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Elevated concentrations of atmospheric CO_2 protect against and compensate for O_3 damage to photosynthetic tissues of field-grown wheat

I. F. McKEE[1]*, B. J. MULHOLLAND[2], J. CRAIGON[3], C. R. BLACK[3] AND S. P. LONG[4]

[1]*Department of Biological Sciences, John Tabor Laboratories, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, UK*
[2]*Horticulture Research International, Department of Soil and Environment Sciences, Wellesbourne, Warwick CV35 9EF, UK*
[3]*School of Biological Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK*
[4]*University of Illinois, Departments of Crop Sciences and Plant Biology*, 190 *Edward R. Madigan Laboratory, 1201 West Gregory Drive, Urbana IL61801, USA*

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SUMMARY

The effects of elevated concentrations of atmospheric carbon dioxide and ozone on diurnal patterns of photosynthesis have been investigated in field-grown spring wheat (*Triticum aestivum*). Plants cultivated under realistic agronomic conditions, in open-top chambers, were exposed from emergence to harvest to reciprocal combinations of two carbon dioxide and two ozone treatments: $[CO_2]$ at ambient (380 μmol mol^{-1}, seasonal mean) or elevated (692 μmol mol^{-1}) levels, $[O_3]$ at ambient (27 nmol mol^{-1}, 7 hr seasonal mean) or elevated (61 nmol mol^{-1}) levels. After anthesis, diurnal measurements were made of flag-leaf gas-exchange and *in vitro* Rubisco activity and content. Elevated $[CO_2]$ resulted in an increase in photoassimilation rate and a loss of excess Rubisco activity. Elevated $[O_3]$ caused a loss of Rubisco and a decline in photoassimilation rate late in flag-leaf development. Elevated $[CO_2]$ ameliorated O_3 damage. The mechanisms of amelioration included a protective stomatal restriction of O_3 flux to the mesophyll, and a compensatory effect of increased substrate on photoassimilation and photosynthetic control. However, the degree of protection and compensation appeared to be affected by the natural seasonal and diurnal variations in light, temperature and water status.

Key words: carbon dioxide, ozone, *Triticum aestivum*, photosynthesis, Rubisco, air pollution, wheat.

INTRODUCTION

The global atmospheric CO_2 concentration has risen by over 30% from pre-industrial levels of *c.* 280 μmol mol^{-1} (Barnola *et al.*, 1995). The upward trend is driven by anthropogenic emissions (Keeling *et al.*, 1995) and, if current rates of emission continue, atmospheric $[CO_2]$ will have risen by *c.* 80% by the end of the twenty-first century (IPCC, 1996). Concurrent emissions of nitrogen oxides and volatile organic compounds have led to rising background concentrations of tropospheric O_3, particularly in the northern hemisphere. European rural concentrations have approximately doubled over the last century and are currently increasing at a rate of 1–2% yr^{-1} (PORG, 1997).

The experimental effects of elevated $[CO_2]$ on crop growth and yield are generally beneficial (Cure, 1985), whereas O_3 is harmful, even at present levels (Heagle, 1989). However, the individual effects of these pollutants on crop growth and yield are not

*Author for correspondence (tel +44 1206 873 325; fax +44 1206 873 416; e-mail Ian.McKee@essex.ac.uk).

simply additive in combined exposures (Barnes & Pfirrmann, 1992; Mulchi *et al.*, 1992; Reinert & Ho, 1995; Fiscus *et al.*, 1997; McKee *et al.*, 1997a; Mulholland *et al.*, 1997a; Heagle *et al.*, 1998).

In combination, ozone damage to photosynthetic tissues is ameliorated by elevated [CO_2], and recent studies have examined the mechanisms behind this interaction (Kramer *et al.*, 1991; Polle *et al.*, 1993; McKee *et al.*, 1995, 1997b; Rao *et al.*, 1995; Booker *et al.*, 1997; Volin *et al.*, 1998; Wieser *et al.*, 1998). In controlled-environment studies, in which spring wheat was exposed to chronically elevated concentrations of CO_2 and/or O_3, the effect of elevated [O_3] on photosynthesis resulted mainly from a loss of Rubisco activity (McKee *et al.*, 1995, 1997b). This led to a decline in photoassimilation rate, which in turn fed back on stomatal conductance. Elevated [CO_2] caused a down-regulation of excess Rubisco activity and partially protected against O_3 damage via a decline in stomatal conductance, restricting the flux of O_3 to the mesophyll. It also compensated for O_3 damage via an increase in photoassimilation rate and a shift in photosynthetic control from Rubisco limitation to RuBP-regeneration limitation at high substrate levels.

Having observed these protective and compensatory mechanisms under stable conditions in controlled-environment experiments, it would not be correct to assume that the same mechanisms pertain under all natural field conditions. It is essential both to confirm their operation in the field and to investigate their efficacy and interaction with other diurnal and seasonal variables. These were the aims of the field experiment presented here.

