Diurnal changes of Rubisco in response to elevated CO_2, temperature and nitrogen in wheat grown under temperature gradient tunnels

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Abstract

Growth at elevated CO_2 and temperature often leads to decreased Rubisco activity. We investigated the effects of increased CO_2, temperature and nitrogen on the diurnal changes in the control of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) activity in wheat (*Triticum aestivum* L.). Spring wheat was grown at ambient and 700 μmol mol^{-1} CO_2, under ambient and 4 ºC warmer temperatures, and with two levels of nitrogen supply in field tunnels in a Mediterranean environment. At ear emergence, elevated CO_2 increased Rubisco activation, but decreased Rubisco protein and, with high nitrogen, Rubisco specific activity, and had no effect on the *rbc*S transcript. Warmer temperatures tended to decrease the *rbc*S mRNA level and Rubisco protein, although the effect on Rubisco activity was small. High nitrogen decreased Rubisco activation or specific activity, depending on the CO_2 concentration. It increased Rubisco protein at the end of the night, but accelerated its diurnal loss. The main changes after anthesis were the disappearance of the decrease in Rubisco specific activity caused by elevated CO_2, an increase in this activity with above-ambient temperatures combined with high nitrogen, and that high temperature and nitrogen did not affect Rubisco protein contents. This study suggests that decreased leaf protein and increased levels of a Rubisco inhibitor, rather than gene repression by sugars, are involved in acclimation to elevated CO_2. High nitrogen increases this down regulation. Changes during growth in levels of leaf metabolites and protein may alter the relative importance of levels of inhibitors and Rubisco amounts for Rubisco regulation.

Key-words: *Triticum aestivum*, carbohydrates, diurnal, elevated CO_2, elevated temperature, nitrogen, protein, Rubisco regulation.

Abbreviations – *rbc*S, Rubisco small subunit; Rubisco, ribulose-1, 5-bisphosphate carboxylase oxygenase; RuBP, ribulose-1,5-bisphosphate.

Introduction

Many studies on acclimation to elevated CO_2 have pointed to a decrease in Rubisco (Drake et al. 1997) which could be accounted for by the acceleration of development and an earlier senescence (Sicher and Bunce 1997), but the decrease in Rubisco precedes any change in senecence or is not affected by it (Nie et al. 1995b). In recent years much emphasis has been placed on the control of Rubisco gene expression by high atmospheric CO_2 levels. The levels of transcripts for the Rubisco subunits show small diurnal variations, from a maximum at approximately dawn to a minimum in the late afternoon (Nie et al. 1995a, Cheng et al. 1998), and are lower at elevated CO_2 (Van Oosten and Besford, 1995), especially before dawn (Nie et al. 1995a) and in the night (Cheng et al. 1998). This diurnal oscillation is antiparallel to the pattern of carbohydrate accumulation (Nie et al. 1995a) and coincides with high levels of hexoses in the first hours of the night under elevated CO_2 (Cheng et al. 1998), suggesting a relationship between high levels of sugars and gene repression. Growth at elevated CO_2 leads to an accumulation of carbohydrates (Nie et al. 1995a) and there is evidence that the expression of photosynthetic genes is inhibited by sugars (Sheen, 1990, Krapp et al. 1993). Despite the relationship between diurnal changes in sugar levels and gene repression, the correspondence of transcript levels to the accumulation of soluble carbohydrates at different stages of development is variable (Nie et al. 1995b, Geiger et al. 1999). Moreover, with a high nitrogen supply sugars increase at elevated CO_2 but no acclimation of photosynthesis or decreased transcripts for Calvin cycle enzymes is observed (Geiger et al. 1999.). The decrease in Rubisco protein at elevated CO_2 is accompanied by lowered total soluble protein (Sicher et al. 1997) and total nitrogen in leaves (Nakano et al. 1997), suggesting that the effect of elevated CO_2 on photosynthesis is due to the nitrogen status (Riviere-Rolland et al. 1996, Nakano et al. 1997, Farage et al. 1998, Geiger et al. 1999).

The control of Rubisco contents by elevated CO_2 may involve multiple effects on mRNA translation and/or protein turnover (Webber et al. 1994, Moore et al. 1999). In addition to this coarse control of Rubisco activity, the k_{cat} of Rubisco decreases with inhibitory phosphorylated compounds that bind tightly to Rubisco during the photoperiod (Parry et al. 1993, Keys et al. 1995, Parry et al. 1997). The catalytic activity of Rubisco increases with elevated CO_2 by 8% in soybean, although it is not affected by CO_2 in rice (Vu et al. 1997). In turn, Rubisco activation decreased at

elevated CO_2 in some studies (Sage et al. 1988, 1989, McKee and Woodward 1994, Vu et al. 1997), though not in others (Nakano et al. 1997).

The increase in atmospheric CO_2 concentration may be associated with a rise in temperature of 1.5-6 ºC (Schneider 1992). Rubisco protein is decreased by 23% in rice and by 17% in soybean as temperatures increase from 32 to 38 ºC and from 28 to 40 ºC, respectively; in addition, the total activity and activation of Rubisco decreases in rice, while only activation decreases in soybean (Vu et al. 1997). That Rubisco protein, but not total activity in soybean, decreases at high temperature indicates that the apparent k_{cat} is up-regulated (Vu et al. 1997). Rubisco catalytic activity increases with temperature and shows values 40-70 % lower in C_3 species from warm than from cool habitats (Sage et al. 2002). It has been observed that growth temperature alters the relative stimulation of photosynthesis by elevated CO_2 in response to temperature, reflecting an acclimation to this environmental parameter (Hikosaka et al. 1999, Bunce 2000 a; Ziska 2001); the apparent specificity of Rubisco for CO_2 (Bunce 2000 b) or long-term adjustments in the potential rate of electron transport and the maximum velocity of RuBP-saturated carboxylation (Ziska 2001) seem to account for this acclimation to temperature.

Studies of the response of Rubisco activity to rising CO_2 and temperature in field crops, with the fluctuations in light, temperature and humidity of the natural environment, particularly under Mediterranean conditions of limiting water and warm temperatures, are sparse. The aim of this work was to assess whether Rubisco activity and its diurnal fluctuation in flag leaves of wheat are affected, at the various control levels described, by combined increases in CO_2 and temperature in the air, and to ascertain whether the nitrogen supply modifies these effects. Carbohydrates were analysed to examine their relationship with Rubisco activity. In order to approach the natural environment of Mediterranean wheat crops, this study was conducted in the field, without the restrictions to root growth imposed by pots, under temperature gradient tunnels – to track the diurnal and seasonal fluctuations in temperature – with a water supply equivalent to the average rainfall during the growing season. The flag leaf at the key growth stage of ear emergence was selected for this study. This was the youngest fully expanded leaf at a time when acclimation to elevated CO_2 is more likely than in younger plants, and the elongating last internode of the stem and the ear provided an active sink for assimilates. Changes

during growth on the control of Rubisco by CO_2, temperature and nitrogen were assessed by comparing leaves sampled at ear emergence and after anthesis.

Materials and Methods

Spring wheat (*Triticum aestivum* L., cv. Areces) was sown in a clay sand soil at a rate of 180 kg ha^{-1} and 0.13 m row spacing on 13 February. Before sowing, N (as NH_4NO_3), P and K fertilizers (80, 40 and 40 kg ha^{-1}, respectively) were applied. The crop was watered weekly through a drip irrigation system providing amounts of water equivalent to the average rainfall in this area during the period of the experiment (198 mm between February and June). The experiment was carried out at the IRNASA farm at Salamanca (41^{o} N, 800 m above sea level).

