

# Leaf photosynthesis and carbohydrate dynamics of soybeans grown throughout their life-cycle under Free-Air Carbon dioxide Enrichment

A. ROGERS<sup>1,4</sup>, D. J. ALLEN<sup>2,3,\*</sup>, P. A. DAVEY<sup>1,2,†</sup>, P. B. MORGAN<sup>2</sup>, E. A. AINSWORTH<sup>1</sup>, C. J. BERNACCHI<sup>2,3</sup>, G. CORNIC<sup>5</sup>, O. DERMODY<sup>2</sup>, F. G. DOHLEMAN<sup>1</sup>, E. A. HEATON<sup>1</sup>, J. MAHONEY<sup>4</sup>, X.-G. ZHU<sup>2</sup>, E. H. DELUCIA<sup>2</sup>, D. R. ORT<sup>1,2,3</sup> & S. P. LONG<sup>1,2</sup>

Departments of <sup>1</sup>Crop Sciences and <sup>2</sup>Plant Biology and the <sup>3</sup>Photosynthesis Research Unit, USDA/ARS, University of Illinois at Urbana/Champaign, IL 61801–4798, USA, <sup>4</sup>Environmental Sciences Department, Brookhaven National Laboratory, Upton, NY 11973, USA and <sup>5</sup>Laboratory D'écophysiologie Végétale, Université Paris XI, Orsay 91405, France

## ABSTRACT

A lower than theoretically expected increase in leaf photosynthesis with long-term elevation of carbon dioxide concentration ( $[\text{CO}_2]$ ) is often attributed to limitations in the capacity of the plant to utilize the additional photosynthate, possibly resulting from restrictions in rooting volume, nitrogen supply or genetic constraints. Field-grown, nitrogen-fixing soybean with indeterminate flowering might therefore be expected to escape these limitations. Soybean was grown from emergence to grain maturity in ambient air ( $372 \mu\text{mol mol}^{-1} [\text{CO}_2]$ ) and in air enriched with  $\text{CO}_2$  ( $552 \mu\text{mol mol}^{-1} [\text{CO}_2]$ ) using Free-Air  $\text{CO}_2$  Enrichment (FACE) technology. The diurnal courses of leaf  $\text{CO}_2$  uptake ( $A$ ) and stomatal conductance ( $g_s$ ) for upper canopy leaves were followed throughout development from the appearance of the first true leaf to the completion of seed filling. Across the growing season the daily integrals of leaf photosynthetic  $\text{CO}_2$  uptake ( $A'$ ) increased by 24.6% in elevated  $[\text{CO}_2]$  and the average mid-day  $g_s$  decreased by 21.9%. The increase in  $A'$  was about half the 44.5% theoretical maximum increase calculated from Rubisco kinetics. There was no evidence that the stimulation of  $A$  was affected by time of day, as expected if elevated  $[\text{CO}_2]$  led to a large accumulation of leaf carbohydrates towards the end of the photoperiod. In general, the proportion of assimilated carbon that accumulated in the leaf as non-structural carbohydrate over the photoperiod was small (< 10%) and independent of  $[\text{CO}_2]$  treatment. By contrast to  $A'$ , daily integrals of PSII electron transport measured by modulated chlorophyll fluorescence were not significantly increased by elevated  $[\text{CO}_2]$ . This indicates that  $A$  at elevated  $[\text{CO}_2]$  in these field conditions was predominantly ribulose-1,5-bisphosphate (RubP) limited rather than Rubisco limited.

There was no evidence of any loss of stimulation toward the end of the growing season; the largest stimulation of  $A'$  occurred during late seed filling. The stimulation of photosynthesis was, however, transiently lost for a brief period just before seed fill. At this point, daytime accumulation of foliar carbohydrates was maximal, and the hexose:sucrose ratio in plants grown at elevated  $[\text{CO}_2]$  was significantly larger than that in plants grown at current  $[\text{CO}_2]$ . The results show that even for a crop lacking the constraints that have been considered to limit the responses of  $\text{C}_3$  plants to rising  $[\text{CO}_2]$  in the long term, the actual increase in  $A$  over the growing season is considerably less than the increase predicted from theory.

*Key-words:* *Glycine max*; atmospheric change; chlorophyll fluorescence; electron transport; elevated carbon dioxide concentration; FACE; global climate change; photosynthesis; stomatal conductance.

*Abbreviations:*  $A$ , net rate of  $\text{CO}_2$  uptake per unit leaf area ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ );  $A_{\text{sat}}$ , light saturated  $A$ ;  $A'$ , daily integral of  $A$ ; DOY, day of year;  $[\text{CO}_2]$ , carbon dioxide concentration; FACE, Free-air  $\text{CO}_2$  enrichment;  $g_s$ , leaf stomatal conductance to water vapour ( $\text{mol m}^{-2} \text{s}^{-1}$ );  $J_{\text{PSII}}$ , rate of photosystem II electron transport ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ );  $J_{\text{PSII}}'$ , daily integral of  $J_{\text{PSII}}$ ; PPF, photosynthetic photon flux density ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ); PPF', daily integral of PPF; Rubisco, ribulose-1,5-bisphosphate carboxylase oxygenase;  $T_{\text{leaf}}$ , leaf temperature ( $^{\circ}\text{C}$ ); TNC, total non-structural carbohydrate ( $\text{mmol m}^{-2}$ ); VPD, vapour pressure deficit (kPa).

## INTRODUCTION

Atmospheric  $\text{CO}_2$  concentration ( $[\text{CO}_2]$ ) is expected to rise from a current  $372 \mu\text{mol mol}^{-1}$  to about  $550 \mu\text{mol mol}^{-1}$  by the middle of the century (Prentice 2001). In the short term, an increase in  $[\text{CO}_2]$  stimulates net photosynthetic rate in  $\text{C}_3$  plants because the present  $[\text{CO}_2]$  is insufficient to saturate Rubisco and because  $\text{CO}_2$  inhibits the competing process of photorespiration (Drake, Gonzalez-Meler & Long

Correspondence: Stephen P. Long. E-mail: stevel@life.uiuc.edu

\*Current address: BASF Plant Science LLC, Research Triangle Park, NC 27709, USA.

†Current address: University of Essex, Colchester, CO4 3SQ, UK.

