Down-regulation of Rubisco activity under combined increases of CO₂ and temperature minimized by changes in Rubisco k_{cat} in wheat

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1 **Abstract**

2 Increases in growth temperature have been observed to affect photosynthesis
3 differently under long-term exposure to ambient- and twice ambient-air CO₂
4 concentrations. This study investigates the causes of this interaction in wheat (*Triticum*
5 *aestivum* L.) grown in the field over two consecutive years under temperature gradient
6 chambers in ambient (370 µmol mol⁻¹) or elevated (700 µmol mol⁻¹) atmospheric CO₂
7 concentrations and at ambient or ambient + 4 °C temperatures, with either a low or a
8 high nitrogen supply. The photosynthesis-internal CO₂ response curves and the activity,
9 activation state, k_{cat} and amount of ribulose-1, 5-bisphosphate carboxylase/oxygenase
10 (Rubisco) were measured, as well as the soluble protein concentration in flag leaves at
11 ear emergence and 8-15 days after anthesis. A high nitrogen supply increased V_{cmax} , the
12 Rubisco amount and activity and soluble protein contents, but did not significantly
13 change the Rubisco k_{cat} . Both elevated CO₂ and above ambient temperatures had
14 negative effects on V_{cmax} and Rubisco activity, but at elevated CO₂, an increase in
15 temperature did not decrease V_{cmax} or Rubisco activity in relation to ambient
16 temperature. The amounts of Rubisco and soluble protein decreased with elevated CO₂
17 and temperature. The negative impact of elevated CO₂ on Rubisco properties was
18 somewhat counteracted at elevated temperatures by an increase in k_{cat} . This effect can
19 diminish the detrimental effects on photosynthesis of combined increases of CO₂ and
20 temperature.

21

22 **Key-words** - *Triticum aestivum*, climate change, elevated CO₂, elevated temperature,
23 photosynthetic acclimation, Rubisco, Rubisco k_{cat} .

24

- 1 **Abbreviations** - $J_{\max}$, maximum rate of photosynthetic electron transport; RuBP,
- 2 Ribulose-1, 5-bisphosphate; $V_{\max}$, maximum Rubisco-limited rate of carboxylation.

1 **Introduction**

2 Nitrogen supply (Schulze et al.1994; Lawlor et al. 1989), temperature (Ford and
3 Thorne 1975; Delgado et al. 1994; Ainsworth and Ort 2010) and atmospheric CO₂
4 concentration (Long et al. 2004) are factors that greatly affect plant productivity and
5 photosynthesis. Continued emissions of CO₂ into the atmosphere stimulate
6 photosynthesis because Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is
7 not substrate-saturated at current atmospheric CO₂ concentrations, and oxygenation is
8 inhibited (Long et al. 2004). This enhancement, however, diminishes over time (days to
9 years), a phenomenon known as CO₂ acclimation or down-regulation of photosynthesis.
10 The most pronounced change in the photosynthetic apparatus with prolonged growth in
11 elevated atmospheric CO₂ is a decrease in Rubisco activity and amount (Long et al.
12 2004). This is often associated with decreased expression of genes encoding the small
13 subunit of Rubisco (*rbcS*) (Drake et al. 1997; Moore et al. 1999). Other studies suggest
14 a strong link between the decrease in Rubisco protein under elevated CO₂ and the plant
15 nitrogen status (Riviere-Rolland et al. 1996; Nakano et al. 1997; Farage et al. 1998;
16 Geiger et al. 1999; Ellsworth et al. 2004; Pérez et al. 2005). Acclimation of
17 photosynthesis to elevated CO₂ is usually more marked in nitrogen-limited plants than
18 in well-fertilized plants (Webber et al. 1994; Drake et al. 1997; Farage et al. 1998; Stitt
19 and Krapp 1999). Acclimation of photosynthesis at low nitrogen has been viewed as an
20 indirect effect resulting from the nitrogen limitation of the development of sinks for
21 photoassimilate (Rogers et al. 1998). We have previously reported that atmospheric CO₂
22 enrichment combined with nitrogen abundance reversed or prevented the diurnal
23 increase in Rubisco k_{cat} observed in wheat plants grown in ambient CO₂ concentrations,
24 which was attributed to a slow release of an inhibitor from the enzyme (Pérez et al.
25 2005).

