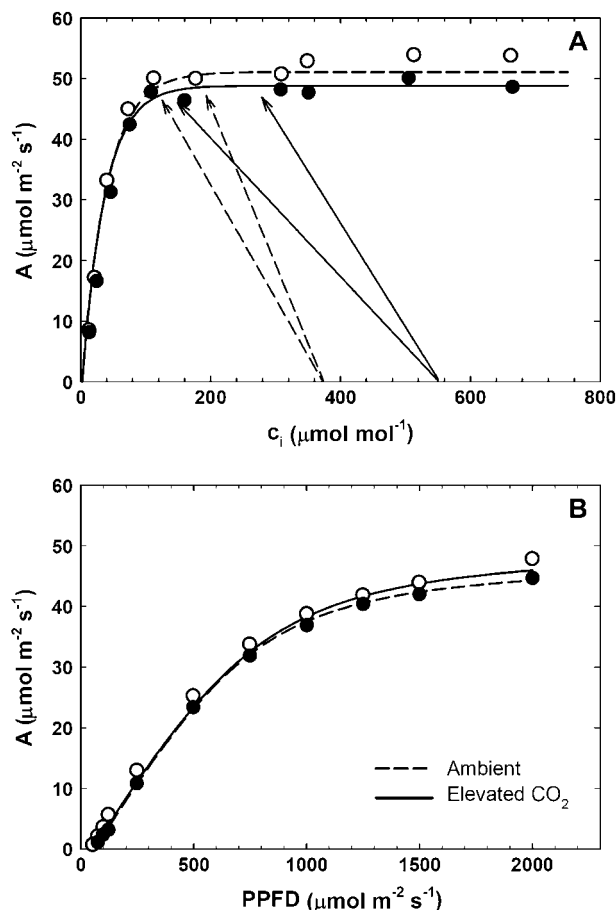
Figure 5. Photosynthetic gas-exchange analysis of the youngest fully expanded leaf of maize growing under ambient (○) and elevated CO_2 (●). A, Representative A/c_i curves fitted with model equations for C_4 photosynthesis (von Caemmerer, 2000) of maize grown at ambient (dashed) and elevated [CO_2] (solid). Arrows indicate the range of c_i measured at midday during in situ measurements of maize grown at ambient (dashed) and elevated [CO_2] (solid). There was no significant CO_2 effect on V_{pmax} (DOY 180: ambient 139 ± 16, elevated [CO_2] 122 ± 13; DOY 212: ambient 109 ± 3, elevated [CO_2] 112 ± 5) or V_{pr} (DOY 180: ambient 51.2 ± 1.8, elevated [CO_2] 48.6 ± 1.2; DOY 212: ambient 44.0 ± 4.6, elevated [CO_2] 40.1 ± 3.0). B, Representative A/Q curves fitted with nonrectangular hyperbolas for maize grown at ambient (dashed) and elevated CO_2 (solid). There was no significant CO_2 effect on maximum apparent quantum yield of photosynthesis (DOY 180: ambient 0.07 ± 0.01, elevated [CO_2] 0.07 ± 0.01) or A_{sat} (DOY 180: ambient 58.9 ± 1.3, elevated [CO_2] 60.8 ± 1.4).

M. Uribelarrea, unpublished data), which have previously been shown to differ in grain yield and composition (Uribelarrea et al., 2004).

Rising temperature can stimulate CO_2-saturated photosynthesis on the plateau of the C_4 A/c_i curve while having little effect on the initial slope (Sage and Kubien, 2003). This means that at high temperatures the CO_2-saturation point increases and C_4 photosynthesis could be more sensitive to direct enhancement by elevated [CO_2]. However, this appears unlikely to regulate the responses to elevated [CO_2] observed at SoyFACE. First, temperatures were relatively low