1997). Therefore, an increase in net photosynthesis in elevated  $[\text{CO}_2]$  is anticipated regardless of whether Rubisco activity or regeneration of ribulose-1,5-bisphosphate (RubP) is limiting assimilation, and regardless of whether light is saturating or limiting (Drake *et al.* 1997). Increased carbon uptake resulting from this initial stimulation of photosynthesis by elevated  $[\text{CO}_2]$  will alter the balance of supply and capacity to use carbohydrates, with the result that non-structural carbohydrate concentrations invariably increase within leaves grown at elevated  $[\text{CO}_2]$  (Drake *et al.* 1997). Such accumulations of carbohydrate may cause a short-term decrease in photosynthetic rate via sequestration of cytosolic inorganic phosphate, and a long-term decrease in photosynthetic capacity by repressing the expression of specific photosynthetic genes, notably *rbcS* (Harley & Sharkey 1991; Socias, Medrano & Sharkey 1993; Drake *et al.* 1997; Moore *et al.* 1999; Pego *et al.* 2000). Acclimation of photosynthesis (i.e. decrease in the stimulation of photosynthesis over time at elevated  $[\text{CO}_2]$ ) is commonly associated with a limitation in the capacity to utilize the additional photosynthate produced under elevated  $[\text{CO}_2]$  (Rogers *et al.* 1998; Ainsworth *et al.* 2003). This limitation may be genetic and/or environmental, in particular, the limitation imposed by insufficient nitrogen supply (Rogers *et al.* 1998; Stitt & Krapp 1999; Hymus, Baker & Long 2001). Soybean, in common with other leguminous crops, might at least in part avoid this limitation, since its nodules provide an additional sink for carbon and the means to increase the supply of N. Further, many soybean cultivars show indeterminate floral initiation, allowing additional sinks for photosynthate to form to utilize additional supply. Does soybean show continued stimulation of photosynthetic rate throughout the day with season-long growth under elevated  $[\text{CO}_2]$ ?

From Rubisco kinetics (Farquhar, von Caemmerer & Berry 1980; Long 1991), a maximum stimulation of light-saturated leaf photosynthesis at 25 °C of 38% upon increasing  $[\text{CO}_2]$  to 550  $\mu\text{mol mol}^{-1}$  and 64% when  $[\text{CO}_2]$  is raised to 700  $\mu\text{mol mol}^{-1}$  is predicted. This assumes that there is no acclimation of leaf photosynthesis, that Rubisco is the major biochemical limitation at light-saturation, and that the ratio of external to intercellular  $[\text{CO}_2]$  is 0.7 (Long 1991; Rogers & Humphries 2000). Ainsworth *et al.* (2002) surveyed all prior studies of soybean grown under elevated  $[\text{CO}_2]$  and found an average increase in  $A_{\text{sat}}$  of 39% for plants grown in approximately doubled  $[\text{CO}_2]$  (mean  $[\text{CO}_2] = 689 \mu\text{mol mol}^{-1}$ , averaged across 78 studies). This is only 60% of the maximum increase predicted from the assumptions outlined above, suggesting either acclimation resulting in a significant loss of photosynthetic capacity, or a predominant limitation by RubP-regeneration. However, Ainsworth *et al.* (2002) also showed that the increase in  $A_{\text{sat}}$  for soybean grown in a large rooting volume (> 9 L) was 58%, and much closer to the theoretically expected increase, compared with 24% for plants grown in a smaller rooting volume (2.5–9 L). Although most of these prior studies considered only light-saturated photosynthesis and none monitored diurnal photosynthesis throughout the

growing season, there is the implication from prior results that field-grown soybean may sustain near-theoretical stimulation of photosynthetic capacity. In irrigated wheat grown using FACE technology there were significant transient decreases in wheat photosynthesis under elevated  $[\text{CO}_2]$  in the late afternoon, despite the lack of any acclimation in Rubisco content, suggesting transient triose-phosphate-utilization-limitation (Nie *et al.* 1995a; Garcia *et al.* 1998).

To date, the measurements of photosynthesis in soybean grown in elevated  $[\text{CO}_2]$  have been limited to plants grown in protected environments. These have ranged from artificially lit cabinets to open-top chambers (Ainsworth *et al.* 2002). Even within open-top chambers, the crop environment is modified by decreased exposure to wind, altered coupling of canopy and atmosphere, increased temperature and humidity, and decreased precipitation and light, such that the long-term effects of enclosure may exceed the effects of elevated  $[\text{CO}_2]$  (McLeod & Long 1999). Free-Air  $\text{CO}_2$  Enrichment (FACE) allows the study of the effects of elevated  $[\text{CO}_2]$  on crops grown under field conditions without any enclosure (Hendrey & Kimball 1994; McLeod & Long 1999). Large areas of undisturbed canopy are available where edge effects and other unnatural disturbances to the growing environment can be avoided. The scale of FACE also allows crops to be managed as typical for the region, with standard agronomic practices and without limitation on rooting volume. In the present study we tested the prediction that under open-field conditions the initial stimulation of leaf photosynthesis by an increase in  $[\text{CO}_2]$  to 552  $\mu\text{mol mol}^{-1}$  persists throughout the life of the crop and throughout the natural diurnal cycle. Diurnal measurements of net leaf  $\text{CO}_2$  uptake ( $A$ ) were supported by simultaneous measurements of leaf carbohydrate dynamics, water vapour flux, modulated chlorophyll fluorescence, and microclimate to aid interpretation of the basis of responses of photosynthesis to elevated  $[\text{CO}_2]$ .

## MATERIALS AND METHODS

### The FACE system and soybean crop

The study was conducted at the soybean FACE facility (SoyFACE) situated on 32 hectares of Illinois farmland within the Experimental Research Station of the University of Illinois at Urbana-Champaign (40°02' N, 88°14' W, 228 m above sea level; <http://www.soyface.uiuc.edu>). It consists of four blocks, each containing two 20-m-diameter octagonal plots. One plot was maintained at current ambient  $[\text{CO}_2]$  of 372  $\mu\text{mol mol}^{-1}$  and one plot was fumigated to an elevated  $[\text{CO}_2]$  of 552  $\mu\text{mol mol}^{-1}$ , constituting a fully randomized block design. Soybean (*Glycine max* L. cv 'Pana') was planted at 0.38 m row spacing on 23 May 2001 (DOY 143). The soil is a Flanagan/Drummer (fine-silty, mixed, mesic Typic Endoaquoll), which is very deep and formed from loess and silt parent material deposited on the till and outwash plains. No nitrogen fertilizer was added to the soybean crop, according to standard regional agronomic practice. The experimental plots were separated by at least

100 m, which has been demonstrated to be sufficient to prevent cross-contamination of CO<sub>2</sub> (Nagy *et al.* 1992).