1 The rise in atmospheric CO₂ concentration will increase temperature by 1.5 °C to 6
2 °C (Schneider 1992), or by 2.5 °C to 4.3 °C, according to the Intergovernmental Panel
3 on Climate Change (IPCC) A1B scenario (Christensen et al., 2007). Photosynthesis is
4 most sensitive to inhibition by elevated temperatures, with inhibition occurring at
5 temperatures only slightly higher than those optimal for growth (Salvucci 2008).
6 Decreased discrimination by Rubisco for oxygen and increased solubility of oxygen
7 relative to CO₂ with rising temperatures inhibit net photosynthesis in C₃ plants due to
8 increased photorespiration (Long 1991; Ainsworth and Ort 2010).

9 Relevant information has been provided by studies on plant responses to separate
10 increases in CO₂ and temperature. However, plant responses to combined increases of
11 CO₂ and temperature have received relatively less attention, particularly in cereals.
12 Research on climate change should include interactions of both environmental factors.
13 An interactive effect of elevation of temperature and CO₂ can be expected because the
14 inhibition of oxygenation by rising atmospheric CO₂, and hence an increase in net
15 photosynthesis, will have its greatest effects where oxygenation is greater, namely, at
16 higher temperatures (Long 1991). It has been reported that temperature increases have
17 variable effects on the photosynthesis response to rising CO₂, with positive effects in
18 soybean (Vu et al. 1997), no effect in several British native species from different
19 functional groups (Stirling et al. 1997), rice (Vu et al. 1997), or *Taraxacum officinale*
20 (Bunce 2000), and negative effects in peanut (Vu 2005) and *Poa annua* (Stirling et al.
21 1997). Soybean leaves showed an increasing stimulation of photosynthesis by CO₂ as
22 growth temperature increased from 25 °C to 35 °C (Ziska and Bunce 1997). The
23 enhancement of photosynthesis by CO₂ in *Albutilon theophrasti* was greater at low
24 measurement temperatures for plants grown at low temperatures, and vice-versa for
25 plants grown and measured at high temperatures, indicating that long-term adjustments

1 in V_{cmax} and J_{max} occurred (Ziska 2001). Furthermore, in *Medicago sativa*, the CO_2
2 stimulation of photosynthesis was suppressed at 30 °C growth temperatures (Ziska and
3 Bunce 1994). We have previously shown that warmer growth temperatures did not
4 modify the photosynthesis response to measurement temperatures from 20 to 35 °C,
5 while growth in elevated CO_2 enhanced the response of both photosynthesis and
6 Rubisco activity to temperature (Alonso et al. 2008).

7 Our previous studies conducted on field crops of wheat over four different years,
8 using temperature gradient chambers, have shown that down-regulation of
9 photosynthesis caused by elevated CO_2 was smaller at 4 °C above ambient temperatures
10 with a high nitrogen supply (del Pozo et al. 2005; Martínez-Carrasco et al. 2005).
11 Photosynthesis rates at 370 and 700 $\mu\text{mol mol}^{-1}$ CO_2 reported in these studies did not
12 shed light on the reasons for the beneficial interaction between elevated CO_2 and
13 warmer temperatures. In this study, the main objective was to assess whether the effects
14 of combined increases in CO_2 and temperature could be due to changes in RuBP
15 regeneration or in Rubisco carboxylation, determined *in vivo* from photosynthesis - CO_2
16 response curves. Furthermore, the activity, activation state, k_{cat} and abundance of the
17 enzyme, together with the amount of soluble protein, were determined in order to
18 evaluate possible changes in Rubisco.

Materials and methods

Experimental setup

The details of this two-year experiment have been described elsewhere (del Pozo et al. 2005). Briefly, wheat (*Triticum aestivum* L. cv. Alcalá) was sown in the field in January-February in two consecutive years. The experiment site (40 ° 95 ' N, 5 ° 5 ' W, 800 m a.s.l.) has a clay-sand soil. The climate is Mediterranean. The long-term (20 year) average for the minimum temperature in the coldest month (January) is 0.0 °C and the maximum temperature for the warmest month (July) is 27.2 °C. Mean annual rainfall is 506 mm.