MATERIALS AND METHODS

Crop growth conditions

Spring wheat (*Triticum aestivum* L. cv. Minaret) was drilled into a cultivated sandy loam of the Astley Hall series at the Sutton Bonington Campus, University of Nottingham, UK (lat 52°N, long 1° 15′W) on 20 March 1995. The mean emergence density was 371 plants m^{-2}. Before emergence, open-top chambers (OTCs) were erected over the crop; these were 2.4 m high with a 45° frustrum, 3.1 m in diameter, and spaced to avoid co-shading. The chambers were clad in 200-mm PVC and the lower, double-skinned section formed a 1-m-high circumferential ventilation plenum distributing air from a fan box (PSA 402/2, Jones & Attwood, Stourbridge, UK). Further details of crop management are given elsewhere (Mulholland *et al.*, 1997a).

This experiment involved four chambered treatments and an unchambered control treatment. All five treatment plots were replicated in three randomized blocks. From the time of emergence, the chambered plots were treated with reciprocal combinations of ambient and elevated [CO_2], and ambient and elevated [O_3]. Air was sampled through PTFE tubing from one replicate per treatment at 1.2 m above ground level, and a roving line was used on each treatment day to cross-check unsampled plots. The air from each treatment was analysed for O_3 and CO_2 using a UV-absorption [O_3] analyser (Model 8810, Monitor Labs, San Diego, CA, USA) and an infrared [CO_2] analyser (Model 225 MK3, ADC, Hoddesdon, UK). CO_2 was supplied from a 25-tonne storage tank (Hydrogas, Middlesex, UK) and O_3 was generated from pure O_2 with an electrical discharge generator (Model LN103, Ozonia, Duebendorf, Switzerland). Pollutant concentrations in elevated treatments were adjusted manually with flow controllers.

The target pollutant concentrations for elevated plots were 680 μmol mol^{-1} [CO_2] and roughly three times ambient [O_3], up to a maximum of 130 nmol mol^{-1}. Elevated [CO_2] was maintained continuously, while elevated [O_3] was maintained 09:00–16:00 hours GMT, for 5 d wk^{-1}. Seasonal means for ambient and elevated [CO_2] were 380 and 692 μmol mol^{-1}, respectively. Seven-hour seasonal means, including nontreatment days, for ambient and elevated [O_3] were 27 and 61 nmol mol^{-1}, respectively.

Sampling intervals

Photosynthetic measurements were made on three dates post anthesis: Day I, which was midsummer day, 93 d after sowing (das), and subsequently at weekly intervals: Day II at 100 das and Day III at 107 das. On days I and II, measurements were made of the diurnal gas-exchange patterns of flag leaves. On days I and III, midday A/C_i curves were plotted. On Day I, flag leaves were sampled for *in vitro* analysis of diurnal patterns of Rubisco activity, activation and content; the remaining mainstem leaves were also sampled for analysis of canopy profiles of Rubisco content. For diurnal time courses, samples were taken at 05:00, 09:00, 12:00, 16:00 and 19:00 hours GMT. In all cases, replication was threefold.

Gas exchange

Gas-exchange measurements were made using three portable gas analysers (CIRAS-I, PP Systems, Hitchen, UK), one per replicate block. The analysers were calibrated against [CO_2] standards (Linde Gas UK Ltd, Stoke-on-Trent, UK). For diurnal time courses, a single flag leaf from each of the five treatments in each block was measured in random order during 100-min periods around each leaf sampling time. Photosynthetic photon fluence rate (PPFR) and temperature within the leaf chamber varied with external conditions, while vapour pressure deficit was controlled at *c.* 0.7 kPa.

On introducing the leaf to the cuvette, the first stable reading was taken for stomatal conductance (g_s), to obtain a measurement as close as possible to growth conditions. The leaf was allowed to acclimatize in the cuvette at growth [CO_2] for 15 min, then a second reading was taken for steady-state assimilation rate (A), intercellular [CO_2] (C_i), and stomatal conductance. A/C_i analysis was conducted between 10:45 and 13:15 hours GMT. Following acclimatization in the cuvette at growth [CO_2], measurements were made at nine CO_2 concentrations from 75–900 μmol mol^{-1}. Data were used to fit models of Rubisco-limited and RuBP-regeneration-limited photoassimilation (von Caemmerer & Farquhar, 1981) to estimate the *in vivo* maximum carboxylation velocity (V_{cmax}) and electron transport rate (J), using kinetic parameters and temperature corrections according to Jordan & Ogren (1984). Boundary-layer conductance was estimated for each treatment plot using blotting paper models and C_i was estimated for the uncuvetted leaf as described by McKee & Woodward (1994) to establish the photosynthetic limitation operating under treatment conditions.

In vitro *analyses*

For each time-point, duplicate flag-leaf samples were taken from each treatment in each replicate block, rapidly frozen and stored in liquid nitrogen. The procedures for the extraction and enzymatic assay of Rubisco activity and activation were based on those outlined by McKee *et al*. (1995). Chemicals were obtained from the Sigma Chemical Company UK, Ltd. (Poole, UK).