The FACE system used has been described in detail previously (Miglietta *et al.* 2001), and is therefore only outlined here. Each plot was surrounded by a segmented octagon of pipes that released CO<sub>2</sub> about 10 cm above the top of the soybean canopy. CO<sub>2</sub> was injected at supersonic velocity into the wind from 300 µm pores spaced at 15–22 mm intervals and then carried back over the crop within the ring. CO<sub>2</sub> was released at a maximum rate along the side of the octagon, upwind and perpendicular to the wind direction, and released at 0.7 of this rate from pipes on the sides flanking the windward side. Via an anemometer and wind vane in the centre of each plot, the control system continuously calculated the rate and position of gas release needed to maintain the desired enrichment within the ring. Infrared gas analysers monitored [CO<sub>2</sub>] at the centre of the enriched plots at 10 cm above the crop canopies, and the system adjusted the CO<sub>2</sub> release to maintain the set-point concentration (Miglietta *et al.* 2001). One-minute-average CO<sub>2</sub> concentrations were within ±10% of the 550 µmol mol<sup>-1</sup> target for more than 85% of the time. Fumigation was operated from planting until harvest during daylight. On those rare instances when wind speeds dropped below 0.2 m s<sup>-1</sup>, CO<sub>2</sub> fumigation cycled around the octagon to maintain the [CO<sub>2</sub>] within the plot as close to the 550 µmol mol<sup>-1</sup> set point as possible. Air temperature, PPFD, and precipitation were recorded at 15-min intervals throughout the growing season.

### Field measurement of leaf CO<sub>2</sub> uptake and transpiration

Measurements were made from pre-dawn to post-dusk on 7 d covering different developmental stages: DOY 164, V1 first node and unifoliate leaf; 176, V3 second trifoliate leaf; 191, V7–V8 seven to eight nodes; 205, R1 beginning bloom; 215, R2 full bloom; 233, R3–R4 beginning to full pods; and 254, R5–R6 beginning to full seeds. V1, V3, etc. denote phenological stage (V = vegetative; R = reproductive), following the system of Ritchie *et al.* (1997). Two teams measured leaf gas exchange and modulated chlorophyll fluorescence, each using a portable open gas-exchange system (LI-6400; Li-Cor, Inc., Lincoln, NE, USA) and fluorometer (FMS; Hansatech, Kings Lynn, UK). The fiberoptic from the fluorometer was held at 45° to the leaf surface at the gas exchange cuvette window. Each day before beginning measurements, the infrared CO<sub>2</sub> and water vapour analysers of these systems were calibrated against a standard mixture of CO<sub>2</sub> in air (Certified Standard Mixture; Smith Welding, Decatur, IL, USA) and a dew-point-controlled water vapour generator (LI-610; Li-Cor, Inc.), respectively. A 2 m<sup>2</sup> area, near the centre of each plot, was reserved for diurnal photosynthesis measurements and simultaneous sampling for carbohydrate analysis. From plants within this area, the youngest fully expanded leaves were selected. At 2 h intervals from 1 h pre-dawn to 1 h post-sunset the teams worked in parallel, one measuring

leaves within the control and one leaves within the treatment plot. Three to five leaves were measured per plot, at each time point. Each measurement system was alternated between plots of the control and treatment to avoid confounding measurement and treatment systems. Measurements within a time point were usually completed within 1 h, and always within 1.5 h.

Net CO<sub>2</sub> assimilation (*A*) and stomatal conductance (*g<sub>s</sub>*) were determined via the equations of von Caemmerer & Farquhar (1981) under near *in situ* conditions approximately 60 s after clamping onto a leaf. Leaves were maintained at nearly ambient light levels by conserving leaf orientation and using a transparent cuvette. Air temperature was measured at the start of each time point, and cuvettes were actively maintained at this temperature using the Peltier-based temperature control of the gas exchange system. The [CO<sub>2</sub>] of the air flowing into the cuvette was controlled to 370 or 550 µmol mol<sup>-1</sup>, to correspond with the plot treatment. The ambient water vapour pressure was used. Leaves remained attached to the plant. Whole chain electron transport through PSII (*J<sub>PSII</sub>*) was estimated by chlorophyll fluorescence, by the procedure of Genty, Briantais & Baker (1989). These measurements were made immediately following the gas-exchange, while the leaf remained in the gas-exchange cuvette. The daily totals of leaf CO<sub>2</sub> uptake (*A'*) and PSII electron transport (*J<sub>PSII</sub>'*) were obtained by integrating under the curve described by the variation of *A* and *J<sub>PSII</sub>*, respectively, with time-of-day for each replicate ring.

### Carbohydrate content and export

Samples for carbohydrate analyses were taken in parallel with the diurnal measurements of gas exchange on four days (DOY 191, 205, 215, 233). On each occasion, samples were taken at four points in a 24 h period: immediately before sunrise, solar noon, immediately following sunset, and just before sunrise the following day. For each leaf sampled, one disc (approximately 3 cm<sup>2</sup>) was removed from a vein-free area of the middle leaflet from the uppermost, fully expanded trifoliate leaf, wrapped in foil, plunged immediately into liquid nitrogen and stored at –80 °C until analysis. On DOY 191 additional samples were taken throughout the photoperiod.

Leaf discs were powdered in liquid nitrogen and transferred to tubes containing 4 mL 90% (v/v) ethanol and incubated at 60 °C for 16 h. Extracts were clarified by centrifugation (4500 g, 10 min), the supernatant decanted into a second tube, and stored at 4 °C. Initial investigations determined that six extractions were necessary to recover more than 98% of the ethanol-soluble carbohydrate fraction. The supernatants from these subsequent extractions were pooled and taken to a known final volume using 90% (v/v) ethanol. A 1.5 mL aliquot of the ethanol extract was purified with activated charcoal as described by Hendrix & Peelen (1987). Three 0.25 mL replicates of the resulting clear alcoholic extract were transferred to a microwell plate and dried using a speedvac system (SC210A, RT4104 and VP100; Savant Instruments Inc., Farmingdale, NY, USA).

The glucose, fructose and sucrose contents were determined from the dried ethanol extract using a continuous enzymatic substrate assay adapted for microwell plates (Stitt *et al.* 1989; Hendrix 1993). The pellet from the final centrifugation of the ethanolic extraction was dried at 60 °C in an oven and starch was extracted using 32% (v/v) perchloric acid as described by Farrar (1993) and assayed using a phenol-sulphuric acid assay (Dubois *et al.* 1956). The total non-structural carbohydrate (TNC) content was calculated as the sum of the starch and ethanol-soluble carbohydrate fractions.

Carbohydrate export was estimated by mass balance. The amount of carbon accumulated in the leaf during the photoperiod was calculated from the dusk and pre-dawn measures of TNC. Export was calculated by subtracting the carbon accumulated from  $A'$  expressed as mmol glucose equivalents.