Temperature gradient tunnels were mounted on the crop on 23 March rather than at sowing to select crop areas with a uniform plant cover. Mean crop densitiy at ear emergence was 534Ä77 shoots m^{-2}. The tunnels were 9 m long, 2.2 m wide, and 1.7 m high at the ridge and followed the design of Rawson et al. (1995). Briefly, each tunnel had transparent walls and roof and comprised three modules separated by horizontally slotted polycarbonate septa to reduce the mixing of air between modules due to convection. Two inlet fans and an outlet fan continuously circulated air through the tunnel at the speed required to maintain a temperature difference between the two extreme modules. Three small fan heaters placed in the outlet plenum were used to help maintain the temperature difference at night and whenever solar radiation was insufficient to raise the temperature. A two-probe Pt-100 system measuring the difference between the inlets of the inlet and outlet fans was connected to proportional integrative differential (PID) controllers with outputs to the fans and the heaters. To raise CO_2 levels in the air, the signal of an infrared gas analyser monitoring the CO_2 concentration at the outlet module was fed into another PID controlling a solenoid valve which injected CO_2 at the two inlet fans. Ventilated temperature and humidity sensors and air probes for CO_2 analysis were placed at the centre of each module. The data were recorded continuously by a computer.

This study was conducted in two tunnels, one kept at the ambient air CO_2 concentration and another at 700 µmol mol^{-1} during the light hours. CO_2 enrichment in the night period may be irrelevant, because direct effects of CO_2 on leaf respiration in the dark seem not to exist, the reports in the literature appearing to be artefactual (Jahnke and Krewitt 2002). The temperature difference between the extreme modules in a tunnel was set at 4 ^{o}C. Additional (40 kg ha^{-1}) nitrogen was added to one of the longitudinal halves of each tunnel 34 days after sowing, such that

two levels of this nutrient (80 and 120 kg ha^{-1}) were compared. The samplings were repeated in four consecutive sections within the two module halves. The experimental design (Fig. 1) and its stastistical analysis are further discussed below.Figure 2 shows that the diurnal courses of air temperature, relative humidity and CO_2 concentration were controlled satisfactorily. Air humidity in the high-temperature module was lower with elevated than ambient CO_2, probably due to decreased transpiration at elevated CO_2. An increase in temperature and an associated decrease in air humidity during the light hours occurred from April to May- early June. No relevant changes in CO_2 concentrations were observed during this period.

On day 3 after the beginning of ear emergence in the whole experiment (22 May) - ear emergence was advanced about 3 days by warm temperatures -, flag leaves (two per replicate) were harvested and immediately plunged into liquid nitrogen just before dawn, 4-6 h later, 1-2 h before dusk, and 2-3 h into the dark period; light intensities were <10, 1700, 100 and <10 μmol m^{-2} s^{-1}, respectively. On day 9 after 100 % anthesis in each temperature regime (3 and 9 June for plants under ambient and ambient + 4 ºC, respectively), another sample of the same size was harvested 4-6 h after the start of the light period. The samples were kept in liquid nitrogen until analysis. After determining the fresh weight in subsamples of frozen leaves, leaf area was determined by image analysis and chlorophyll was extracted in 80 % acetone and measured (Arnon 1949). This allowed the results to be expressed on a leaf area basis.

For Rubisco activity assays, a procedure based on that described by Lilley and Walker (1974), as modified by Ward and Keys (1989) and Sharkey et al. (1991), was followed. Aliquots of the frozen leaves were ground in a mortar with liquid nitrogen and extracted with 100 mM Bicine-NaOH (pH 7.8), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 2% PVPP (w/v). An aliquot of the whole extract was used to determine chlorophyll contents (Arnon 1949) and the remainder was centrifuged at 13000 *g*. The total time from extraction to the assay of initial Rubisco activity was less than 2.5 min. Activity was assayed by adding extract to a mixture of 100 mM Bicine (pH 8.2), 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM KCl, 1 mM ribulose-1,5-bisphosphate (RuBP), 0.2 mM NADH, 5 mM ATP, 5 mM creatine phosphate, 52 units/ml phosphocreatine kinase, 12 units/ml phosphoglycerate kinase, 11 units/ml glyceraldehyde 3-phosphate dehydrogenase and recording the decrease in absorbance

at 340 nm minus 400 nm for 40-60 s, at a stoichiometry of 2:1 between NADH oxidation and RuBP carboxylation. To assay total Rubisco activity, an aliquot of the extract was incubated with $NaHCO_3$ and $MgCl_2$ for 10 min at room temperature prior to the addition of coupling enzymes and NADH; the reaction was started by adding RuBP. The activation state was estimated as initial activity as a percentage of total activity. Commercial coupling enzymes suspended in ammonium sulphate were precipitated by centrifugation and dissolved in 20 % glycerol (Sharkey et al. 1991). With the assay buffer described, the initial lag in the reaction reported by others (Ward and Keys 1989, Sharkey et al. 1991) was not observed. The amount of Rubisco in a subsample of frozen leaf material was determined by densitometric scanning of SDS-PAGE gels, as described previously (Martín del Molino et al. 1995). Rubisco specific activity was determined by dividing total activity by Rubisco active site contents.

For analysis of RNA, frozen leaf material was homogenized with guanidine buffer. The supernatant was extracted with phenol/chloroform (1:1, v:v) and after phase separation the aqueous phase was washed with chloroform. Total nucleic acids were precipitated with sodium acetate and ethanol. The pellet was washed with ethanol, dried and finally resuspended in DEPC water. Purity was checked by measuring absorption at 230, 260, 280 and 320 nm and the absorption at 260 nm was used for quantification (Logemann et al. 1987). For Northern blots, 10 µg of RNA were separated electrophoretically on 1.2% agarose gels containing 7% formaldehyde (Sambrook et al. 1989) and transferred to membranes (Hybond N, Amersham Pharmacia Biotec). The membrane was baked and prehybridization was performed as described by Krapp et al. (1993). The ^{32}P-labelled (radiolabelling was by random primer using the Boehringer kit) Rubisco small subunit (*rbc*S) cDNA of wheat kindly provided by Dr. Christine Raines (Dept. of Biological Sciences, John Tabor Laboratories, University of Essex, UK) was added to the prehybridization mixture and hybridization was carried out for at least 12 h. The filters were washed and autoradiography was carried out at –80ºC with Kodak Xomat films, using a single intensifying screen. The relative levels of *rbc*S expression were determined by image analysis using the Scion ImagePC (Scion Corp., Maryland, USA)) software.

Subsamples of leaves stored in liquid nitrogen were extracted three times in 1 ml 80 % ethanol - 10 mM Hepes-KOH (pH 7.5) at 80 ºC for 30 min and the extracts were pooled. Then, the residue was extracted three times in 1 ml water at 80 ºC for

30 min and the extracts were pooled. Chlorophyll in the extracts was analysed as described above. Glucose, fructose and sucrose in ethanol extracts were determined according to Jones et al. (1977). Since most commercial invertases hydrolyze oligofructans (Koroleva et al. 1998), sucrose contents were estimated from glucose rather than from fructose released by invertase. For fructan analysis, aliquots from both the ethanol and water extracts were rendered 360 mM HCl, heated at 60 ºC for 30 min and then neutralized with KOH before analysing glucose and fructose; fructan (fructose equivalents) in the hydrolyzed ethanol extract was estimated by subtracting the amounts of free- and sucrose-fructose; fructose in excess of glucose in the sucrose analysis was assumed to correspond to fructans and was added to these. Fructose in the hydrolyzed water extract was added to obtain total fructan contents. Starch in the residue from ethanol-water extractions was digested with amyloglucosidase and amylase and determined according to ap Rees et al. (1977).