After emergence, two temperature gradient chambers were placed on the crop on different field sites each year. The chambers were adapted from Rawson et al. (Rawson et al. 1995) and have been described previously (Pérez et al. 2005; Aranjuelo et al. 2005). The chambers were 9 m long, 2.2 m wide and 1.7 m high at the ridge, with a polyethylene sheet roof, rigid polycarbonate walls and containing three longitudinal modules separated by slotted polycarbonate septa to reduce the mixing of air between modules through convection. Inlet fans and outlet fans and heaters kept the inlet module at temperatures similar to those outside, and the final outlet module at a temperature 4 °C higher, whilst the central module was left as a spacer. One chamber was kept at ambient CO₂ (370 µmol mol⁻¹), while in the other CO₂ was increased to 700 µmol mol⁻¹ (elevated CO₂) by injecting pure CO₂ at the two inlet fans during daylight hours. Since there appear to be no or only minor positive (Davey et al. 2004) direct effects of growth CO₂ on leaf dark respiration, with reports in the literature even suggesting these might be attributed to experimental artefacts (Jahnke and Krewitt 2002), the lack of CO₂ enrichment during the night is probably irrelevant. In addition, two amounts of nitrogen (ammonium nitrate) were applied to the longitudinal halves of the chambers; these were

52 and 122 kg ha⁻¹ in the first year and none and 70 kg ha⁻¹ in the second, when fertilizer was applied 22 days later than in the first year (2 and 30 April for the first and second year, respectively). The crop was watered weekly through a drip irrigation system providing the average rainfall in the area during the period of the experiment (198 mm). Light intensity inside the tunnels was $89 \pm 3\%$ that outside. CO₂ concentration and temperature in the chambers were very close to set values (del Pozo et al. 2005).

Gas exchange measurements

The photosynthesis-internal CO₂ concentration response curves were measured in flag leaves at ear emergence and 8-15 days after anthesis in both years. An infrared gas analyser (CIRAS-2, PP Systems, Hitchin, Herts., UK) was used with differential operation in an open system. Measurements were taken from 3 to 8 h after the start of the photoperiod using a leaf chamber with a 1.7 cm² window, 300 ml min⁻¹ air flow rate, 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance, 25 °C and 1.6 ± 0.23 kPa vapour pressure deficit. CO₂ concentrations were increased in steps from 60 to 1900 $\mu\text{mol mol}^{-1}$. Four consecutive areas of equal length were marked out in the three-metre long chamber modules, with a leaf randomly chosen from each area being measured. A replicate leaf from each treatment was measured before the next leaf, with treatments in random order, so that differences during the day could be absorbed within the replicated leaves. V_{cmax} and J_{max} were estimated from the response of photosynthesis to intercellular CO₂ concentration in leaves using the model proposed by Farquhar et al. (1980), with the help of the Photosynthesis Assistant software ([http:// www.scientific.force9.co.uk](http://www.scientific.force9.co.uk)).

1 *Rubisco activity*

2 At mid-morning on the photosynthesis measurement dates, four leaves from each of
3 the four sampling areas in a treatment plot were harvested and rapidly plunged *in situ*
4 into liquid nitrogen and then stored at -80 °C until analysed. The fresh weight, leaf area
5 and chlorophyll content of sub-samples of frozen leaves were determined as described
6 (Pérez et al. 2005). This allowed the results to be expressed on a leaf area basis.

7 Rubisco (EC 4.1.1.39) activity before (initial activity) and after (total activity) active
8 site carbamylation was assayed at 23 °C by a spectrophotometric procedure coupled to
9 NADH oxidation (Pérez et al. 2005). Rubisco activation was estimated as the percent
10 ratio of initial to total activities for each sample. The amounts of soluble protein and
11 Rubisco in frozen sub-samples were measured after extraction, spectrophotometric
12 analysis and gel electrophoresis followed by densitometry, using BSA as protein
13 standard, as described (Aranjuelo et al. 2005). To calculate the molar concentration of
14 Rubisco subunits, molecular masses of 52 and 15 kDa were assumed for the large and
15 small subunits, respectively. The k_{cat} of Rubisco was determined for each sample by
16 dividing total activity by the number of moles of active sites, which was taken as
17 equivalent to the moles of the large Rubisco subunit. This would include any Rubisco
18 site bound to inhibitors and would yield low k_{cat} as compared to methods determining
19 free Rubisco sites (Sharkey et al. 1991).