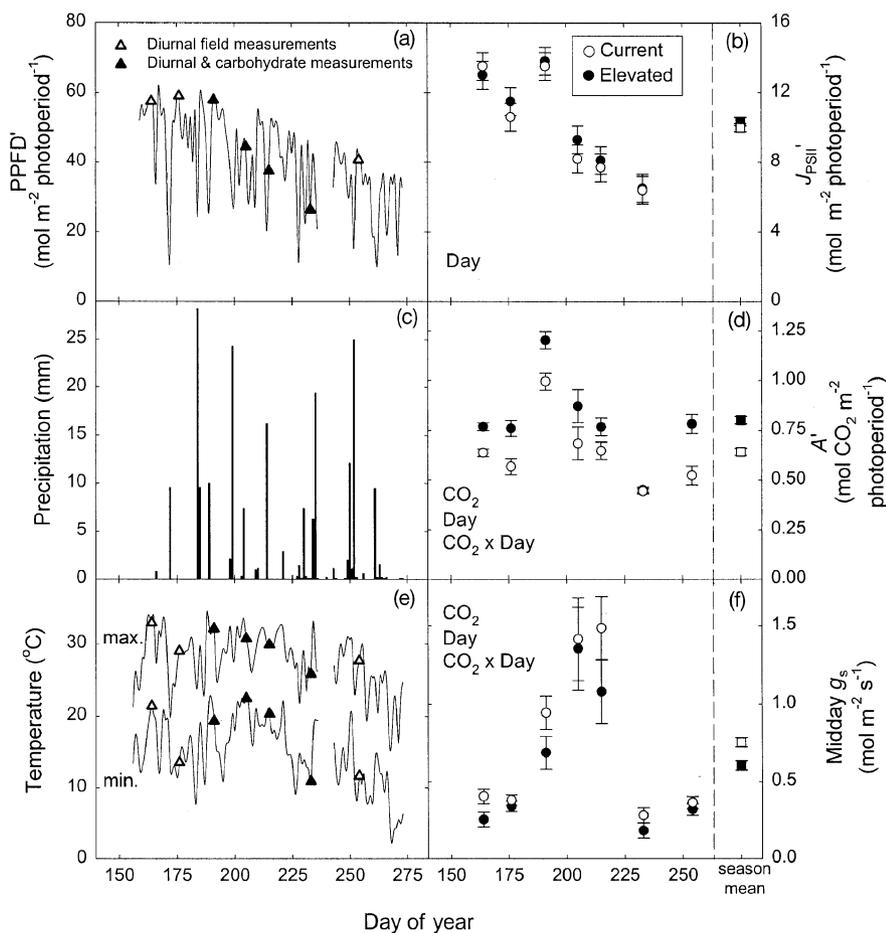
### Statistical analysis

All statistics are based on the plot as the sample unit; thus, although three to five leaves were measured in each plot at each time point, these values were averaged to provide the sample estimate for that replicate. For all comparisons of measured parameters across the season (see Figs 1, 4, 5 & 6), a mixed-model repeated-measures analysis was used

with day of year (DOY), treatment, and the DOY-by-treatment interaction as fixed effects. For comparisons within each day it was necessary to account for correlations between time points; therefore, a repeated-measures approach was used for all variables (see Figs 2 & 3) with time of day, treatment and time of day-by-treatment interaction as fixed effects. For all comparisons, variance/covariance matrices were constructed and for each variable Akaike's information criterion (AIC) was used in selecting the appropriate matrices (Akaike 1974; Keselman *et al.* 1998; Littell, Henry & Ammerman 1998; Littell, Pendergast & Natarajan 2000). The covariance structure was modelled using SAS software (SAS Institute, Cary, NC, USA). *A priori* pairwise comparisons of elevated versus ambient [CO<sub>2</sub>] within days were made via linear contrasts of the least-square means (ESTIMATE; SAS Institute).

### RESULTS

Diurnal measurements were made on dates representative of the range of weather experienced (Fig. 1), with measurements on both clear sky days in which the integral of incident photon flux ( $PPFD'$ ) was maximal for the time in the growing season (e.g. DOY 176), and on overcast days with a  $PPFD'$  that was minimal for the point in the growing season (e.g. DOY 233; Fig. 1a). Measurements were also