The restriction of the number of available tunnels to just two, with the consequent lack of replication for CO_2, was a drawback in the experimental design. In addition, randomisation of treatments was limited with temperature gradient tunnels, which unavoidably placed in a same tunnel module the two combinations of nitrogen with a given temperature. With a lay-out of samples within samples, analyses of variance were performed as in a nested design according to Snedecor and Cochran (1967), with temperature and nitrogen as a stratum included in CO_2, and replicates as a stratum included in that for temperature and nitrogen. Details of one of such analyses are summarized in Table 1.The variance ratio for CO_2 was obtained by dividing the mean square for CO_2 (1 degree of freedom) by the nitrogen and temperature within CO_2 mean square (6 degrees of freedom). Thus, the variability of the CO_2 levels was compared with the variability of individual nitrogen and temperature treatments after removing any overall similarity between treatments belonging to the same CO_2. Although debatable, this method of obtaining the variance ratio following the Snedecor and Cochran (1967) model seemed more adequate than comparing the CO_2 mean square against a residual mean square representing the random variability between treatments after removing CO_2 as well as nitrogen and temperature effects. Similarly, the nitrogen, temperature and interactive effects (1 degree of freedom each) were compared against the replicates within CO_2-temperature-nitrogen mean square (24 degrees of freedom). Time effects were evaluated by including the hour of day as a further stratum in the analysis (3 against 9 degrees of freedom). The

standard errors of differences, rather than simply the standard error of means, are shown in the figures, since the former are better estimates of the treatment effects.

Results

Flag leaf photosynthesis during the morning hours (data not shown) was higher in plants grown at elevated than ambient CO_2. When measured at the same CO_2 concentration, however, elevated CO_2 plants had decreased photosynthesis rates, except that plants grown at above ambient temperatures and abundant nitrogen supply showed smaller or no significant decreases in photosynthesis.

Ear emergence

Rubisco

Rubisco activity

In general, initial Rubisco activity (Fig. 3 A, B) showed a decrease from the end of the night to the end of the light period, and then increased during the two first hours of the night. Elevated CO_2 decreased initial Rubisco activity at the end of the night and mid morning, and then the effect disappeared due to a faster decline with ambient than elevated CO_2. With a high nitrogen supply, the initial activity of the enzyme was higher at the end of the night, as compared to low nitrogen; at other times of the day, nitrogen had no significant effect on initial activity. Growth temperatures did not affect initial Rubisco activity.

Compared to the values obtained at the end of the night, the activation state of Rubisco (Fig. 3 C, D) decreased to a greater extent at ambient than elevated CO_2 in the light period. Two hours into the dark period, Rubisco activation displayed an increase in plants grown at ambient, but not at elevated, CO_2. A similar increase in Rubisco activation after dusk was observed by Parry et al. (1993), and no pre-dawn decrease in activity was observed in a number of species (Vu et al. 1984, Servaites et al. 1986). The activation state of Rubisco was similar at ambient and elevated CO_2 by the end of the night, while it was higher at elevated CO_2 during the day. A high nitrogen supply decreased Rubisco activation, although in the morning this decrease occurred only at ambient CO_2; as an exception, nitrogen had no effect on the degree of activation of Rubisco by the end of the night. Temperature had no effects on Rubisco activation.

In plants grown at ambient CO_2, total Rubisco activity (Fig. 3 E, F) showed an increase from the end of the night to mid-morning; from there to the end of the day, it changed little in plants at the current temperatures and increased with 4 ºC higher temperatures. Two hours after dusk, in contrast, total Rubisco activity increased

somewhat at the current temperatures, but decreased to values close to those at the end of the night at 4 ºC above ambient temperatures. By contrast, at elevated CO_2 total Rubisco activity generally decreased from the end of the night to the first part of the light period and then increased until 2 hours after dusk. Compensating in part the increased activation of Rubisco, total activity was decreased by elevated CO_2 at the end of the night and the decrease was even greater during the light hours. Abundant nitrogen did not prevent the decrease in total Rubisco activity caused by elevated CO_2; rather, high nitrogen increased total Rubisco activity in plants grown under ambient CO_2, but not under elevated CO_2. The increase in temperature had no significant effect on total Rubisco activity.

Rubisco specific activity

At ambient CO_2, the specific activity of Rubisco (Fig. 3 G, H), as total Rubisco activity, generally increased from the end of the night to the end of the day. Two hours into the dark period, Rubisco specific activity changed relatively little at the current temperatures, but underwent a marked decrease at 4 ºC higher temperatures. In plants grown at elevated CO_2, Rubisco specific activity also increased from the end of the night to mid-morning in plants with a low nitrogen supply, but, in contrast, it decreased in this interval in plants with an ample nitrogen supply. At elevated CO_2 there was no homogeneous pattern of change in specific activity from the morning to the end of the day, and this was generally followed by an increase two hours into the dark period.

During the light hours, elevated CO_2 decreased Rubisco specific activity in plants with abundant nitrogen and did not affect it with low nitrogen; two hours after the start of the night, elevated CO_2 continued to decrease the specific activity of the enzyme in plants with high nitrogen and ambient temperatures, but not with above-ambient temperatures. High temperatures increased the specific activity at the end of the day in plants at ambient CO_2 and decreased it at the beginning of the dark period in plants at ambient CO_2 and high nitrogen. Nitrogen showed the described interaction with CO_2 on Rubisco specific activity.

Rubisco protein content

The diurnal change in Rubisco protein (Fig. 3 I, J) depended on the supply of nitrogen. Thus, with abundant nitrogen Rubisco protein generally decreased from the end of the night to the end of the day. With low nitrogen, Rubisco protein showed little change from the end of the night to the final light hours at ambient CO_2, while

at elevated CO_2 it decreased at mid-morning and changed little thereafter at the current temperatures, but increased throughout the light hours with higher temperatures. With both nitrogen levels, Rubisco protein increased in the first hours of the night. Elevated CO_2 decreased the amount of Rubisco protein. High temperatures decreased the amount of Rubisco in plants with high nitrogen during the light hours, but the effect disappeared or was reversed two hours into the dark period. With low nitrogen, however, this effect of high temperatures only occurred under elevated CO_2 during the night hours. A high nitrogen supply increased the amount of Rubisco in the night hours at high temperature, and at the end of the night and mid-morning at the current temperatures, but did not modify Rubisco contents at the end of the day.

*rbc*S transcript levels

Northern-blots were carried out with two of the four replicates of all treatments and sampling times (Fig 4 A). The mRNA level for the Rubisco small subunit did not change significantly during the day (Fig. 4 B). High nitrogen increased the *rbc*S mRNA level. However, this increase was not judged as reliable because the high- and low-nitrogen samples were arranged on separate membranes. There were no significant effects of CO_2 on *rbc*S transcript levels. In contrast, high temperatures tended to decrease *rbc*S expression.

Leaf Carbohydrates

Fructose (Fig. 5 C-D) increased during the first part of the light period and decreased thereafter; although glucose and fructans (Fig. 5 A-B, G-H) followed the same pattern, it did not reach significance. Sucrose (Fig. 5 E, F) increased from the end of the dark period to the first part of the day, remained at this level until the end of the light period, and then declined during the first hours of the night. Starch (Fig. 5 I, J) increased throughout the light period and was mobilized at night. Therefore, total non-structural carbohydrate contents increased during the day compared to the end of the night and decreased two hours into the night.