(maximum approximately 26°C) on July 11, 2002, when the greatest [CO_2] effect on photosynthesis was observed (Leakey et al., 2004). The crop experienced higher temperatures on all other measurement dates in 2002 and also on several dates in this study without a [CO_2] effect on photosynthesis. Second, the initial slopes of the A/c_i curves for maize in this study were steep (at 30°C, CO_2-saturation point <150 μmol mol^{-1}) compared to those for *Amaranthus retroflexus* reported by Sage and Kubien (2003; at 32°C, CO_2-saturation point >200 μmol mol^{-1}). Therefore, in maize grown at SoyFACE, any increase in the V_{pr} with temperature would have less effect on the CO_2-saturation point. Notably, the CO_2-saturation point of the A/c_i curve is reported to increase with increasing PPFD, N, and water supplies as a result of changes in PEPc/Rubisco activity ratio and bundle sheath leakiness (Leegood and von Caemmerer, 1989; Ghannoum et al., 2000). This would make C_4 photosynthesis more sensitive to direct stimulation by elevated [CO_2] and suggests that the favorable growing conditions at SoyFACE in 2004 make this study a relatively conservative test for direct CO_2 effects on C_4 photosynthesis. Nonetheless, it only infers that elevated [CO_2] will not directly stimulate the large fraction of global maize supply produced in the U.S. Corn Belt. Projecting the future performance of maize crops grown in tropical latitudes, where stress is more severe and elevated [CO_2] might provide greater benefits, requires further study.

It is possible that A might not change in situ if counteracting acclimations to elevated [CO_2] occur, e.g. direct stimulation of A by elevated [CO_2] offset by a decrease in capacity for PEP carboxylation or PEP regeneration. However, there was no effect of growth at elevated [CO_2] on these activities in vivo or in vitro. Neither PEP carboxylation or PEP regeneration capacity calculated from the A/c_i response, nor the in vitro activity at 25°C of the key photosynthetic enzymes Rubisco, PEPc, and PPDK, were altered by elevated [CO_2]. Similarly, the A/Q responses suggest a complete absence of acclimation in both light-limited and light-saturated photosynthetic capacity. The in vitro photosynthetic enzyme activities did not match the photosynthetic rates measured in situ. In vitro conditions did not mimic in vivo temperatures, and it is likely that some activity or protein was lost during the extraction and in vitro assay procedure. However, these effects should impact samples from each treatment to the same degree and should not prevent comparisons between treatments on a relative basis. Previously, incomplete extraction of Rubisco protein from pine needles reduced in vitro measures of activity below estimates from in vivo assays but did not alter the magnitude of CO_2 treatment effects (Rogers et al., 2001). There was also no change in c_i/c_a, suggesting an absence of any acclimation of stomatal response. Therefore, the lower g_s in elevated [CO_2] likely resulted from an instantaneous response and not any long-term response to growth at elevated [CO_2].

Table III. *Biomass of stover and grain, kernel number, individual kernel weight, total leaf area, and DOY of anthesis and silking for maize grown at ambient (370 $\mu mol\ mol^{-1}$) or elevated [CO_2] (550 $\mu mol\ mol^{-1}$) upon harvest at the end of the growing season in 2004 at SoyFACE in Urbana, IL*

Parameter	[CO_2] 370	[CO_2] 550	*P*
Stover biomass R6 (g plant^{-1})	134 ± 11	131 ± 9	0.68
Grain biomass R6 (g plant^{-1})	140 ± 6	142 ± 6	0.8
Kernel number (plant^{-1})	598 ± 38	609 ± 29	0.37
Kernel weight (mg)	248 ± 7	247 ± 5	0.83
Total leaf area (cm^2 plant^{-1})	6,280 ± 471	6,304 ± 365	0.48
Anthesis date	188.9 ± 0.3	188.7 ± 0.2	0.53
Silking date	188.3 ± 0.3	188.1 ± 0.3	0.63

Increased activities of enzymes involved in Suc and starch synthesis have been reported in maize grown at elevated [CO_2] with stimulated photosynthetic rates (e.g. Maroco et al., 1999). There was no evidence of increased starch or Suc content at elevated [CO_2] in this study even during the vegetative stage of crop