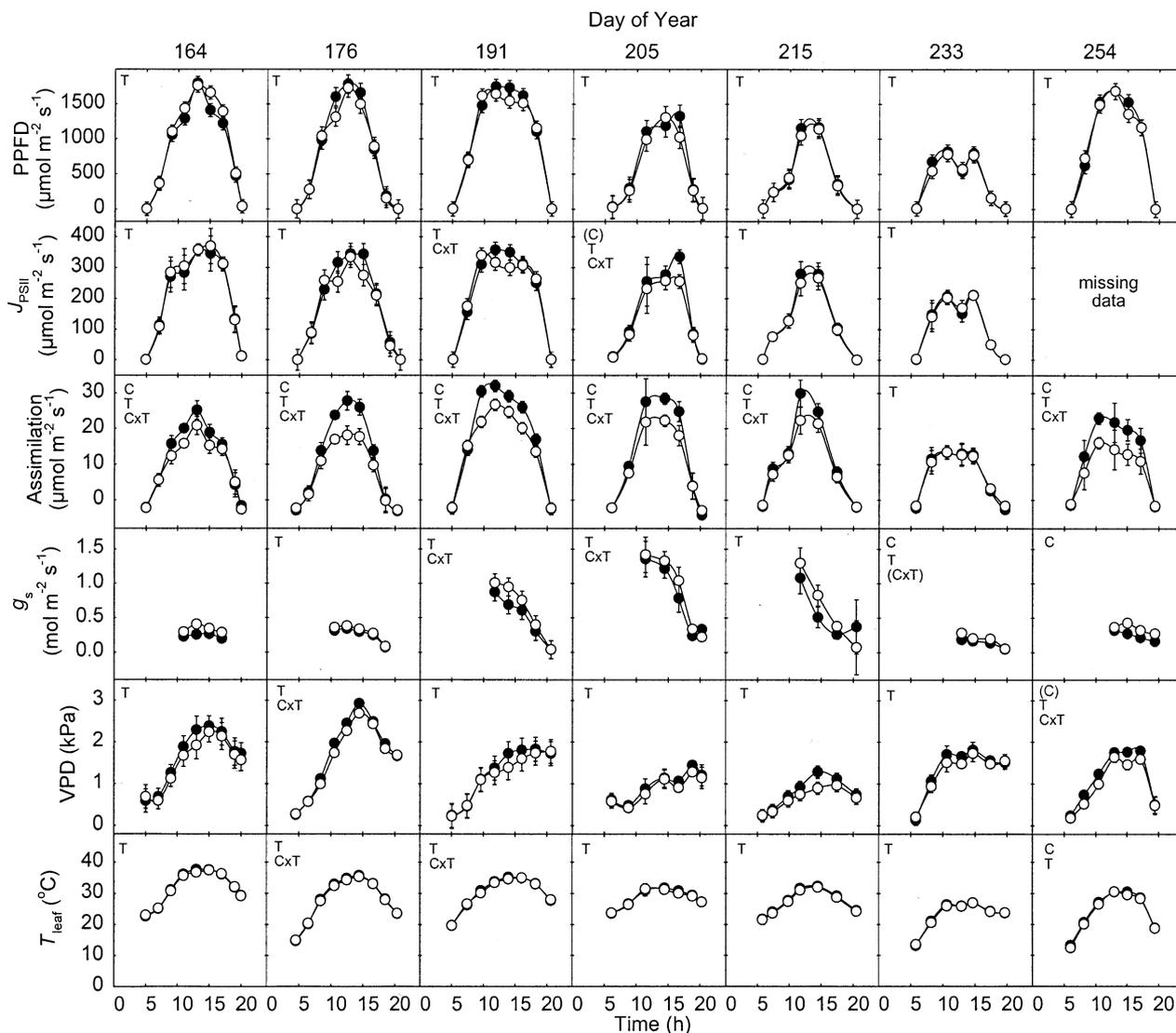


**Figure 1.** The daily integral of photosynthetic photon flux density ( $PPFD'$ ), a), daily precipitation (c) and minimum and maximum daily temperature (e) recorded at the field site. Open triangles indicate days upon which measurements were made throughout a photoperiod [presented in Fig. 2 and in panels (b), (d) and (f)], filled triangles show the days when these diurnal measurements were supplemented by carbohydrate analysis (presented in Figs 3–6). (b), (d) and (f) show the daily integral of photosystem II electron transport ( $J_{PSII}$ ), b), the daily integral of net CO<sub>2</sub> assimilation ( $A'$ ), d) and midday stomatal conductance (Midday  $g_s$ ), f). These measurements were made *in situ* on the terminal leaflets of the most recently fully expanded trifoliate leaves of soybeans grown in the field at elevated [CO<sub>2</sub>] (552  $\mu\text{mol mol}^{-1}$ , ●) and current [CO<sub>2</sub>] (372  $\mu\text{mol mol}^{-1}$ , ○). The season mean for elevated [CO<sub>2</sub>] and current [CO<sub>2</sub>] are shown at the right-hand-side of panels (b), (d) and (f) as filled and open squares, respectively. Significant ( $P < 0.05$ ) effects of treatment (CO<sub>2</sub>), day of year (DOY) and the interaction (CO<sub>2</sub> × DOY) are indicated in the panels. Data are least-square means  $\pm$ SE of the difference in means.

made both immediately after (e.g. DOY 254) and several days after (e.g. DOY 164) a significant precipitation event (Fig. 1c). Mean air temperature declined from about 27 °C on the first measurement day to 19 °C by the last, when minimum temperature had dropped close to 10 °C (Fig. 1e). Although maximum air temperature did not exceed 32 °C, average leaf temperature peaked at 40 °C on DOY 164 and peaked at over 30 °C even as late as DOY 254 (Fig. 2). Leaf temperature averaged over all dates and treatments was 27.5 °C.

Integrated over the photoperiod, the mean leaf CO<sub>2</sub> assimilation (*A'*) for the season was 24.6% higher in elevated [CO<sub>2</sub>] (Fig. 1d). With the exception of DOY 233, *A* was always higher in elevated [CO<sub>2</sub>] for the measurements

made around mid-day, with little evidence of any treatment effect in the early morning and late afternoon, as reflected in the significant interaction between treatment and time of day (Fig. 2). Across all time points when mean PPFD exceeded 1000 μmol m<sup>-2</sup> s<sup>-1</sup> (Fig. 2), mean *A* before solar noon was 18.2 and 24.2 μmol m<sup>-2</sup> s<sup>-1</sup> for current ambient and elevated [CO<sub>2</sub>], respectively, and 16.7 and 21.5 μmol m<sup>-2</sup> s<sup>-1</sup> after solar noon. There was no evidence that this afternoon decline was any greater in elevated [CO<sub>2</sub>]. There was considerable variation in stimulation of *A'* across dates, ranging from no stimulation on DOY 233 to a 49.5% increase on DOY 254. There was a significant interaction between DOY and CO<sub>2</sub>, but with no obvious seasonal trend (Figs 1d & 2). An average value of stomatal conductance