Elevated CO_2 significantly increased glucose and fructans during the dark period; in the day time this increase did not reach significance. Elevated CO_2 also increased fructose, sucrose and starch at all day and night samplings. Thus, total non-structural carbohydrates always increased at elevated CO_2. The higher carbohydrate levels at the end of the night under elevated CO_2 indicate that the extra carbohydrate accumulated during the day was not fully mobilized during the dark period. High

temperatures decreased the contents of fructose, except in plants at elevated CO_2 and low nitrogen, and, in the light hours, they decreased sucrose contents. High temperatures also decreased starch contents in plants with ample nitrogen in the light hours and, in plants with ample nitrogen and elevated CO_2, also two hours after the start of the night. Nitrogen deficiency did not affect glucose, fructose and sucrose contents; it increased fructan contents only in the light hours and starch contents throughout the day in elevated CO_2 plants and, at mid-morning and the beginning of the night, also in ambient CO_2 and high-temperature plants.

After anthesis

Rubisco

Rubisco activity

At mid morning 9 days after anthesis, elevated CO_2 decreased initial Rubisco activity (Fig. 6 A) as was observed at ear emergence, except in plants under current temperatures and low nitrogen, which also showed high total and specific Rubisco activity and protein compared to other combinations of factors. Initial Rubisco activity showed a positive response to increased temperatures after anthesis – except for elevated CO_2 and low nitrogen -, in contrast with the temperature insensitivity displayed at ear emergence. Nitrogen increased initial Rubisco activity at ambient CO_2, significantly so at above ambient temperatures; at elevated CO_2 and current temperatures, nitrogen decreased initial Rubisco activity due to the high value with the elevated CO_2, current temperature and low nitrogen combination.

While elevated CO_2 increased Rubisco activation state at ear emergence, it decreased activation after anthesis (Fig 6 B), except at warm temperature and low nitrogen. A temperature increase, which had no significant effect on the activation of Rubisco at ear emergence, caused an increase in activation after anthesis which was small in all treatment combinations except in elevated CO_2 and low nitrogen. At the current temperatures, high nitrogen did not affect the activation state of Rubisco, while at high temperatures it decreased this activation, especially at elevated CO_2, an effect which was observed at both temperatures, but only at ambient CO_2, at ear emergence.

At above ambient temperatures, elevated CO_2 decreased total Rubisco activity (Fig. 6 C), an effect observed at both temperatures at ear emergence; at ambient temperature and low nitrogen, total activity was high at elevated CO_2, as already

indicated. Except for this treatment, a temperature rise increased total Rubisco activity after anthesis, especially at ambient CO_2 and high nitrogen, in contrast with its lack of effect at ear emergence. Apart from the high activity in plants at elevated CO_2, ambient temperature and low nitrogen, a high nitrogen supply increased total Rubisco activity, and the interaction of nitrogen and CO_2 on this activity at ear emergence disappeared after anthesis.

Rubisco specific activity

Elevated CO_2 increased the specific activity of Rubisco (Fig. 6 D); this effect of CO_2 tended to be higher at current than warmer temperatures, which accounted for the unaltered total Rubisco activity under elevated CO_2 at the current temperatures. Therefore, the decrease in specific activity caused by elevated CO_2 with a high nitrogen supply at ear emergence disappeared after anthesis. Above ambient temperatures increased Rubisco specific activity with high nitrogen, while under elevated CO_2 and low nitrogen this activity was high at ambient temperature.

Rubisco protein contents and *rbc*S transcript levels

Except for the high Rubisco protein under ambient CO_2 and temperature with low nitrogen, elevated CO_2 decreased the amount of Rubisco protein (Fig. 6 E) 9 days after anthesis, as it did at ear emergence. Warmer temperatures had no effect on Rubisco protein, in contrast with the decrease induced in its contents in plants with high nitrogen at ear emergence. Abundant nitrogen did not affect the contents of Rubisco protein after anthesis, and thus the positive effect of high nitrogen at ambient temperatures observed at mid morning at ear emergence, disappeared after anthesis.

The mRNA level for the Rubisco small subunit was not affected by the treatments after ear emergence (data not shown).

Leaf Carbohydrates

Nine days after anthesis elevated CO_2 had no significant effect on the contents of glucose and fructose (Fig. 7 A, B) and increased starch contents (Fig. 7 E), except at current temperatures and high nitrogen. With low nitrogen, elevated CO_2 increased sucrose contents (Fig. 7 C), but with high nitrogen it decreased these contents. Under ambient temperatures, elevated CO_2 decreased the levels of fructan (Fig. 7 D), while at warmer temperatures it increased these levels. Thus, the overall increase in non-structural carbohydrate contents caused by elevated CO_2 at ear emergence had disappeared after anthesis. Above ambient temperatures decreased fructan contents

and, with elevated CO_2 and low nitrogen, as well as with ambient CO_2 and high nitrogen, also the starch contents. Consequently, a rise in temperature tended to decrease the contents of non-structural carbohydrates, as was generally observed at ear emergence. Nitrogen deficiency did not affect the contents of glucose, fructose and fructan. Starch contents were higher with low nitrogen, elevated CO_2 and current temperatures than with other combination of factors.

Discussion

With the modifications discussed below, initial Rubisco activity at ear emergence showed a decline during the day which was due to a deactivation of Rubisco, since total Rubisco activity generally showed an increase from before dawn or mid-morning, depending on growth CO_2 level. Although Rubisco activation state often increases with light (Portis, 1992), the higher activation of Rubisco at night hours in our experiment is consistent with previous results showing an increase in Rubisco activation in the dark (Parry *et al.*, 1993, Anwaruzzaman & Yokota, 1999) and no pre-dawn decrease in Rubisco activity in some species, including Gramineae (Vu *et al.*, 1984; Servaites *et al.*, 1986). The increase during the day in total Rubisco activity contrasts with the afternoon decrease reported in previous studies (Kobza and Seemann 1989) and was due – with some exceptions, discussed later - to increasing specific activities of the enzyme, since Rubisco protein contents displayed a decreasing pattern. This inhibition of catalysis, which did not disappear *in vitro* (He et al. 1997, Sharkey et al. 2001), pointed to the *in vivo* presence of tight-binding Rubisco inhibitors (Kobza and Seemann 1988, Parry et al. 1997). A day-time inhibitor of Rubisco with properties similar to those of pentodiulose-bisphosphate (Kane et al. 1998) has been found in wheat (Keys et al. 1995, Parry et al. 1997); the inhibitor contents could increase in response to an accumulation of its precursors, as has been confirmed for the nocturnal inhibitor 2-carboxyarabinitol 1-phosphate (Andralojc et al. 2002) and can play a major role in regulating Rubisco activity in the light (Parry et al. 1993, 1997, Medrano et al. 1997). The increase up to the end of the day in Rubisco specific activity in most plants grown at ambient CO_2 revealed that the putative inhibitor was released only slowly from Rubisco active sites. In turn, Rubisco protein displayed diurnal decreases - similar in magnitude to those observed for Nitrate Reductase (Scheible et al. 1997) and soluble protein (Häder et al. 1997) - which showed a low correspondence with the diurnal changes in Rubisco mRNA levels, suggesting that gene transcription was not the only regulator of the abundance of this enzyme (Webber et al. 1994, Moore et al. 1999).