The extraction buffer contained: 100 mol m^{-3} 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) adjusted to pH 8; 20 mol m^{-3} $MgCl_2$; 50 mol m^{-3} DTT; 1% BSA; 0.2% polyvinylpolypyrrolidone (PVPP) by mass. Leaf samples with measured areas of *c*. 500 mm^2 were extracted in 5000 mm^3 of buffer, in liquid nitrogen, in a mortar. Each homogenate was rapidly thawed by agitation and immediately centrifuged for 5 min at 15000 ***g*** and 0°C. A sample of the supernatant was carried on ice for immediate photometric assay of Rubisco activity and activation state.

The assay buffer contained: 100 mol m^{-3} N,N-bis(2-hydroxyethyl)glycine (BICINE) adjusted to pH 8.2; 50 mol m^{-3} DTT; 10 mol m^{-3} $NaHCO_3$; 20 mol m^{-3} $MgCl_2$; 20 mol m^{-3} NaCl; 0.66 mol m^{-3} D-ribulose 1,5-bisphosphate (RuBP); 42 mol m^{-3} NADH; 5 mol m^{-3} ATP; 5 mol m^{-3} phosphocreatine; 100 nkat 3-phosphoglyceric phosphokinase (PGK; EC 2.7.2.3); 100 nkat glyceraldehyde-3-phosphate dehydrogenase (GAPdH; EC 1.2.1.12); 200 nkat phosphocreatine phosphokinase (EC 2.7.3.2). For each extract, two assays were conducted, one to measure the initial activity of Rubisco (i.e. the maximum activity measured at, as near as possible, the *in vivo* state of activation), the other to measure the activated activity (i.e. the maximum activity measured after activation of the enzyme with optimal [Mg^{2+}] and [CO_2]). The two assays were conducted at 25°C, in stirred quartz cuvettes. For the initial activity assay, 40 mm^3 of extract was added to 960 mm^3 of buffer. For the activated activity assay, the extract was preincubated with the BICINE, DTT, $NaHCO_3$ and $MgCl_2$ components for 7 min at 25°C before starting the reaction with the other constituents. In each case, the activity of Rubisco was measured using the absorbance change at 340 nm to calculate the rate of NADH depletion which, in this coupled reaction, is double the rate of carboxylation.

From each flag-leaf extract, a subsample was taken for SDS-PAGE. At 12:00 hours GMT, samples were also taken for extraction of all remaining mainstem leaves on the same plants. Protein concentration of the supernatant was estimated by the bicinchonic acid assay system (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. The proportion of protein attributable to Rubisco was determined by SDS-PAGE separation on 10–17.5%-gradient polyacrylamide gels, which were stained with Fast Green stain and scanned using a laser-scanning densitometer (Model 300A, Molecular Dynamics, Sunnyvale, CA, USA). The densitometer was calibrated against Rubisco protein standard curves for each gel, and the gels were loaded so as to keep Rubisco sample densities within the approximately linear calibrated range.

Statistical analysis

Data were analysed using Statview® (SAS Institute Inc., Cary, NC, USA). Where duplicate samples were taken from each replicate plot, for the *in vitro* analyses, the mean was used in statistical analyses. Replication was threefold and consequently, to control for type II error, an alpha value of 0.08 was adopted. For the data from diurnal measurements, the four OTC treatments represented two factors, each with two levels (2 $CO_2 \times 2$ O_3), and the five sampling times represented repeated measures; main factors and interactions were analysed according to a repeated-measures model, using multivariate analysis of variance (MANOVA). At each sampling time, between-factors effects were analysed using Scheffé's *F* procedure. For the data from measurements which were not repeated, multiple comparisons were conducted using Scheffé's *F* procedure and, in the case of V_{cmax} data, factorial ANOVA was performed.

RESULTS

In terms of the commonly reported ozone-exposure index, accumulated ozone exposure over a threshold of 40 nmol mol^{-1} (AOT40), the elevated [O_3] treatments provided a mean accumulated exposure