20

21 *Statistical analyses*

22 The experiment was analysed as a randomized-block strip-plot design, with year as
23 the blocking factor, CO₂ allocated to whole-plots within blocks, temperature and
24 nitrogen allocated, respectively, to rows and columns within whole-plots, and sampling
25 date in a subplot stratum within rows and columns. The variance was estimated using

1 the method of residual maximum likelihood (REML; Genstat 6.2). As there were only
2 two blocks, the threshold for significance was chosen as $P < 0.055$ to avoid the
3 possibility of a Type II error.

1 **Results**

2 The effects of elevated CO₂, temperature and nitrogen supply plus sampling date on
 3 photosynthesis and Rubisco characteristics were analyzed. In a previous article (del
 4 Pozo et al. 2005), we have shown that photosynthesis rates measured at 370 and 700
 5 μmol mol⁻¹ CO₂ were decreased by growth in elevated CO₂. The photosynthesis-internal
 6 CO₂ response curves for each treatment combination averaged over years and sampling
 7 dates (Fig. 1) show that in plants grown in ambient CO₂ photosynthesis with
 8 intercellular CO₂ concentrations of 182 ± 36 and 383 ± 77 μmol mol⁻¹ (corresponding to
 9 air CO₂ levels of 370 and 700 μmol mol⁻¹, respectively) was limited by Rubisco and
 10 RuBP regeneration, respectively. By comparison, photosynthesis at the respective
 11 intercellular CO₂ concentrations of 166 ± 29 and 352 ± 39 μmol mol⁻¹ in plants grown
 12 in elevated CO₂ was limited by Rubisco activity. The reported effects on photosynthesis
 13 measured at ambient or elevated CO₂ (del Pozo et al. 2005) induced by CO₂ enrichment
 14 or by combined increases in CO₂ and temperature were therefore due to changes in
 15 Rubisco activity.

16 Elevated CO₂ did not change the gas exchange-determined J_{max} (Tables 1 and 2),
 17 while it significantly decreased V_{cmax}, initial and total Rubisco activities (Table 3), and
 18 Rubisco activation. Rubisco protein contents (Table 4) significantly decreased in
 19 elevated CO₂, and total soluble protein had a similar response to CO₂. As a fraction of
 20 soluble protein, Rubisco was similar with both CO₂ levels at ear emergence, while it
 21 was lower with elevated rather than ambient CO₂ after anthesis (Tables 1 and 4). This
 22 pointed to a specific decrease with time of Rubisco in elevated CO₂. In ambient, but not
 23 high temperature, elevated CO₂ decreased Rubisco k_{cat} (Table 3).

24 Warmer temperatures had no significant effects on photosynthesis measured at 370
 25 or 700 μmol mol⁻¹, probably because they increased stomatal conductance (del Pozo et

1 al. 2005). Above-ambient temperatures decreased $J_{\max}$ (Tables 1 and 2) and $V_{\max}$,
 2 except with high nitrogen and elevated CO_2 (non-significant interactions). Initial
 3 Rubisco activity (Tables 1 and 3) decreased as the temperature increased under ambient
 4 CO_2 , but not under elevated CO_2 ; total Rubisco activity followed the same trend,
 5 although it did not reach statistical significance. Rubisco protein contents significantly
 6 decreased with above-ambient temperatures (Table 4). Therefore, a temperature
 7 increase under elevated CO_2 did not decrease activity, but reduced the amount of
 8 Rubisco. Warmer temperatures also decreased total soluble protein contents (Table 4)
 9 and did not affect Rubisco as a percentage of soluble protein, which suggested that
 10 temperature effects on Rubisco protein were due to an alteration of soluble protein
 11 contents, rather than being a specific change in this enzyme. Rubisco k_{cat} (Table 3)
 12 increased with temperature under elevated CO_2 , but not under ambient CO_2 .

13 A high nitrogen supply generally increased photosynthesis (del Pozo et al. 2005). In
 14 turn, $J_{\max}$ (Tables 1 and 2) was higher with a high rather than a low nitrogen supply at
 15 anthesis, but the difference disappeared 8-15 days later. $V_{\max}$ (Table 2), initial and total
 16 Rubisco activities (Table 3), as well as Rubisco and soluble protein contents (Table 4),
 17 increased with nitrogen supply, but enzyme activation (Table 3) was reduced with high
 18 rather than low nitrogen. Nitrogen did not significantly change Rubisco as a percentage
 19 of soluble protein or the Rubisco k_{cat} . Most experimental parameters decreased from ear
 20 emergence to 8-15 days after anthesis (Tables 1 to 4); exceptions were Rubisco
 21 activation and k_{cat} (Table 3), which did not vary significantly between dates.