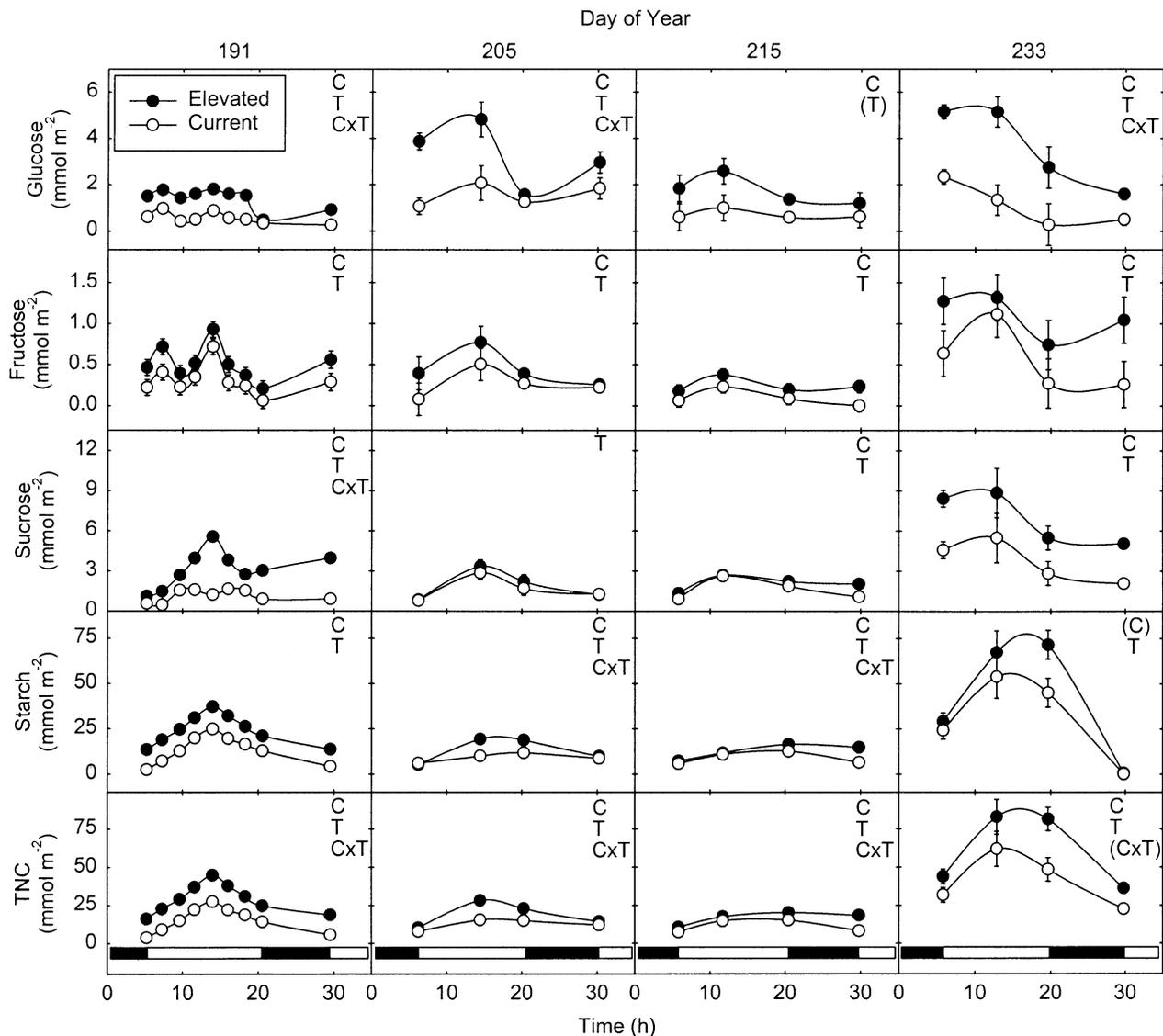
**Figure 2.** Diurnal measurements of photosynthetic photon flux density (PPFD), the rate of photosystem II electron transport ( $J_{\text{PSII}}$ ), net CO<sub>2</sub> assimilation (Assimilation) stomatal conductance ( $g_s$ ), vapour pressure deficit (VPD) and leaf temperature ( $T_{\text{leaf}}$ ), in the plants described in Fig. 1. Diurnal measurements were made on seven occasions during the development of the crop (day of year 164, 176, 191, 205, 215, 233 and 254; measurement days are indicated by triangles in Fig. 1). Stomatal conductance could not be reliably estimated before 1000 hours due to dew on the leaves in the early morning. Data are least-square means  $\pm$ SE of the difference in means (in some cases obscured by the symbol). Significant ( $P < 0.05$ ) effects of CO<sub>2</sub> treatment, time and the interaction of CO<sub>2</sub> and time are indicated on the individual panels by C (CO<sub>2</sub>), T (Time) and CxT (interaction), respectively. Letters in parentheses indicate a significant effect at  $P < 0.1$ .

( $g_s$ ) for each diurnal period could not be calculated because dew precluded measurement of water vapour flux on most mornings (Fig. 2). However, mid-day  $g_s$  was 21.9% lower, averaged over all dates (Fig. 1f). Although a 4.5% increase in  $J_{PSII}$  was indicated across all dates, this was not significant (Fig. 1b). However, there was a significant interaction between treatment and time of day on two dates, with an increase in  $J_{PSII}$  around mid-day in elevated  $[CO_2]$  (Fig. 2).

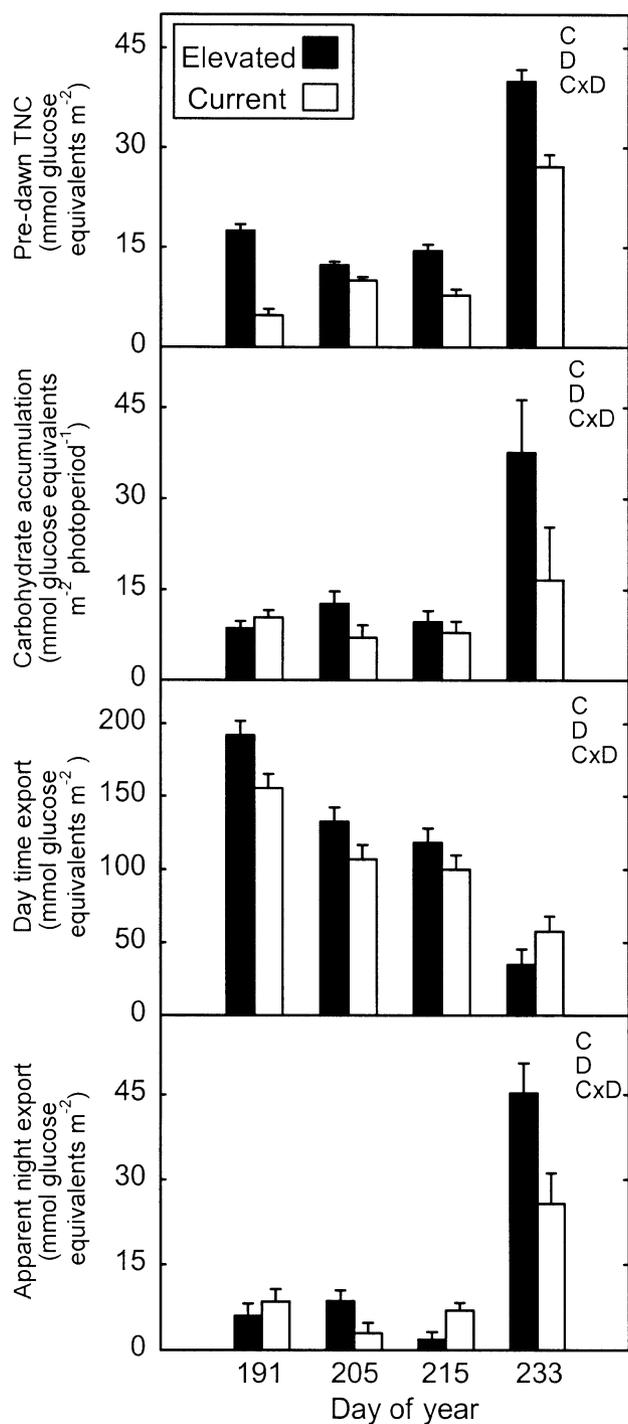
Elevated  $[CO_2]$  resulted in a significant increase in the levels of all foliar carbohydrate fractions examined (Fig. 3). Starch was the principal carbohydrate stored, comprising approximately 80% of the total non-structural carbohydrate content (Fig. 3). The significant effect of increased  $[CO_2]$  on foliar carbohydrate levels was also evident in leaves harvested before the beginning of the photoperiod.