The activation state of Rubisco was higher at elevated CO_2, especially with an abundant nitrogen supply, due to a slower deactivation of Rubisco during the day. Therefore, the decrease in initial activity of Rubisco at elevated CO_2 must be a consequence of a lowered total activity, which was not prevented, but was

exacerbated by a high nitrogen supply, in contrast to previous reports (Riviere-Rolland et al. 1996, Geiger et al. 1999, Stitt and Krapp 1999). This occurred, firstly, because when combined with abundant nitrogen, elevated CO_2 reversed or prevented the increase in specific activity observed at ambient CO_2. This change points to an increase in the level of an inhibitor of Rubisco parallel to the greater accumulation of carbohydrates - particularly of hexoses and sucrose with high nitrogen - and probably also phosphorylated intermediates caused by elevated CO_2. Secondly, elevated CO_2 reduced the amount of Rubisco protein, in agreement with earlier studies reporting an inhibition of gene expression for this enzyme (Van Oosten and Besford 1995, Nie et al. 1995a, Cheng et al. 1998). However, we observed no significant changes in *rbcS* expression associated with the accumulation of carbohydrates at elevated CO_2. The decrease in Rubisco protein was associated, in agreement with others (Nakano et al. 1997, Geiger et al. 1999), with a decrease in leaf nitrogen, attributable in part to a dilution on a leaf area basis and to a change in allocation within the plant rather than to decreased nitrogen uptake (I. Martín del Molino, P. Pérez, R. Martínez-Carrasco, R. Morcuende, unpublished data), which is consistent with the results reported by Makino et al. (1997). In contrast with other studies (Geiger et al. 1999), however, decreased leaf nitrogen at elevated CO_2 was not relieved by a high nitrogen supply, because the latter did not alter the allocation of nitrogen to the flag leaf (unpublished data). The main changes in effects of elevated CO_2 on Rubisco after anthesis were a decrease in the activation state and no decrease in Rubisco specific activity. The latter may be due to the fact that levels of carbohydrates, and possibly also of photosynthetic intermediates, were no longer increased by elevated CO_2, probably due to enhanced carbohydrate export, as the increased ear dry weight suggests (data not shown).

Higher temperatures did not affect the activation or the total activity of Rubisco at ear emergence as elevated CO_2 did. In contrast, high temperatures altered the specific activity of Rubisco at certain hours of the day. The paralellism of this effect with carbohydrate levels was poor. In addition, high temperatures with abundant nitrogen, decreased the amount of Rubisco protein, in agreement with previous studies (Vu et al. 1997). Here we show that a trend towards decreased *rbcS* transcript is involved in the loss of Rubisco at high temperature. After anthesis, the activation state of Rubisco and its specific activity with high nitrogen were stimulated by above-ambient temperatures, while Rubisco protein and *rbcS* levels were unaffected. Again,

the change in specific activity with temperature was not closely related to carbohydrate levels. With abundant nitrogen, a slower loss of Rubisco protein from ear emergence to 9 days after anthesis with warmer temperatures accounted for the disappearance of the negative effect of rising temperatures on Rubisco protein and associated levels of mRNA (Geiger et al. 1999).

The greater decrease in the morning of initial Rubisco activity with a high than with a low nitrogen supply at ambient CO_2 was due to a faster decrease in Rubisco activation state, in agreement with other studies (Mächler et al. 1988, Cheng and Fuchigami 2000), possibly because the ATP/ADP ratio decreases with high nitrogen (Mächler et al. 1988). At variance with this, at elevated CO_2 a high nitrogen supply did not decrease Rubisco activation, but tended to decrease total Rubisco activity at mid morning as a consequence of decreased specific activity, as already discussed. By contrast, abundant nitrogen generally increased the amount of Rubisco protein at the end of the night and/or mid morning at both ambient and elevated CO_2, as previously reported (Cheng and Fuchigami, 2000, Nakano et al. 1997), although it accelerated the loss of protein during the light period. This increase in Rubisco protein could be associated with higher levels of *rbcS* transcripts. After anthesis, abundant nitrogen increased the specific activity of Rubisco at high temperatures, a change that was not associated with lower carbohydrate contents. More Rubisco protein was lost between the two samplings with high than with low nitrogen, probably as a consequence of increased export to sinks, and thus the positive effect of nitrogen on Rubisco contents and on the nitrogen-dependent amount of RNA (Geiger et al. 1999) disappeared after anthesis.

In conclusion, this study suggests that decreased leaf protein and increased levels of a Rubisco inhibitor, rather than gene repression by sugars, are involved in acclimation to elevated CO_2. Warmer temperatures may decrease Rubisco gene expression with little consequence for Rubisco activity. High nitrogen does not prevent, but rather increases down regulation of Rubisco under elevated CO_2, probably due to increased levels of Rubisco inhibitors. Changes during growth in levels of leaf metabolites and protein may alter the relative importance of Rubisco amounts and levels of inhibitors for Rubisco regulation under a changing climate.

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Figure legends

Fig. 1. Lay-out of the experiment in temperature gradient tunnels at ambient (360 μmol mol^{-1}) and elevated (700 μmol mol^{-1}) CO_2, ambient and ambient + 4 ºC temperatures, and 80 (low nitrogen) and 120 (high nitrogen) kg nitrogen ha^{-1}.

Fig. 2. Mean daily courses of air temperature, humidity and CO_2 concentration in temperature gradient tunnels set at either ambient (360 μmol mol^{-1}, open symbols) or elevated (700 μmol mol^{-1}, closed symbols) CO_2, and ambient (circles) or ambient + 4 ºC (squares) temperatures.

Fig. 3. Diurnal changes in Rubisco activity, activation state, specific activity and Rubisco protein content of flag leaves of wheat in response to CO_2, temperature and nitrogen 3 days after ear emergence. Plants were grown in the field under temperature gradient tunnels at either ambient (360 μmol mol^{-1}) or elevated (700 μmol mol^{-1}) CO_2, ambient (circles) or ambient + 4 ºC (squares) temperatures, and 80 (open symbols) or 120 (closed symbols) kg nitrogen ha^{-1}. Light intensities before dawn, 4-6 h later, 1-2 h before dusk, and 2-3 h into the dark period were <10, 1700, 100 and <10 μmol $m^{-2}s^{-1}$, respectively. Each point is the mean of four replicates. Vertical bars represent twice the standard error of the difference for main effects of CO_2 (a), temperature and nitrogen (b) and time (c). The standard errors of the differences for treatment interactions are omitted for clarity.

Fig 4. (A) Diurnal changes in *rbcS* mRNA levels 3 days after ear emergence in flag leaves of wheat grown in the field under temperature gradient tunnels either at ambient (360 μmol mol^{-1}) or elevated (700 μmol mol^{-1}) CO_2, ambient or ambient +4 ºC temperatures and 80 (low N) or 120 (high N) kg nitrogen ha^{-1}. Numbers (1, 2) represent replicate samples. Equal amounts of RNA were applied to each lane. (B) Quantified signals (arbitrary units) of diurnal changes in *rbcS* transcripts. The signal intensity indicates the transcript abundance relative to total RNA Symbols as in Fig. 2.

Fig. 5. Diurnal changes in carbohydrate contents of flag leaves of wheat in response to CO_2, temperature and nitrogen 3 days after ear emergence. Plants were grown in

the field under temperature gradient tunnels. The levels of fructan and starch are in hexose equivalents. Each point is the mean of four replicates. Symbols as in Fig. 2.

Fig. 6. Effects of CO_2, temperature and nitrogen on Rubisco activity, activation state, specific activity and Rubisco protein content of flag leaves of wheat at mid morning 9 days after anthesis. Plants were grown in the field under temperature gradient tunnels at either ambient (360 μmol mol^{-1}) or elevated (700 μmol mol^{-1}) CO_2, ambient (white, dark grey) or ambient + 4 ºC (pale grey, black) temperatures, and 80 (white, pale grey) or 120 (dark grey, black) kg nitrogen ha^{-1}. Each point is the mean of four replicates. Vertical bars represent twice the standard error of the difference for main effects of CO_2 (a) and temperature and nitrogen (b). The standard errors of the differences for treatment interactions are omitted for clarity.