Figure 6. Soil H$_2$O% at depths of 5 to 25 cm (A) and 25 to 55 cm (B) in plots of maize growing under ambient (O) and elevated CO_2 (●) during 2004 at SoyFACE. Each point is the mean (±SE) of the replicate plots measured at that time (n = 4). C, The ratio of H$_2$O% in elevated [CO_2] compared to ambient [CO_2] treatments, at 5 to 25 cm (dashed line) and 25 to 55 cm (solid line). *P* values indicate statistical significance of CO_2 × time interaction effects.

development, when sink capacity in maize is relatively low compared to sink capacity during grain filling. It is also possible that lower water use, indicated by consistently lower g_s, might reduce nitrate uptake by mass flow, reduce leaf N, and counteract any enhancement of *A* by elevated [CO_2]. Total leaf N, soluble protein, and chlorophyll contents are markers of leaf N status, while total leaf free-amino acid content reflects whole-plant N status (Hirel et al., 2005). Total free-amino acid content, which is the hub around which the processes of N assimilation and associated carbon metabolism revolve (Foyer et al., 2003), was higher in young vegetative plants, as reported previously (Hirel et al., 2005). Nonetheless, there was no [CO_2] effect on markers of plant or leaf N status. In summary, despite a consistent decrease in g_s, there was no evidence of the stimulation of *A* and acclimation in the photosynthetic apparatus in response to the elevated [CO_2] that has been observed in some studies within chambers.

A number of factors may explain this difference in results; these include genotype, developmental stage, and the treatment [CO_2]. Maize has been grown at approximately 3 times current [CO_2] (Maroco et al., 1999) and sorghum at approximately 2 times current [CO_2] (Watling et al., 2000), compared to approximately 1.5 times current [CO_2] in this experiment. But there was no evidence of the physiological acclimation to elevated [CO_2] observed in these chamber studies in this field study. Therefore, the difference could only be explained by a threshold effect, i.e. acclimation occurring when a threshold concentration is exceeded. The cultivar used in this experiment is a major production line currently used in the Corn Belt and closely related to the germ plasm used in most of the region's current production lines. Although effects could be specific to developmental stages not covered by this study, this has included vegetative and reproductive stages of the crop, including those where any effect on *A* would have its maximum impact on yield (Ritchie et al., 1993). Alternatively, maize crops in the deep soils of the Corn Belt can root to 2 m. Therefore, their root systems extend far beyond that allowed by even the largest pots. Compared to the field, the root system of any potted plant will be highly restricted, and this would slow water uptake of even the best watered pots. As a result, subtle improvements in plant water status may occur in container-grown maize under
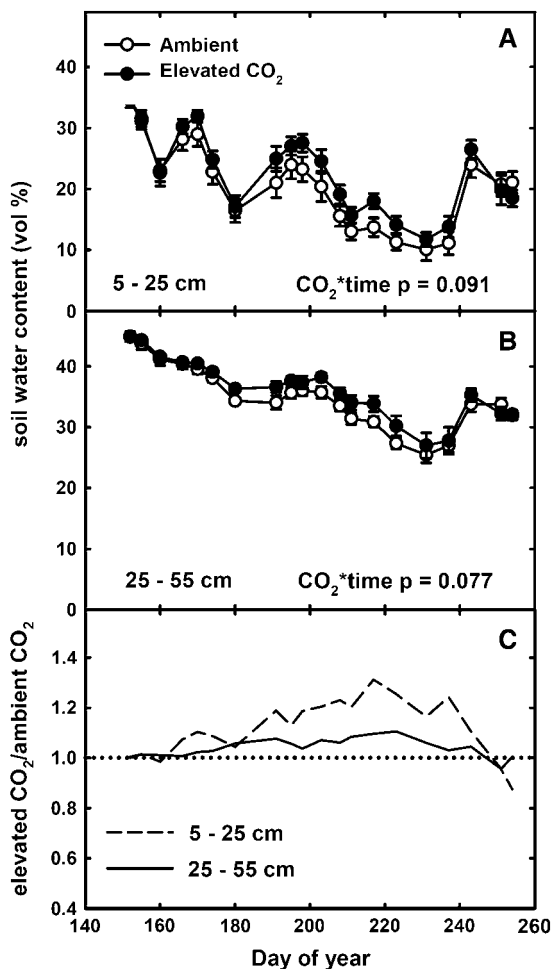
elevated [CO_2] that would be absent in the open field under ample water conditions.