Levels of pre-dawn TNC showed a significant increase in soybeans grown in elevated  $[CO_2]$  (Fig. 4). Levels of leaf carbohydrate were generally higher towards the end of the season (Fig. 3, DOY 233), and examination of pre-dawn TNC content showed a significant effect of DOY on pre-dawn TNC content. Levels at both current and elevated  $[CO_2]$  were markedly greater on DOY 233 than earlier in the season (Fig. 4). In addition to higher absolute levels of TNC, plants measured on DOY 233 also showed a greater accumulation of foliar carbohydrate; this trend was most marked in soybeans grown in elevated  $[CO_2]$ , in which carbohydrate accumulation on DOY 233 was over three-fold greater than earlier in the season (Fig. 4).

There was a significantly greater apparent export of photosynthate from soybean leaves grown at elevated  $[CO_2]$



**Figure 3.** Glucose, fructose, sucrose, starch and total non-structural carbohydrate (TNC) content in the plants described in Fig. 2, measured during a 24 h period on four occasions during development (day of year 191, 205, 215 and 233) indicated by filled triangles in Fig. 1. The photoperiod is indicated by a white/black bar. Statistically significant ( $P < 0.05$ ) effects of  $CO_2$  treatment (C), time of day (T) and the interaction of  $CO_2$  treatment and time (CxT) are indicated in the top right-hand corner of each panel. Data are least-square means  $\pm$  SE of the difference in means.

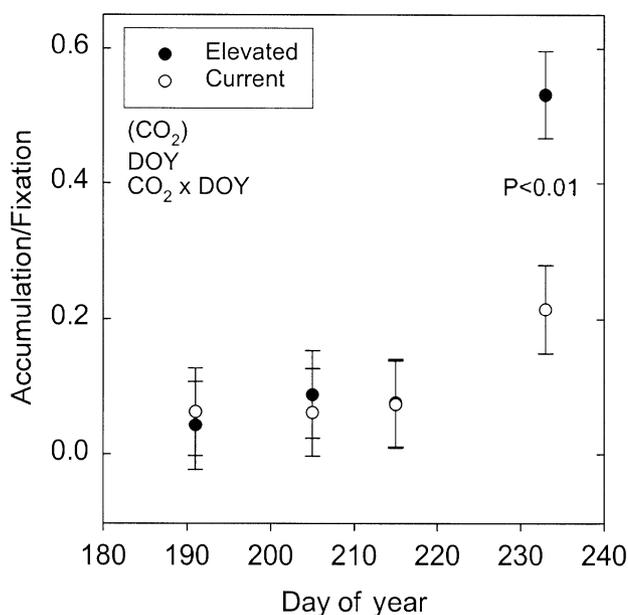


**Figure 4.** Pre-dawn total non-structural carbohydrate content (Pre-dawn TNC), carbohydrate accumulation, daytime carbohydrate export and apparent night export rate in the plants described in Fig. 3. Apparent night export rate is not corrected for respiratory carbon losses. Significant ( $P < 0.05$ ) effects of  $\text{CO}_2$  treatment, day of year and the interaction of  $\text{CO}_2$  and day of year are indicated on the individual panels by C ( $\text{CO}_2$  treatment), D (day of year) and CxD (interaction), respectively. Data are least-square means  $\pm$ SE of the difference in means.

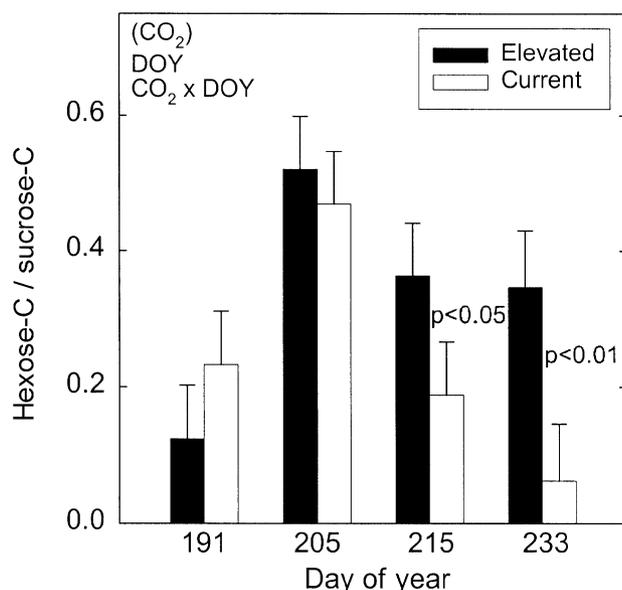
(Fig. 4). Total daytime export decreased with advancing date in the season, in parallel with declines in  $PPFD'$ ,  $J_{PSII}'$ , and  $A'$  (Fig. 1). Most of the fixed carbon was exported early in the measurement period; however, on DOY 233 more carbohydrate was accumulated during the photoperiod and less was exported. Despite this large daytime accumulation, respiration and night-time export had removed all this accumulation by dawn (Figs 3 & 4). Figure 5 shows that in the earlier phases of growth more than 90% of carbon fixed was respired and/or exported during the photoperiod, and there was no effect of  $\text{CO}_2$  treatment on the fraction of carbon exported. However, on DOY 233 when fixation over the day was lower (Fig. 1) and accumulation greatest (Fig. 4), there was a significant increase at elevated [ $\text{CO}_2$ ] where over 50% of fixed carbon was stored in the leaf during the photoperiod at elevated [ $\text{CO}_2$ ], compared with 21% at current [ $\text{CO}_2$ ] (Fig. 5). There was also a significantly higher ratio of hexose-carbon to sucrose-carbon at elevated [ $\text{CO}_2$ ] in leaves sampled at the end of the photoperiod (Fig. 6). This increase in the hexose-C to sucrose-C ratio at elevated [ $\text{CO}_2$ ] on DOY 233 occurred at the same time as an apparent sink limitation of carbon export (Figs 4 & 5) and the loss of stimulation in photosynthesis (Figs 1 & 2).

## DISCUSSION

Photosynthetic  $\text{CO}_2$  assimilation in soybean under Free-Air  $\text{CO}_2$  Enrichment shows a significant and sustained increase across the growing season, except at one point in time.