1 **Discussion**

2 The cause of the smaller down-regulation of photosynthetic capacity with elevated
 3 CO₂ in warmer rather than ambient temperatures in these experiments (del Pozo et al.
 4 2005) and in previous ones (Martínez-Carrasco et al. 2005) had not been identified
 5 beyond doubt. The photosynthesis-CO₂ responses reported here (Fig. 1) show that
 6 photosynthesis in plants grown in elevated CO₂ was limited by Rubisco activity up to
 7 CO₂ concentrations above those prevailing during growth. This was due, first, to the
 8 low stomatal conductance and intercellular CO₂ concentrations in the warm, dry
 9 environment of the experiments (del Pozo et al. 2005), and, second, to the decrease in
 10 Rubisco activity with long-term growth in elevated CO₂ (Tables 1 and 2) that has also
 11 been observed in preceding studies (Drake et al. 1997; Long et al. 2004; Pérez et al.
 12 2005). Accordingly, the smaller decrease in photosynthetic capacity caused by elevated
 13 CO₂ at warmer rather than ambient temperatures is attributable to changes in Rubisco-
 14 limited carboxylation. We have also found that growth in elevated CO₂ increases the
 15 response of photosynthesis and Rubisco activity to temperature (Alonso et al. 2008).

16 To further explore the involvement of changes in Rubisco activity in responses to
 17 growth in elevated temperature and CO₂, we estimated V_{cmax} using gas exchange
 18 measurements and the *in vitro* activity of Rubisco. Separately, both elevated CO₂ and
 19 above-ambient temperatures had negative effects on V_{cmax} and Rubisco activity, with
 20 CO₂ generally having a greater impact than temperature. When applied together,
 21 elevated CO₂ and warmer temperature did not have a cumulative negative effect; that is,
 22 increasing the temperature by 4 °C for plants grown in elevated CO₂ did not decrease
 23 V_{cmax} and Rubisco activity to a larger extent than in plants grown at ambient
 24 temperature. Thus, with combined increases in CO₂ and temperature, V_{cmax} and Rubisco

1 activity decreased in relation to ambient CO₂, but not in relation to ambient temperature
2 under elevated CO₂.

3 The decrease in Rubisco activity under CO₂ enrichment was a result of the well
4 established decrease in enzyme amount (Long et al. 2004) and - at low nitrogen - of the
5 decarbamylation of the enzyme that is observed in some studies (Sage et al. 1988),
6 though not in others (Nakano et al. 1997). It is conceivable that at low nitrogen, with
7 low Rubisco amounts and activities, RuBP may be in excess and therefore bind tightly
8 to any uncarbamylation sites. Consistent with studies relating down-regulation
9 of Rubisco in elevated CO₂ to a low plant nitrogen status (Riviere-Rolland et al. 1996;
10 Nakano et al. 1997; Farage et al. 1998; Geiger et al. 1999; Ellsworth et al. 2004; Pérez et
11 al. 2005), the loss of Rubisco amount in elevated CO₂ was part of a general decrease in
12 soluble protein, although after anthesis there was a preferential loss of Rubisco. When
13 not applied at a high temperature, CO₂ enrichment decreased Rubisco k_{cat} . Decreases in
14 Rubisco catalysis not associated with deactivation (decarbamylation) have been
15 observed when carbohydrates build up upon the transfer of plants from low to high light
16 intensity (Grub and Machler 1990), after blocking export from leaves by cold-girdling
17 (Krapp and Stitt 1995), when grown in elevated CO₂ (Pérez et al. 2005) and in anti-
18 Rubisco activase plants, particularly in high CO₂ (He et al. 1997). Diurnal changes in
19 the activity of carbamylated Rubisco have also been found, but these were similar in
20 ambient and elevated CO₂ (Mckee et al. 2000). Tightly bound Rubisco inhibitors could
21 decrease the carbamylated enzyme turnover rate, although He et al. (1997) found that
22 most of this decrease disappeared after rapid extraction for *in vitro* assays of Rubisco
23 activity; in our experiment, the extraction procedure preserved the inhibitor binding to
24 Rubisco. We take the variations in Rubisco k_{cat} as evidence of the presence of a daytime
25 inhibitor of the enzyme, as reported for wheat and other plants (Keys et al. 1995; Parry