Only one other FACE experiment has assessed the photosynthetic response of a C_4 crop to elevated [CO_2]. Sorghum was grown under elevated [CO_2] in Arizona, with irrigation. These plants were suggested to display C_3-like photosynthesis in young leaves and some suppression of photorespiration, along with increases in energy use efficiency (Cousins et al., 2001). The analysis of A/c_i curves indicated that this phenomenon did not occur in maize at SoyFACE. In addition, it was concluded that the direct effects of CO_2 enrichment on A of sorghum under FACE in Arizona were minor, and indirect enhancement of A by improved water relations was cited as the primary mechanism of response (Wall et al., 2001).

Direct Effects of Elevated [CO_2] on Water Use

Given no change in leaf area, the decrease in g_s at elevated [CO_2] would favor reduced whole-plant water use. This is consistent with the observation that, at soil depths of 5 to 55 cm, soil in the elevated [CO_2] plots retained progressively more moisture compared to ambient plots until maximum leaf area was reached. Water conservation under elevated [CO_2] has been observed in chamber experiments on C_4 species (Owensby et al., 1997; Nelson et al., 2004). However, in these cases, forced canopy-atmosphere coupling, caused by fumigation with forced air circulation, may have artificially increased the extent to which plant water use was controlled by g_s. Sorghum canopy evapotranspiration, measured across two growing seasons by energy balance techniques in the FACE experiment in Arizona, was reduced by elevated [CO_2] under both ample water supply (-10%) and severe drought stress (-4%; Conley et al., 2001). However, in the "wet" treatment, with ample water supply, the soil was drier under elevated [CO_2] throughout both growing seasons (Wall et al., 2001), creating some ambiguity as to the basis for the result.

The growing conditions of 2004 in Central Illinois were so close to ideal that the observed improvements in water use efficiency did not alter plant water status. However, greater soil water would in most growing seasons be expected to delay or prevent the onset of drought stress during the periods of low rainfall. The episodic enhancement of A in maize during periods of drought at SoyFACE in 2002 is consistent with this phenomenon (Leakey et al., 2004).

Should an increase in A be expected due to the reduced evaporative cooling caused by lower g_s at elevated [CO_2]? The season-long average g_s over the five diurnal cycles of measurement was 0.21 mmol m^{-2} s^{-1} for ambient [CO_2] and 0.15 mmol m^{-2} s^{-1} for elevated [CO_2], with a daytime average air temperature (T_{air}) of 22.7°C and PPFD of 880 μmol m^{-2} s^{-1}, approximating to a solar radiation flux of 420 J m^{-2} s^{-1}. Assuming an absorptance of 0.9, typical of healthy leaves, mean daytime relative humidity of 70%, and wind speed of 4.1 m s^{-1}, the average increase in temperature caused by the lower g_s in elevated [CO_2] would be 0.26°C, calculated from the energy balance equations of Grace (1983). Using the relationship of A to leaf temperature for maize defined by Hofstra and Hesketh (1969), this would cause an increase in leaf photosynthesis of 0.3 μmol m^{-2} s^{-1}. Even this may be an overestimate since it is based on a mean temperature (22.7°C) below the optimum; during periods when the optimum (approximately 34°C) is approached or reached, there will be no increase in A. Therefore, growth at elevated [CO_2] probably favors greater photosynthesis due to increased leaf temperature, but the effect is small relative to A_{sat}. As a result, the effect had no detectable impact on biomass accumulation and yield over the growing season.