Fig. 7. Effects of CO_2, temperature and nitrogen on carbohydrate contents of flag leaves of wheat at mid morning 9 days after anthesis. Plants were grown in the field under temperature gradient tunnels. The levels of fructan and starch are in hexose equivalents. Each point is the mean of four replicates. Symbols as in Fig. 6.

Table 1.- Example of the analysis of variance used in this study with two CO_2 concentrations, two air temperatures, two nitrogen levels, four replicate samples and four sampling times in a nested design, with nitrogen and temperature in a stratum within CO_2, replicates in a stratum within temperature and nitrogen, and sampling hour as a further stratum within replicates. Details are shown on the distribution of degrees of freedom and error terms used to estimate variance ratios.

	Sum of Squares	Degrees of freedom	Mean Square	Variance ratio
CO2 (C)	1467.9	1	1467.9	27.323
SubTN stratum	322.3	6	53.7	
Temp. (T)	1.4	1	1.4	0.008
Nitro. (N)	0.8	1	0.8	0.005
CT	47.8	1	47.8	0.276
CN	199.2	1	199.2	1.150
TN	22.2	1	22.2	0.128
CTN	50.9	1	50.9	0.294
Sub Replicate stratum	4157.2	24	173.2	
Sub Hour stratum	11606.0	96	120.9	
Hour	2144.8	3	714.9	19.560
Error	328.9	9	36.5	
Total	17553.5	127		

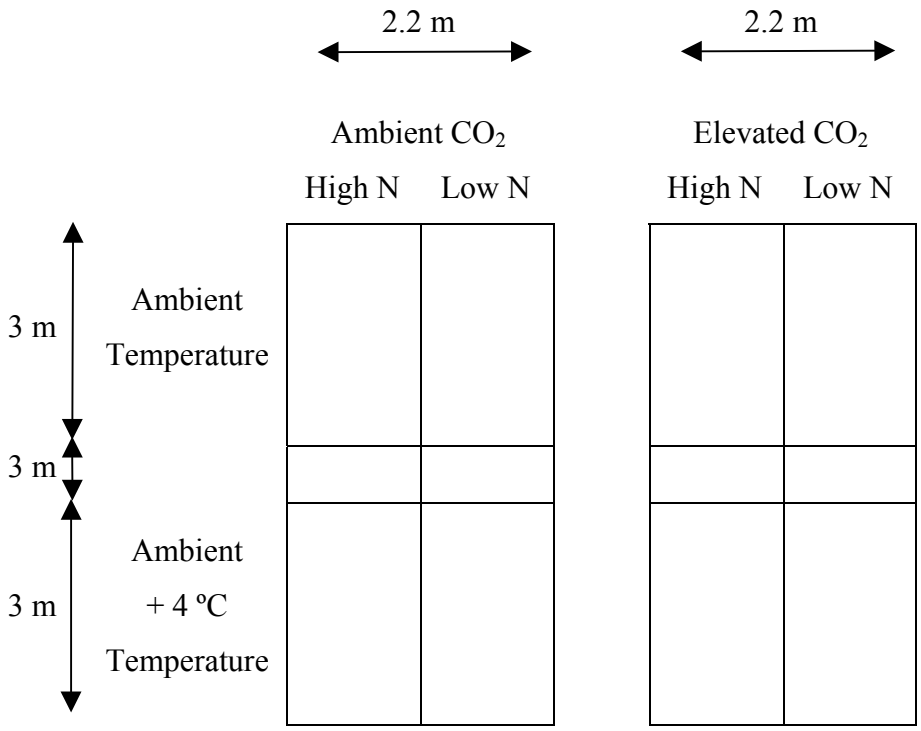
2.2 m
2.2 m
Ambient CO_2
Elevated CO_2
High N
Low N
High N
Low N
3 m
Ambient
Temperature
3 m
3 m
Ambient
+ 4 ºC
Temperature

Fig. 1

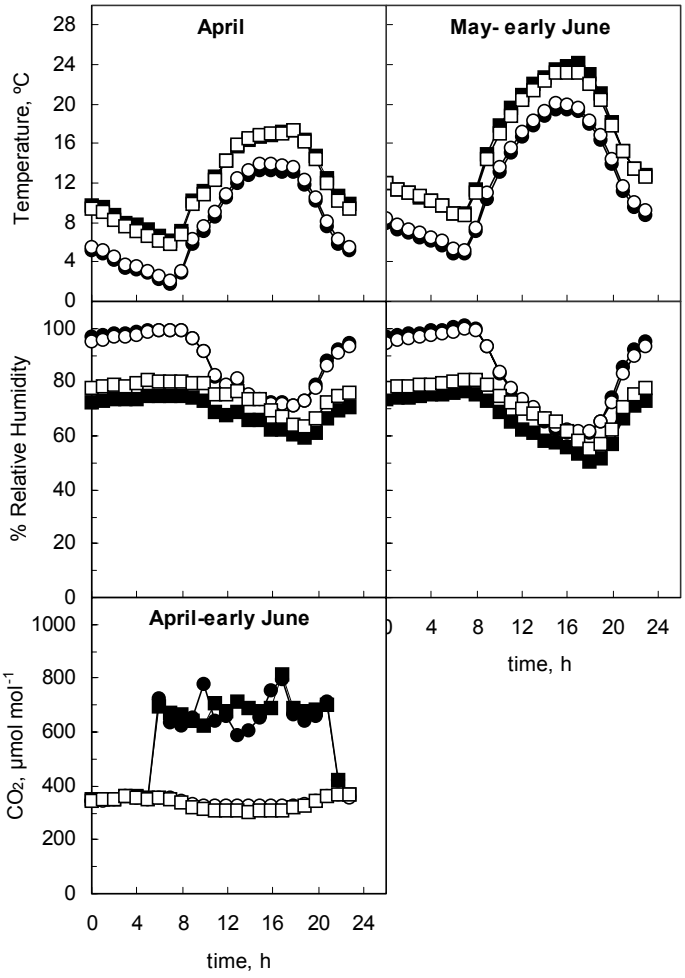
April
May- early June
April-early June
Temperature, °C
% Relative Humidity
CO2, µmol mol-1
time, h
time, h