1 et al. 1997). This inhibitor has properties similar to those of pentodiulose-bisphosphate
2 (Kane et al. 1998), which is produced by the oxygenation of the enediol intermediate
3 formed from RuBP in Rubisco catalysis (Pearce and Andrews 2003). It has been
4 speculated that the inhibitor may be formed non-enzymatically from RuBP in the
5 chloroplast, given its favourable conditions for this process (Kane et al. 1998). In this
6 case, the levels of the inhibitor could increase in response to an accumulation of
7 precursor metabolites, as reported for the nocturnal inhibitor of Rubisco, carboxy-
8 arabinitol 1-phosphate (Andralojc et al. 2002). Consistent with this, we have found that
9 elevated CO₂ increased the levels of carbohydrates and, possibly, of phosphorylated
10 metabolites (Pérez et al. 2005).

11 Rubisco activity did not decrease with temperature in elevated CO₂ as it did in
12 ambient CO₂. However, warmer temperatures in both ambient and elevated CO₂
13 decreased the amounts of Rubisco, in agreement with other studies (Yamasaki et al.
14 2002; Pérez et al. 2005), and total soluble protein. There was no compensating increase
15 in Rubisco activation to account for the interaction of elevated CO₂ and temperature
16 over enzyme activity. In elevated CO₂, the decrease in Rubisco amount caused by
17 ambient + 4 °C temperature was counteracted by an enhancement of the (apparent) k_{cat}
18 of Rubisco (Tables 1 and 2). This finding is consistent with the observation of a
19 decrease in Rubisco protein in soybean at high temperatures, but not in total activity,
20 implying that the k_{cat} of Rubisco was up-regulated (Vu et al. 1997). The catalytic
21 activity of Rubisco is known to increase with temperature (Sage 2002), but this would
22 not explain the observed change in k_{cat} measured *in vitro* at the same temperature in all
23 treatments. The smaller decrease in Rubisco catalysis in elevated CO₂ caused by warm
24 temperatures is probably due to decreases in the daytime inhibitor of the enzyme. This

1 may be associated with less accumulation of metabolites at warm temperatures, as the
2 decreased levels of carbohydrates suggest (Pérez et al. 2005).

3 Both the modeling of the photosynthetic responses to internal CO₂ concentration and
4 Rubisco activity measurements indicate that Rubisco is a major determinant of
5 photosynthetic inhibition at elevated CO₂ and at elevated temperature. We have shown
6 how these two factors interact on photosynthesis, whereby warm temperatures reduce
7 the inhibition of Rubisco k_{cat} caused by elevated CO₂. This interaction is different from
8 the displacement of the temperature optimum for photosynthesis to higher values in
9 elevated CO₂, due to the decrease in photorespiration (Long 1991). The reported effect
10 on Rubisco k_{cat} diminishes the negative impact on photosynthesis of combined increases
11 in CO₂ and temperature.

1 **Acknowledgments**

2 The technical cooperation of A. L. Verdejo in gas exchange measurements and
3 Rubisco activity assays is acknowledged. L. Hernández analyzed Rubisco and soluble
4 protein. We thank the staff at this Institute's experimental farm for their technical
5 assistance in crop husbandry. This work was funded by the Spanish Research and
6 Development Plan (Projects no. BFI2000-0871 and AGL2006-13541-C02-02/AGR)
7 and the CSIC intramural contract no. 200640I148. R. Morcuende had I3P and Ramon y
8 Cajal research contracts, and A. Alonso an I3P pre-doctoral fellowship, from the CSIC-
9 European Social Fund. G. Zita was the recipient of a grant from the Autonomous
10 University of Mexico. We thank the anonymous referee whose constructive comments
11 were very helpful in improving the quality of this work.