CONCLUSION

Maize is predicted to become the world's most important crop, in terms of human food supply, by 2050 (Pingali, 2001). While the results of our study are limited to one location and one hybrid line, farming practice and crop performance at SoyFACE are typical of the surrounding area and the genotype shares lineage with many other production lines. Champaign County is centrally located in the U.S. Corn Belt and is consistently high yielding (http://www.usda.gov/nass/graphics/county04/crpmap04.htm#corn). So the results of this study should at least relate to the Corn Belt, which generates 40% of global maize production (USDA, 2005). The absence of any photosynthetic, growth, or yield response of maize to elevated [CO_2] in 2004 at SoyFACE is inconsistent with some earlier cabinet studies, and suggests that including a direct and consistent CO_2 fertilization effect on C_4 crop performance is currently a significant source of error in estimating future food security. It appears that elevated [CO_2] will only enhance performance by reducing crop water use. Therefore, improvements in A, growth, and yield will only occur if stress is ameliorated in times or places of drought. Unfortunately, the indirect nature of this mechanism, combined with considerable uncertainty regarding future soil water availability (Cubasch et al., 2001), makes predicting future crop performance difficult. Total precipitation in North America is projected to increase slightly this century (Giorgi et al., 2001), but there is also predicted to be an increase in the frequency and magnitude of droughts as climate becomes more variable (Gregory et al., 1997; Beersma and Buishand, 1999). Therefore, projections of crop performance will need to explicitly deal with water stress, and its interaction with elevated [CO_2], if they are to be reliable. In 2002, A of maize at SoyFACE was stimulated, on average, by 10% (Leakey et al., 2004). Years with episodic droughts, such as 2002, occur every 2 to 3 years in Central Illinois. Therefore, future elevated [CO_2] may often indirectly enhance A and possibly yield. However, the

impact on growth and yield will vary with the duration and timing of water stress in the growing season. Additionally, FACE studies of C_3 crops indicate that the benefits of growth at elevated $[CO_2]$ are greatest for A, lower for productivity, and least for yield (Ainsworth and Long, 2005; Long et al., 2005; Morgan et al., 2005). Likewise, in a FACE experiment on amply irrigated sorghum, elevated $[CO_2]$ stimulated A by 9%, but did not enhance total biomass or grain yield (Ottman et al., 2001). Therefore, it appears that elevated $[CO_2]$ will increasingly have a role in determining C_4 crop performance via amelioration of drought stress. However, in the absence of any direct stimulation of photosynthesis, it is unclear that this will be sufficient to override, or even negate, the detrimental effects of increasing temperature and drought on yield.

MATERIALS AND METHODS

Field Site, Cultivation, and FACE System

The study was conducted in a 16-ha field of maize (*Zea mays*) at the SoyFACE facility in Champaign, IL. The facility operational procedures and crop cultivation were repeated from a previous experiment (Leakey et al., 2004). Maize cv 34B43 (Pioneer Hi-Bred International) was planted on April 29, 2004, emerged on May 9, 2004, and was harvested on September 10, 2004. The infrastructure for CO_2 enrichment was installed immediately after planting in four experimental blocks ($n = 4$ for statistical tests). In each block, one plot was at current ambient $[CO_2]$ of 376 μmol mol^{-1}, while a second plot was fumigated during daylight hours to an average elevated $[CO_2]$ of 542 μmol mol^{-1}. The target $[CO_2]$ for simulating the conditions in 2050 was 550 μmol mol^{-1}, midpoint of different projections varying in assumptions about population and economic development (Prentice et al., 2001). The $[CO_2]$ enrichment achieved during the growing season was within ±20% of the target 93% of the time.

Meteorological and Soil Water Data

An on-site weather station measured T_{air}, relative humidity, incident PPFD, and rainfall throughout the season. H_2O% was measured in 10-cm increments between depths of 5 and 105 cm using a capacitance probe (Diviner-2000; Sentek Sensor Technologies). Measurements were taken every 3 to 7 d at four positions in a 1-m^2 area near the center of each plot. Weekly records of the PCMI from 1973 to 2004 for East Central Illinois were provided by the Climate Operation Branch of NOAA (http://www.usda.gov/oce/waob/jawf/).