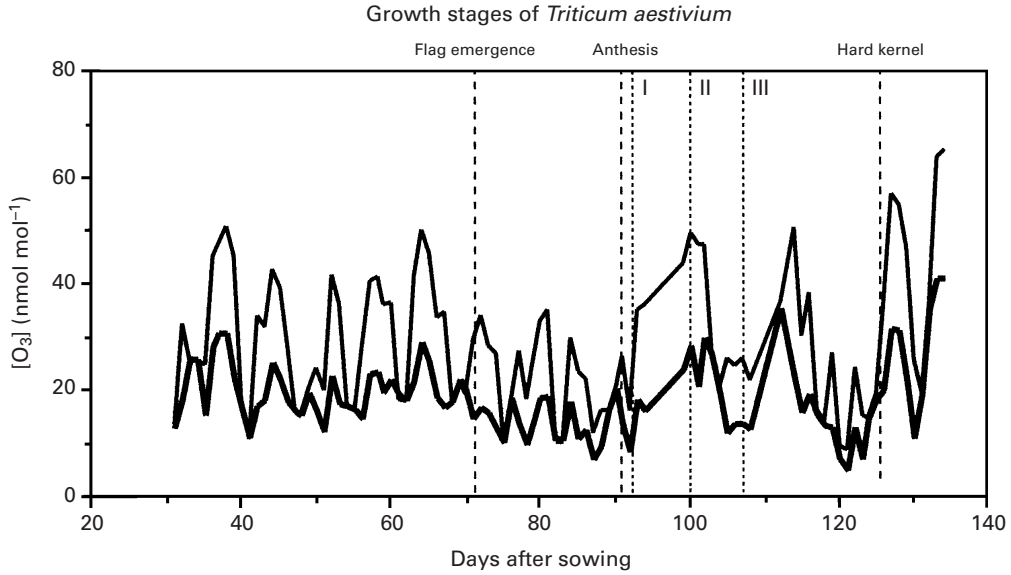

Fig. 1. The time course of daily mean [O_3] within the OTCs at Sutton Bonington, UK over the measurement period: ambient chambers, thick line; elevated chambers, thin line. I, first measurement day, 93 d after sowing (das); II, second measurement day, 100 das; III, third measurement day, 107 das.

of 19084 nmol mol^{-1} h, calculated between dawn and dusk throughout the growth season, compared with 1473 nmol mol^{-1} h for the ambient chambers. Interpretation of the effect of a given AOT40 value requires caution (Grunhage *et al.*, 1999), particularly in OTCs where air is blown through the canopy. As the elevated [O_3] was controlled relative to ambient, a period of comparatively low ozone exposure directly preceded the measurement period, between flag-leaf emergence and anthesis (Fig. 1).

The two days of diurnal measurement, 93 and 100 das, were mostly cloud-free, with two short periods of variable, light cloud before and after noon on Day I (Fig. 2a). Ambient air temperature differed significantly after 08:00 hours, rising to less than 20°C on Day I but over 27°C on Day II (Fig. 2b).

The O_3 treatment had no significant effect on flag-leaf photoassimilation rate, stomatal conductance, or *in vitro* Rubisco activity on either day of diurnal measurement. Therefore, although the full statistical analysis is given, only CO_2 effects are illustrated for clearer presentation (Figs 3, 4).

On both days of diurnal measurement, CO_2 enrichment significantly increased the rate of flag-leaf photoassimilation, the effect being greatest around noon: 43% on Day I and 67% on Day II (Fig. 3a,b). The analysis for Day II revealed a significant interaction between [CO_2] and time (Fig. 3b). On Day I, CO_2 enrichment significantly reduced flag-leaf conductance to water vapour (Fig. 3c); at noon the decrease was 47%. A similar CO_2 effect on conductance was not observed on Day II (Fig. 3d). A significant CO_2 effect on flag-leaf temperature ($P = 0.071$) was detected only at 12:00 hours on Day I (data not shown).

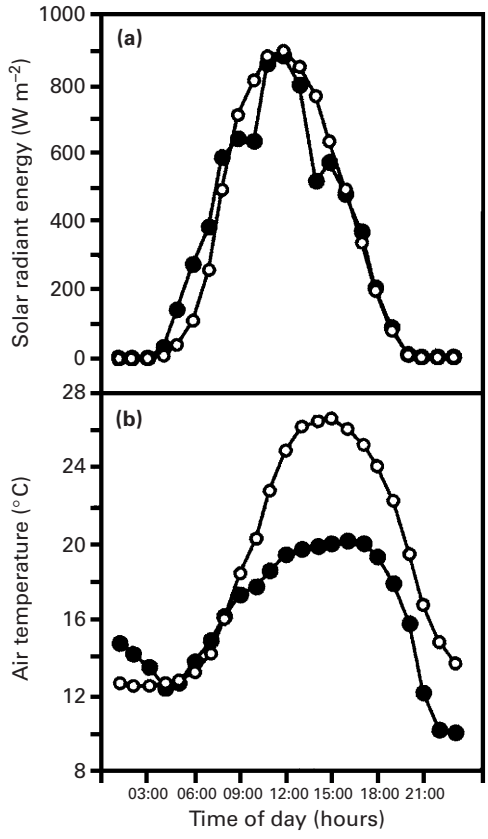

Fig. 2. The diurnal pattern of (a) solar radiant energy incident upon the wheat canopy, and (b) air temperature above the canopy on Day I, first diurnal-measurement day, 93 d after sowing (das) (filled circles) and Day II, second diurnal-measurement day (100 das) (open circles).