**Figure 5.** Ratio of foliar carbon accumulation to carbon fixation for the plants described in Fig. 3. Significant ( $P < 0.05$ ) effects of  $\text{CO}_2$  treatment, day of year and the interaction of  $\text{CO}_2$  and day of year are indicated by  $\text{CO}_2$ , DOY and  $\text{CO}_2 \times \text{DOY}$ , respectively. Parentheses indicate a significant effect at  $P < 0.1$ . Data are least-square means  $\pm$ SE of the difference in means.  $P < 0.01$  indicates a highly significant difference between means on day of year 233, based on a linear pairwise contrast.



**Figure 6.** Ratio of leaf hexose-carbon to sucrose-carbon, in leaves sampled at dusk from the plants described in Fig. 3. Significant ( $P < 0.05$ ) effects of  $\text{CO}_2$  treatment, day of year and the interaction of  $\text{CO}_2$  and day of year are indicated by  $\text{CO}_2$ , DOY and  $\text{CO}_2 \times \text{DOY}$ , respectively. Parentheses indicate a significant effect at  $P < 0.1$ . Data are least-square means  $\pm$  SE of the difference in means.  $P$  values indicate significant differences on individual days, based on linear pairwise contrast.

Rather than diminishing with time, the greatest stimulation was actually observed on the final date of measurement, which was during late seed filling. Similarly, the expected decrease in stomatal conductance persisted throughout the season. The soybeans were heavily nodulated and the cultivar examined was indeterminate. Both factors should maximize the ability of the plant to use additional photosynthate, and nodulation should minimize the possibility of N-limitation. There was no obvious evidence that relative stimulation declined in the early afternoon relative to the late morning, as would be expected had increased accumulation of carbohydrates at elevated  $[\text{CO}_2]$  led to limitation of triose phosphate utilization (Fig. 2). This contrasts with a similar study with wheat (Garcia *et al.* 1998), a strongly determinate crop that lacks any nitrogen-fixing association. Lack of any afternoon loss of stimulation in the soybean crop is also consistent with the fact that there was no increased accumulation of carbohydrates (except DOY 233) relative to total assimilation in response to elevated  $[\text{CO}_2]$  (Fig. 5); again in contrast to wheat grown in FACE (Nie *et al.* 1995b). Nevertheless, stimulation of photosynthesis was evidently lost on DOY 233 (phenological stage R3–R4, i.e. the stages of pod formation, but preceding rapid seed growth) when apparent daytime export rate (Fig. 4) and the accumulation:fixation ratio (Fig. 5) suggested a marked reduction in sink capacity. There may be multiple causes for this loss of stimulation. First, photon flux was lower on this date than on any other measurement date (Fig. 1a). In general, stimulation of  $A$  was minimal on all dates at times when photon flux was less than  $1000 \mu\text{mol}$

$\text{m}^{-2} \text{s}^{-1}$  (Fig. 2), and photon flux was less than this value throughout DOY 233. Second, the possibility that the plants were transiently sink limited at this stage is supported by the high pre-dawn carbohydrate concentration and a large daytime accumulation of carbohydrate relative to fixation (Figs 4 & 5). There was also a five-fold greater ratio of hexose-C : sucrose-C compared with control plants on this day (Fig. 6), which is hypothesized to indicate sink limitation (Moore *et al.* 1999).

Observations of increased foliar carbohydrate content in plants grown in elevated  $[\text{CO}_2]$  are well documented, including soybean, in which growth at elevated  $\text{CO}_2$  resulted in a 45% significant increase in  $TNC$  (Ainsworth *et al.* 2002). Although the large increases in starch (approximately 110%) reported by these authors exceed the maximum increase observed in this study (approximately 60%, Fig. 3), this may be explained by the growth of the crop in our study at  $552 \mu\text{mol mol}^{-1}$ , compared with an average close to  $700 \mu\text{mol mol}^{-1}$  across the studies included in the meta-analysis by Ainsworth *et al.* (2002).

Integrated over the growing season,  $A$  was increased by 24.6%. The mean leaf temperature over all measurements was  $27.5^\circ\text{C}$ . Applying the temperature corrections of Bernacchi *et al.* (2001) and Bernacchi, Pimentel & Long (2003) to the model of Farquhar *et al.* (1980), an increase in  $[\text{CO}_2]$  from 372 to  $552 \mu\text{mol mol}^{-1}$  at  $27.5^\circ\text{C}$  would increase  $A$  by between 19.7 and 44.5%, depending on whether RubP or Rubisco were limiting, respectively. This assumes no acclimation in the capacities for Rubisco- or RubP-limited photosynthesis. The mean increase observed here is between these values, suggesting either a reduction in carboxylation capacity or that RubP-limitation may have prevailed. Another alternative might be that the control of photosynthesis shifted from Rubisco limited in current  $[\text{CO}_2]$  towards RubP-limited in elevated  $[\text{CO}_2]$ . The lack of an effect of elevated  $[\text{CO}_2]$  on whole chain electron transport ( $J_{\text{PSII}}$ ) is consistent with a RubP-regeneration limitation of photosynthesis.  $J_{\text{PSII}}$  would increase with increase in  $[\text{CO}_2]$  if Rubisco were limiting, since the enzyme is not saturated at current ambient  $[\text{CO}_2]$ . If RubP-limited, an increase in  $[\text{CO}_2]$  simply increases the partitioning of RubP and electron transport toward carboxylation and away from oxygenation and photorespiratory metabolism, such that  $J_{\text{PSII}}$  will remain constant (Hymus *et al.* 2001). It is notable that while theory predicts that a stimulation of  $A$  would be expected at all light levels, stimulation was apparent only when photon flux was above  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Fig. 2). In early morning, evening and on overcast days (DOY 233), stimulation was not apparent. This has also been observed in diurnal measurements of  $A$  in both *Triticum aestivum* and *Lolium perenne* crops with long-term growth in FACE (Garcia *et al.* 1998; Ainsworth *et al.* 2003).

In conclusion, the expected significant increase in leaf photosynthesis and decrease in stomatal conductance at elevated  $[\text{CO}_2]$  were observed in field-grown soybean across the growing season. Despite the fact that soybean has a nitrogen-fixing association and an indeterminate growth pattern, and that  $\text{CO}_2$  elevation was under open-air

conditions and without any limitation on rooting volume, there was a complete loss of the stimulation of photosynthesis by elevated CO<sub>2</sub> at one point in the growing season, and overall a lower than predicted stimulation of photosynthesis. This indicates that even highly productive plants, in the absence of any restrictions on root growth, can show a loss of photosynthetic capacity during certain conditions under elevated [CO<sub>2</sub>].

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