In Situ Gas Exchange and Tissue Sampling

The diurnal course of gas exchange and chlorophyll fluorescence of the youngest fully expanded leaf in each plot was measured on five dates across the season, using four open gas-exchange systems with integrated modulated chlorophyll fluorometers (LI-6400 and LI-6400-40; LI-COR). Full expansion was judged by emergence of the ligule. The dates corresponded to five discrete stages of crop development, including vegetative growth, silking, and grain filling (Table I). On each date, four gas-exchange systems were used simultaneously at intervals of approximately 2 h from early morning to sunset. At each interval, one gas-exchange system was operated within each of the four experimental blocks. Each block consisted of one ambient and one elevated $[CO_2]$ plot. Two gas-exchange systems were first used in ambient $[CO_2]$ plots, while the other two gas-exchange systems were first used in elevated $[CO_2]$ plots. Each gas-exchange system was then moved to the alternate $[CO_2]$ treatment within the block. The gas-exchange systems were rotated among blocks and starting $[CO_2]$ treatment at each time point. These procedures ensured that measurements were not biased by differences in microclimate over time, or differences between gas-exchange systems. Three plants were measured in each plot at each time interval. Measurements of

chlorophyll fluorescence and gas-exchange parameters on all plants were made at growth $[CO_2]$, T_{air}, and PPFD. Leaf A, g_s, and c_i were calculated using the equations of von Caemmerer and Farquhar (1981). The formation of dew on leaves precluded measurement of g_s at dawn and dusk and at other periods of the day on occasion. Transpiration per unit leaf area (E), measured by the gas-exchange system, is affected by chamber humidity, which may differ from that of the external atmosphere. Therefore, a better measure of transpiration was calculated as the product of leaf conductance and the leaf vapor pressure deficit, which was determined from measured leaf temperature and the external ambient air humidity. Chlorophyll fluorescence parameters (qP, Φ_{PSII}, F_v'/F_m', NPQ, and Φ_{CO2}) were defined and calculated as described by Naidu and Long (2004).

Directly after the photosynthetic measurements, leaf discs (approximately 1.2 cm^2) were excised, plunged immediately into liquid N, and then stored at −80°C until analyzed for carbohydrate, protein, free-amino acid, and chlorophyll contents. Additional discs were removed and sealed into scintillation vials for RWC analysis (approximately 3.6 cm^2 per plant) or sealed in stainless steel psychrometer chambers (approximately 2.4 cm^2 per plant; C-30; Wescor) for water potential analyses. Finally, leaf discs (approximately 3.6 cm^2 per plant) were removed and dried in an oven at 70°C to constant weight and weighed for calculation of SLA.

Foliar Biochemical, Nitrogen, and Water Analyses

Foliar contents of carbohydrates, protein, and total free-amino acids were determined from 80% (v/v) ethanol extracts as by Geigenberger et al. (1996). Glc, Fru, and Suc were determined using a continuous enzymatic substrate assay (Rogers et al., 2004). For protein and starch determination, pellets of the ethanol extraction were solubilized by heating to 95°C in 0.1 M NaOH. Protein content was determined using a commercial kit (Protein assay kit; Pierce) with bovine serum albumin as a standard. The NaOH solution containing the dissolved pellet was then acidified to pH 4.9 and the starch content was determined as by Hendriks et al. (2003). Total free-amino acid contents were determined using a fluorescamine assay (Bantan-Polak et al., 2001).