On Day I, both the initial and activated activities of Rubisco extracted from flag leaves in the elevated [CO_2] treatment were significantly lower than those

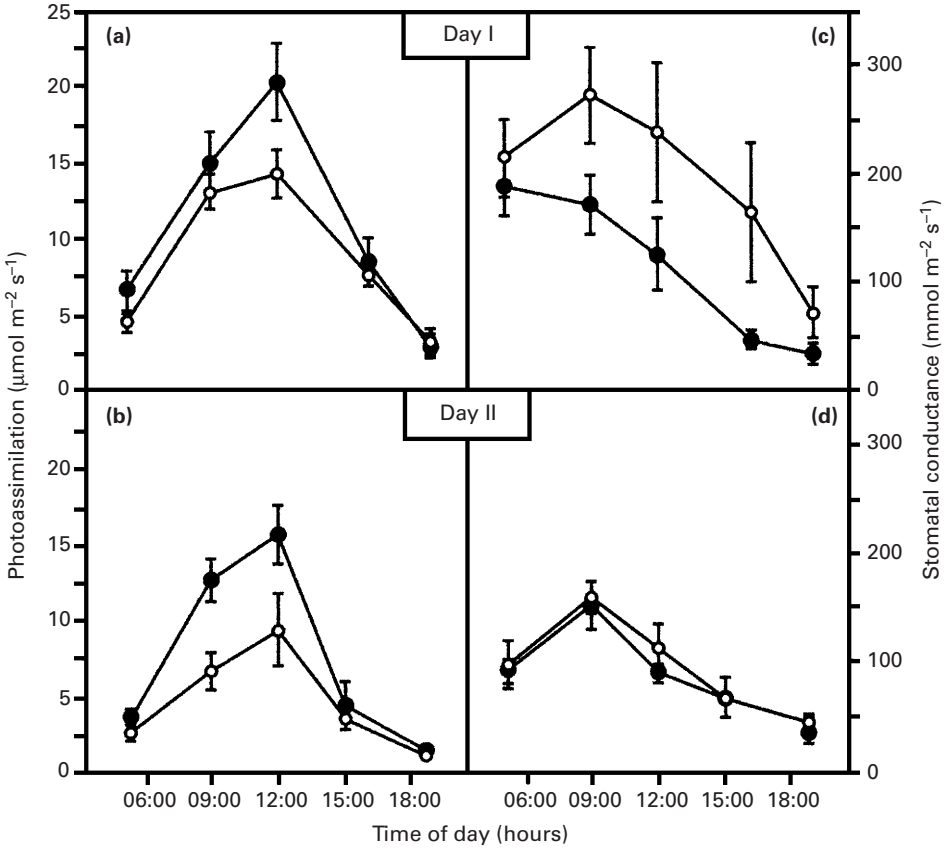

Fig. 3. The effect of elevated [CO_2] on diurnal patterns of photoassimilation (a,b) and stomatal conductance (c,d) in flag leaves of *Triticum. aestivum* on Day I, first diurnal-measurement day, 93 d after sowing (das) (a,c) and Day II, second diurnal-measurement day, 100 das (b,d). Plants were grown throughout their development under ambient (filled circles) or elevated (open circles) [CO_2]. Bars represent SE. (a) MANOVA ($n = 3$): P[CO_2] = 0.045, P Time <0.0001, P[O_3] and all interaction terms were nonsignificant. Scheffé's F test indicated a significant CO_2 effect ($P = 0.067$) at 12:00 hours. (b) MANOVA ($n = 3$): P[CO_2] = 0.025, PTime <0.0001, P[CO_2] × Time = 0.017, P[O_3] and other interaction terms were nonsignificant. Scheffé's F test indicated a significant CO_2 effect at 09:00 hours ($P = 0.009$) and 12:00 hours ($P = 0.063$). (c) MANOVA ($n = 3$): P[CO_2] = 0.055, P Time <0.0001, P[O_3] and all interaction terms were nonsignificant. Scheffé's F test indicated a significant CO_2 effect at 09:00 hours ($P = 0.035$). (d) MANOVA ($n = 3$): P Time <0.0001, P[CO_2], P[O_3] and all interaction terms were nonsignificant. Scheffé's F test indicated no significant CO_2 effect at any time.

from the ambient treatment (Fig. 4). Although the content of Rubisco did not vary through the day in either treatment (data not shown), the activated activity varied significantly with time (Fig. 4).

Flag-leaf Rubisco content was slightly lower in the chambered treatments than in the unchambered control (Fig. 5). In the elevated [CO_2] treatment, Rubisco content was significantly lower than ambient in the flag leaf and in the leaf below it. A significantly negative effect of elevated [O_3] was observed in leaf 5, but not when combined with elevated [CO_2] (Fig. 5).

On Day I, because variable light resulting from intermittent cloud around midday interfered with some A/C_i measurements, replication was insufficient for the data to be used. On Day III, A/C_i curves revealed no significant CO_2 effect on either modelled maximum carboxylation velocity (V_{cmax}) or electron transport rate (J). However, CO_2 enrichment did cause a shift in photoassimilatory control; C_i for the ambient treatment lay in the Rubisco-limited region of the A/C_i curve while C_i for the elevated [CO_2] treatment lay in the RuBP-regeneration-limited region (Fig. 6).