Fig. 2

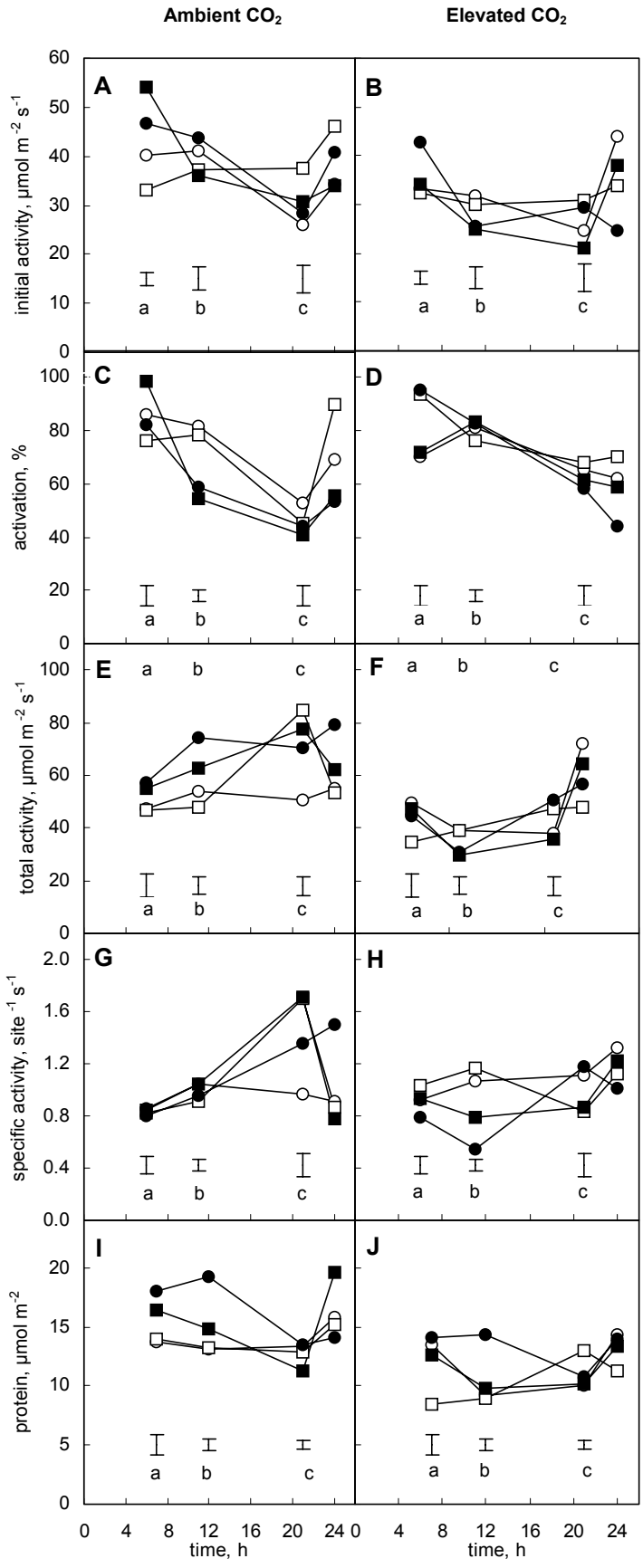
Ambient CO_2
Elevated CO_2
A
B
C
D
E
F
G
H
I
J
initial activity, µmol m^{-2} s^{-1}
activation, %
total activity, µmol m^{-2} s^{-1}
specific activity, site^{-1} s^{-1}
protein, µmol m^{-2}
a
b
c
time, h
0
4
8
12
16
20
24

Fig. 3

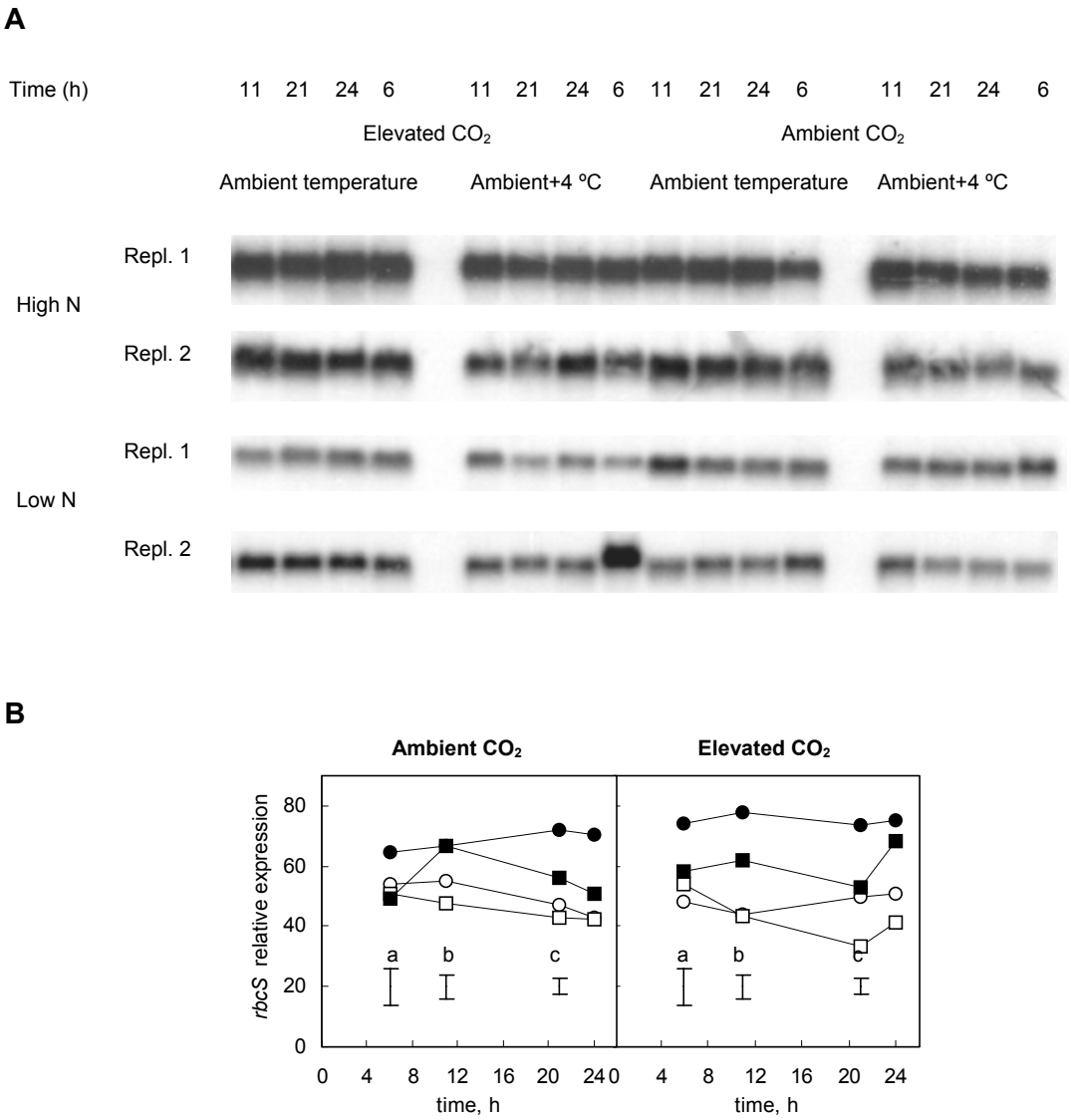
A
Time (h)
11 21 24 6 11 21 24 6 11 21 24 6 11 21 24 6
Elevated CO2
Ambient CO2
Ambient temperature
Ambient+4 ºC
Ambient temperature
Ambient+4 ºC
Repl. 1
High N
Repl. 2
Repl. 1
Low N
Repl. 2
B
Ambient CO2
Elevated CO2
rbcS relative expression
80
60
40
20
0
a
b
c
a
b
c
0 4 8 12 16 20 24 0
4 8 12 16 20 24
time, h
time, h