The in vitro activities of Rubisco, PPDK, and PEPc were all measured indirectly as the rate of oxidation of NADH (specific absorption coefficient of 6.22 mM^{-1}) using linked enzyme assays in a dual-beam spectrophotometer (Cary I; Varian) at 340 nm and 25°C. The extraction of Rubisco followed the procedure outlined by Sharkey et al. (1991), with the following modifications. One protease inhibitor cocktail tablet (Roche Applied Science) per 10 mL of extraction solution was added to inhibit enzyme degradation. Leaf tissue was rapidly ground (60–120 s) in 2.0 mL of extraction solution at 0°C using an ice-chilled glass tissue homogenizer. The extract was then centrifuged for 15 s at 15,000g. To fully activate Rubisco, a 1-mL aliquot of the supernatant was added to 20 mM MgCl$_2$ and 10 mM NaHCO$_3$ (Sharkey et al., 1991). The crude extract was incubated at room temperature until maximum activity was stable (approximately 8 min). A 50-μL aliquot of the crude extract was added to a cuvette containing 700 μL of assay medium and assayed for 1 min. The assay medium was prepared according to Sharkey et al. (1991) with the following modifications: 1.8 units (2.6 units mL^{-1}) of creatine phosphokinase, 1.8 units (2.6 units mL^{-1}) of phosphoglycerate kinase, and 9.2 units (13.1 units mL^{-1}) of glyceraldehyde-3-P dehydrogenase were used. Leaf tissue was prepared as for the Rubisco assay, before PPDK was extracted as previously described (Crafts-Brandner and Salvucci, 2002). The crude extract was allowed to incubate in the assay medium for 5 min at 25°C, and the reaction was initiated by the addition of 15 μL of 100 mM pyruvate (2.2 mM final concentration) and 12 μL (6 units) of purified maize PEPCase (Bio-Research Products) and then assayed for 1 min. Incubation in this manner was found to increase the in vitro activity by 10% to 20%. Leaf tissue was prepared as for the Rubisco assay, before PEPCase was extracted by the method of Crafts-Brandner and Salvucci (2002), with the exception of using 5 mM dithiothreitol in place of β-mercaptoethanol. A 35-μL aliquot of the supernatant was added to a cuvette containing 665 μL of assay medium and assayed for 1 min. The assay medium was prepared as described previously (Giglioli-Guivarc'h et al., 1996), with the addition of 5 mM Glc-6-P and 2 mM dithiothreitol (Ashton et al., 1990). Malate dehydrogenase was increased to 6.5 units per assay (9.4 units mL^{-1}).

Dried leaf material was powdered and analyzed for N content using an elemental combustion system (model 4010; Costech Analytical Technologies). RWC was measured as by Ghannoum et al. (2002). A dew point micro-voltmeter (HR-33T; Wescor) measured ψ_{leaf} after psychrometer chambers (C-30; Wescor) containing leaf discs (2.4 cm^2) were equilibrated in a controlled environment cabinet at 25°C.

A/c_i and A/Q Curves

Predawn on DOY 180 and 212, the youngest fully expanded leaf of two plants per plot were cut from the plant and then immediately recut under water and kept immersed. The objective was to reveal any effect of elevated $[CO_2]$ on the potential photosynthetic capacity of the leaves, through measurement of A/c_i and A/Q curves. Sampling leaves predawn and performing measurements under controlled conditions avoided the short-term decreases in water potential, chloroplast inorganic phosphate concentration, and maximum PSII efficiency that can occur in the field and may transiently limit photosynthetic performance. Using the gas-exchange and fluorescence apparatus described above, A/c_i curves were determined in the laboratory at a PPFD of 1,750 μmol m^{-2} s^{-1} and A/Q curves were determined at growth $[CO_2]$, as by Bernacchi et al. (2005). All measurements were performed at 30°C. The response of A to c_i at $c_i < 50$ μmol mol^{-1} was used to solve for V_{pmax} (von Caemmerer, 2000). V_{pr} was estimated from the horizontal asymptote of a nonrectangular hyperbolic function for each A/c_i curve. From A/Q curves, Φ_{CO_2} and A_{sat} were calculated as by Naidu and Long (2004).

Crop Development, Biomass, and Yield

Silking dates were defined at the point in time when 50% of plants had visible silks and anthesis dates when 50% of plants shed pollen. At flowering, the area of every leaf on four randomly sampled plants from each plot was determined from the linear dimensions and a pre-established relationship with area (McKee, 1964). At the end of the growing season, four plants were sampled from each plot and separated into grain and stover (i.e. the remainder of the shoot). These fractions were oven dried at 75°C to constant weight and their mass determined.

Statistics

In all cases, statistics were performed on plot means using the MIXED procedure of SAS, with the Satterthwaite option (SAS Institute, Cary, NC). The Akaike's criterion was used to choose the best model of variance-covariance. In all tests, $[CO_2]$ treatment was a fixed effect and block a random effect. In tests of physiological processes, time of day and DOY were fixed effects. For the overall comparison of H$_2$O% between treatments over the growing season, a mixed model was fitted to repeated measures of time.

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