On Day III, a significant decline in modelled V_{cmax} was observed in the elevated [O_3] treatment (Fig. 7), though O_3 had not affected J. Although V_{cmax} for the combined $CO_2 + O_3$ treatment was not significantly different from that in either individual treatment, a protective effect of elevated [CO_2] against ozone damage was demonstrated by a significant interaction term in the ANOVA (Fig. 7).

DISCUSSION

The large increases in midday flag-leaf photoassimilation rate observed under elevated [CO_2] (Fig. 3a,b) were consistent with earlier studies involving large rooting volumes and high nitrogen input (Drake *et al.*, 1997), and were reflected in biomass and yield increases for this crop (Mulholland *et al.*, 1997a). The increase in photo-

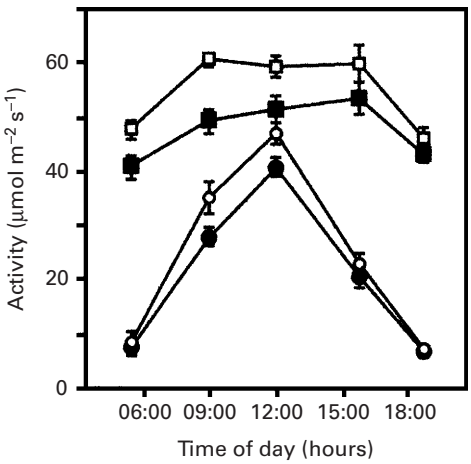

Fig. 4. The effect of elevated [CO_2] on diurnal *in vitro* Rubisco activity in flag leaves of *Triticum aestivum* on Day I (day of first diurnal measurement, 93 d after sowing (das)). Initial activities for plants grown at ambient [CO_2], open circles, or elevated [CO_2], filled circles. Activated activities for plants grown at ambient [CO_2], open squares, or elevated [CO_2], filled squares. Bars represent SE. MANOVA (n = 3) for initial activities: P[CO_2] = 0.012, PTime <0.0001, P[O_3] and all interaction terms were nonsignificant. Scheffé's F test indicated a significant CO_2 effect at 09:00 hours (P = 0.057) and 12:00 hours (P = 0.033). MANOVA (n = 3) for activated activities: P[CO_2] = 0.0003, P Time <0.0001, P[O_3] and all interaction terms were nonsignificant. Scheffé's F test indicated significant CO_2 effects at 05:00 hours (P = 0.035), 09:00 hours (P = 0.0009) and 12:00 hours (P = 0.032).

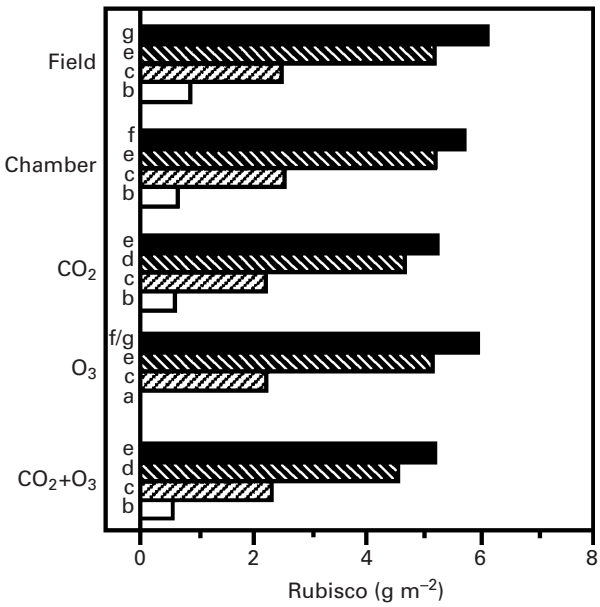

Fig. 5. The effect of elevated [CO_2] and/or elevated [O_3] on the Rubisco content of *Triticum aestivum* on Day I (first diurnal measurement day, 93 d after sowing): leaf 8 (flag), solid bars; leaf 7, dark-hatched bars; leaf 6, light-hatched bars; leaf 5, open bars. Each mean represents samples from three replicate chambers. Different letters indicate significant differences according to Scheffé's F test, P <0.08.

assimilation occurred despite a slight loss of Rubisco content and *in vitro* activity (Figs 4, 5), which represented acclimation rather than damage, because

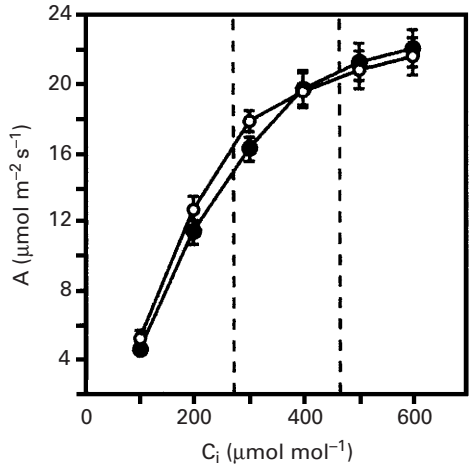

Fig. 6. The effect of elevated [CO_2] on the response of photoassimilation rate (A) to intercellular [CO_2] (C_i) in the flag leaves of *Triticum aestivum* on Day III (third day of measurement, 107 d after sowing). The plants were grown throughout their development under ambient (open circles) or elevated (filled circles) [CO_2]. Each mean represents the modelled interpolation of measurements from three replicate OTCs. Bars represent SE. Dashed lines indicate C_i calculated for ambient and elevated [CO_2] under OTC conditions.