Fig. 4

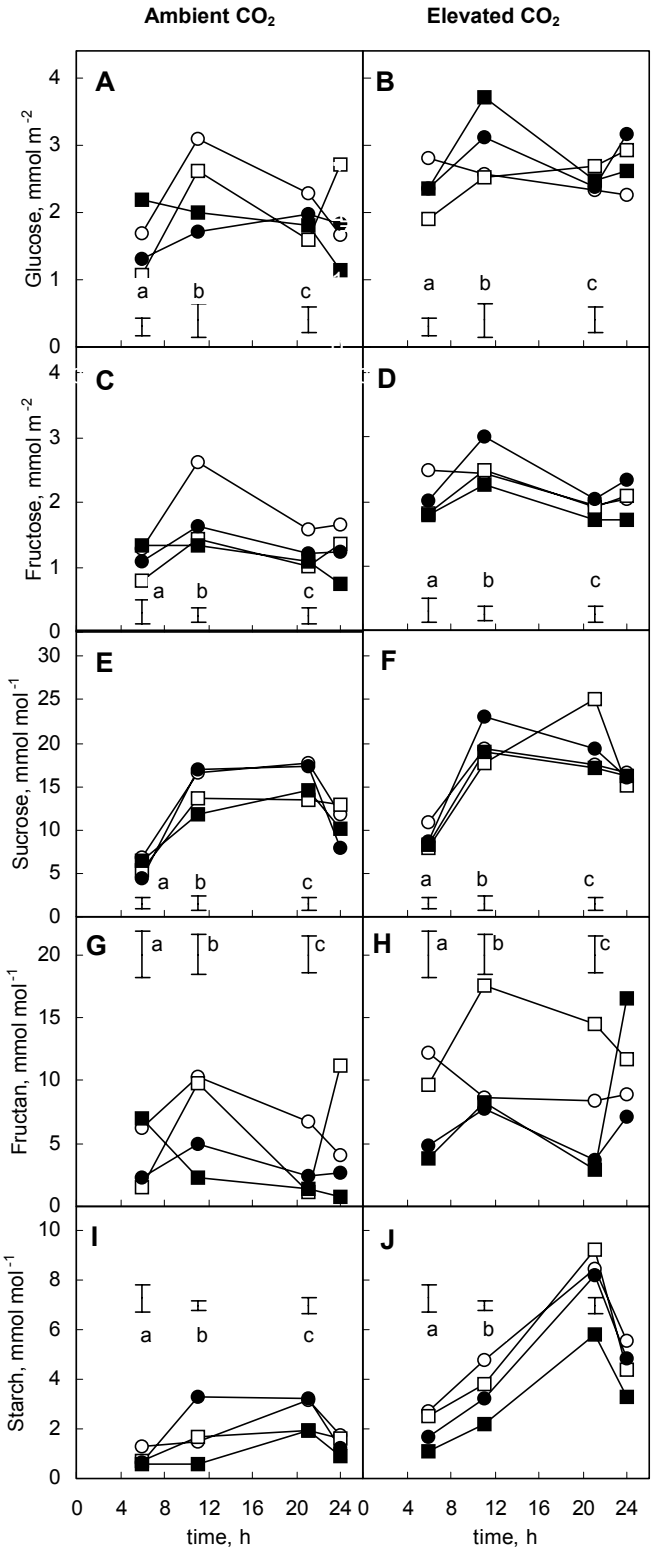
Ambient CO_2
Elevated CO_2
A
B
C
D
E
F
G
H
I
J
Glucose, mmol m^{-2}
Fructose, mmol m^{-2}
Sucrose, mmol mol^{-1}
Fructan, mmol mol^{-1}
Starch, mmol mol^{-1}
a
b
c
0 4 8 12 16 20 24
time, h

Fig. 5

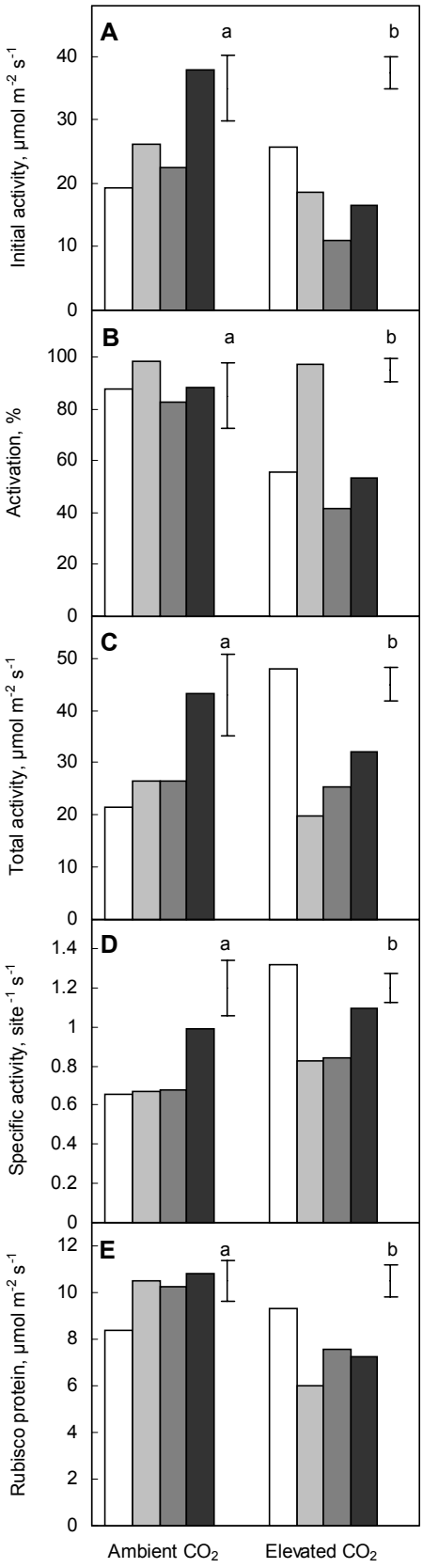
A
B
C
D
E
a
b
Initial activity, µmol m-2 s-1
Activation, %
Total activity, µmol m-2 s-1
Specific activity, site-1 s-1
Rubisco protein, µmol m-2 s-1
Ambient CO2
Elevated CO2

Fig. 6

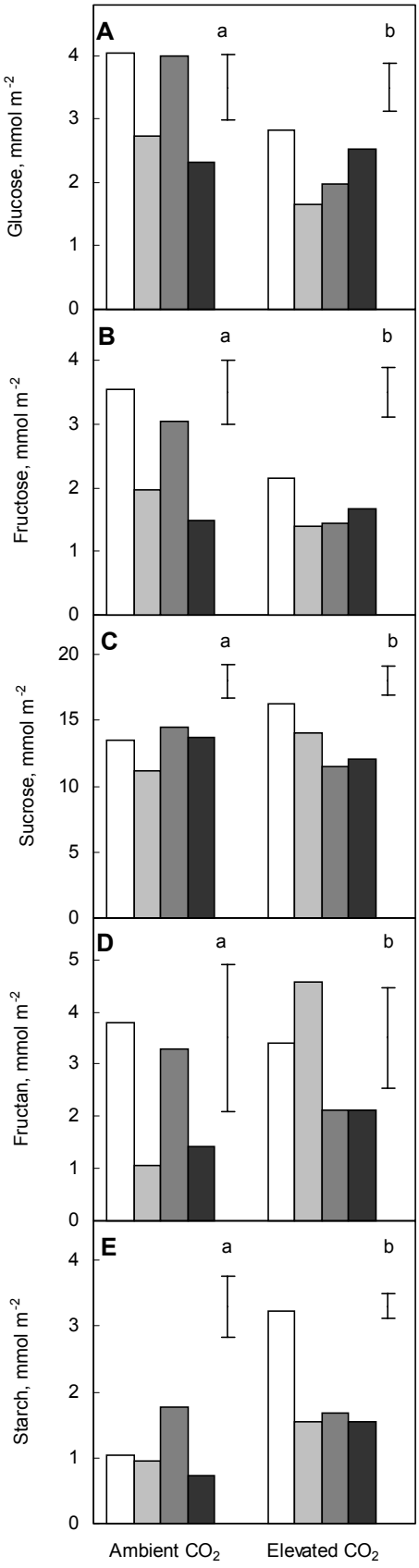
A
B
C
D
E
a
b
Glucose, mmol m-2
Fructose, mmol m-2
Sucrose, mmol m-2
Fructan, mmol m-2
Starch, mmol m-2
Ambient CO2
Elevated CO2

Fig. 7