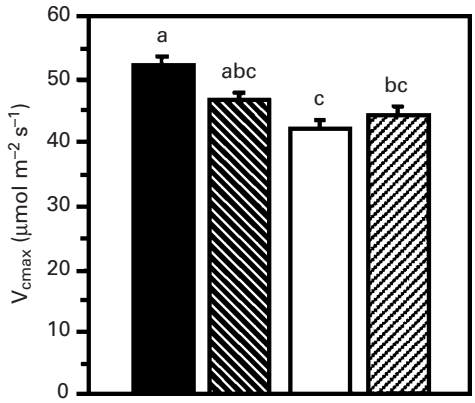

Fig. 7. The effect of elevated [CO_2] and/or elevated [O_3] on the *in vivo* maximum activity of Rubisco (V_{cmax}) in the flag leaves of *Triticum aestivum* modelled from gas-exchange measurements on Day III (third day of measurement, 107 days after sowing): solid bars, ambient chambers; dark-hatched bars, elevated [CO_2]; open bars, elevated [O_3]; light-hatched bars, elevated [CO_2] and [O_3]. Different letters indicate significant differences according to Scheffés's F test, P <0.08. ANOVA (n = 3): P[CO_2] was nonsignificant; P[O_3] = 0.005; P[CO_2]×[O_3] = 0.047.

the *in vivo* activity remained in slight excess at midday (Fig. 6). The lower Rubisco activity measured on Day I was not reflected in a significantly lower V_{cmax} modelled from measurements on Day III. This might partly relate to the inherently greater variability of V_{cmax} compared with the *in vitro* measurements, though it has previously been observed that the relative effect of elevated [CO_2] on V_{cmax} in spring wheat declined with leaf age (McKee & Woodward, 1994; McKee *et al.*, 1995).

It was important to confirm the occurrence of acclimation in Rubisco activity of field-grown wheat exposed to elevated [CO_2] since, following the assimilate feedback hypothesis (Stitt, 1991), it has been suggested that the Rubisco acclimation under elevated [CO_2] observed in many studies was associated with root restriction imposed on pot-grown plants (Arp, 1991; Thomas & Strain, 1991). However, this explanation has been questioned (McConnaughay *et al.*, 1993) and lower steady-state levels of rbcS and rbcL transcripts were observed throughout development in the flag leaves of field-grown wheat under free-air CO_2 enrichment (Nie *et al.*, 1995). Under field conditions, it is not yet clear whether the causes of Rubisco acclimation relate mainly to assimilate feedback (van Oosten *et al.*, 1994), to developmental changes (Sicher & Bunce, 1997), or to nitrogen use (Farage *et al.*, 1998).

For Day II photoassimilation data, there was a significant interaction term for [CO_2] with time (Fig. 3b) and on both days the greatest absolute and relative [CO_2] effect on photoassimilation rate was observed around noon. This pattern is consistent with photosynthetic models which predict larger [CO_2] effects under high light and at higher temperatures within the temperate range (Long, 1991; Harley *et al.*, 1992).

In vitro initial Rubisco activities on Day I also followed the diurnal light pattern, with activation state peaking at *c.* 80% around noon (Fig. 4). Although Rubisco content did not vary through the day, interestingly, its activated activity did (Fig. 4). This might have been expected in species which accumulate the tight-binding inhibitor 2-carboxy-D-arabinitol-1-phosphate (CA1P) in the dark. For instance, Servaites *et al.* (1991) reported lower activated activities (total activity) in *Beta vulgaris* at low irradiances during a simulated natural photoperiod. Although *Triticum aestivum* does not accumulate CA1P, Parry *et al.* (1997) observed higher activated activities (maximal activity) in wheat grown at low irradiance following SO_4^{2-} treatment to displace tight-binding inhibitors. They suggested that this might have resulted from the presence of the tight-binding inhibitor of wheat Rubisco which had previously been partially identified by Keys *et al.* (1995). Kane *et al.* (1998) noted suggestive similarities between this inhibitor and D-glycero-2,3-pentodiulose-1,5-bisphosphate, an oxidation product of RuBP. Whatever the reason for this apparent regulation, it does not appear to have been affected by growth under elevated [CO_2].

The elevated [CO_2] treatment had a greater relative effect on photoassimilation rate on Day II than on Day I (Fig. 3a,b). This partly reflected the higher air temperature on Day II (Fig. 2b), as predicted by models of the photosynthetic response to [CO_2] and temperature (Long, 1991; Harley *et al.*, 1992), but it must also relate to the observed stomatal conductance response. Whereas a significant and consistent reduction in stomatal conductance has been observed throughout flag-leaf development under controlled-environment conditions (McKee *et al.*, 1997b), a similar consistency was not found in this experiment (Fig. 3c,d).

Although the experimental plots were watered manually at weekly intervals to avoid drought stress, Day II followed a hot period with no precipitation and vapour pressure deficit reaching 1.8 kPa. Unlike photoassimilation, which tracked PPFR, stomatal conductance declined after 09:00 hours on both diurnal-measurement days (Fig. 3c,d), suggesting that water status was then the dominant influence on stomatal conductance. Field data were used to parameterize an evapotranspiration model with a simple soil-stomatal feedback, based on the analysis of Monteith (1995); this model (data not shown) is consistent with the hypothesis that declining water potential, in response to water loss, resulted in both the decline in stomatal conductance and the convergence between CO_2 treatments. During this dry period, higher transpiration rates in ambient than in elevated [CO_2] plots would have led to more rapid water loss, feeding back on stomatal conductance, and leading to the convergence in conductances observed in Fig. 3d. This short-term water status effect has two main implications. First, under field conditions, elevated [CO_2] will partly protect against the detrimental effects of natural drying cycles (Sionit *et al.*, 1981). Second, because of the convergence in stomatal conductances, the relative exclusion of ozone and other pollutants under elevated [CO_2] will be reduced during drying periods, which, by their nature, may coincide with photochemical episodes.

Neither on Day I nor on Day II was a significant O_3 effect observed in any of the flag-leaf parameters measured. Mulholland *et al.* (1997a) suggested that the flag leaf might have been more ozone-tolerant than earlier leaves, because accelerated senescence was not observed. However, by Day III, elevated [O_3] had caused a significant decline in V_{cmax} (Fig. 7). This late response could be explained by the pattern of variation in ambient [O_3] and the method of [O_3] enhancement (Fig. 1). Between flag-leaf emergence and anthesis, ambient ozone concentrations were relatively low. Following the protocol of the European Stress Physiology And Climate Experiment (ESPACE) required to facilitate comparison of responses between different European sites, [O_3] in the treatment chambers was enriched proportionately to the ambient concentration rather than being applied as ambient plus a fixed increment; thus, before Day I the flag leaves in the elevated [O_3] chambers had experienced a relatively low ozone exposure. After Day I, [O_3] increased, but a response to O_3 was not apparent until Day III, perhaps because the relatively low conductances measured

around Day II restricted O_3 flux to the mesophyll. The flag leaf contributes a large proportion of the photosynthate allocated to the grain and this lack of O_3 response until late in the life of the flag leaf was reflected in the absence of a yield response (Mulholland *et al.*, 1997a).

Ozone damage was apparent in the negligible Rubisco content of leaf 5, measured on Day I, and elevated [CO_2] clearly ameliorated this damage (Fig. 5). This effect was observed late in the life of the leaf and was probably attributable to accelerated senescence rather than to a direct photosynthetic response. However, the lower V_{cmax} measured in the flag leaves from the elevated [O_3] treatment on Day III (Fig. 7) was not associated with accelerated senescence. Here, the combined response to elevated [CO_2] and [O_3] was less clear, because the mean was not significantly different from that in either of the individual treatments. However, the interaction term was significant, demonstrating that CO_2 enrichment ameliorated the detrimental effect of elevated [O_3] on flag-leaf photosynthesis under field conditions.

Three main mechanisms have been proposed to explain the amelioration of O_3 damage by elevated [CO_2]. First, lower stomatal conductance in elevated [CO_2] may restrict the flux of O_3 to the mesophyll (McKee *et al.*, 1995, 1997b; Fiscus *et al.*, 1997); this protective effect, as shown in this study, might be less consistent under variable field conditions. Second, greater availability of photosynthate under elevated [CO_2] might enhance detoxification capacity (Rao *et al.*, 1995), although some studies suggest that this may be of minor importance (Polle *et al.*, 1993; McKee *et al.*, 1997b). Third, there may be compensation for loss of Rubisco activity at higher substrate levels under elevated [CO_2]. McKee *et al.* (1995) observed an O_3-induced decline in Rubisco activity but not in RuBP-regeneration capacity; under elevated [CO_2], photosynthetic control shifted from O_3-sensitive Rubisco limitation to the relatively insensitive RuBP-regeneration limitation. In this experiment, a compensatory shift in photosynthetic control was also observed in the flag leaf at noon on Day III (Fig. 6). However, C_i in the elevated [CO_2] treatment lay close to the transition in the A/C_i curve from Rubisco limitation to RuBP-regeneration limitation; thus the degree of compensation is likely to vary depending on the balance between pollutants, and diurnal and seasonal changes in the environment.

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