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Table 1. REML analysis (χ^2 probability) for $J_{\max}$, $V_{\max}$, activity, activation, content and k_{cat} of Rubisco, and soluble protein concentration in flag leaves of wheat grown in the field under temperature gradient chambers with ambient ($370 \mu\text{mol mol}^{-1}$) or elevated ($700 \mu\text{mol mol}^{-1}$) CO_2 , ambient or ambient + 4 °C temperatures, and high or low nitrogen supply, at ear emergence and 8-15 days after anthesis. Mean values for two years. Numbers in bold represent significant effects ($P < 0.055$)

	$J_{\max}$, $\mu\text{mol m}^{-2} \text{s}^{-1}$	$V_{\max}$, $\mu\text{mol m}^{-2} \text{s}^{-1}$	Initial Rubisco activity, $\mu\text{mol m}^{-2} \text{s}^{-1}$	Rubisco activation, %	Total Rubisco activity, $\mu\text{mol m}^{-2} \text{s}^{-1}$	Rubisco protein $\mu\text{mol m}^{-2}$	Rubisco, % soluble protein	Soluble protein, g m^{-2}	k_{cat} , s^{-1}
CO_2 (C)	0.631	0.005	<0.001	0.053	<0.001	0.054	0.002	<0.001	0.119
Temperature (T)	0.028	<0.001	0.005	0.847	0.098	0.01	0.337	0.043	0.011
Nitrogen (N)	0.032	0.012	<0.001	0.024	<0.001	<0.001	0.067	0.014	0.893
Date (D)	<0.001	<0.001	<0.001	0.22	<0.001	<0.001	0.049	<0.001	0.218
C.T	0.163	0.581	0.003	0.761	0.102	0.966	0.827	0.863	0.032
C.N	0.556	0.903	0.435	0.157	0.931	0.939	0.326	0.879	0.745
T.N	0.279	0.088	0.697	0.678	0.61	0.537	0.219	0.934	0.716
C.D	0.078	0.167	0.063	0.346	0.899	0.987	0.004	0.737	0.983
T.D	0.171	0.276	0.365	0.909	0.591	0.395	0.175	0.522	0.357
N.D	0.01	0.655	0.164	0.952	0.317	0.111	0.554	0.243	0.546
C.T.N	0.639	0.238	0.322	0.783	0.535	0.591	0.249	0.892	0.233
C.T.D	0.415	0.918	0.587	0.897	0.884	0.956	0.675	0.852	0.687
C.N.D	0.15	0.604	0.351	0.543	0.377	0.752	0.44	0.752	0.641
T.N.D	0.083	0.987	0.852	0.981	0.729	0.752	0.27	0.909	0.891
C.T.N.D	0.035	0.431	0.203	0.873	0.624	0.936	0.383	0.921	0.793

Table 2. Photosynthetic parameters $J_{\max}$ and $V_{\max}$ in flag leaves of wheat grown in the field under temperature gradient chambers with ambient ($370 \mu\text{mol mol}^{-1}$) or elevated ($700 \mu\text{mol mol}^{-1}$) CO_2 , ambient (T) or ambient + 4 °C (T+4) temperatures, and high or low nitrogen supply. Leaves were measured at ear emergence and 8-15 days after anthesis. Only significant interactions are shown. Mean values for two years. sed, standard error of the difference between means.

		$J_{\max}$, $\mu\text{mol m}^{-2} \text{s}^{-1}$	$V_{\max}$, $\mu\text{mol m}^{-2} \text{s}^{-1}$
CO_2 (C)	Amb. CO_2	426.2	121.3
	Elev. CO_2	431.8	102.4
	sed	11.60	6.75
Temperature (T)	T	463.3	119.7
	T+4	394.7	104.0
	sed	31.20	4.61
Nitrogen (N)	Low N	395.5	106.1
	High N	462.5	117.7
	sed	31.20	4.61
Date (D)	Ear emergence	476.4	122.1
	After anthesis	381.6	101.6
	sed	23.03	4.82
Nitrogen. Date (N·D)	Low N· Ear emer.	413.2	
	Low N· After anth.	377.9	
	High N· Ear emer.	539.7	
	High N· After anth.	385.2	
	sed	36.71	

Table 3. Rubisco activity, activation and k_{cat} in flag leaves of wheat grown in the field under temperature gradient chambers with ambient ($370 \mu\text{mol mol}^{-1}$) or elevated ($700 \mu\text{mol mol}^{-1}$) CO_2 , ambient (T) or ambient + 4°C (T+4) temperatures, and high or low nitrogen supply. Leaves were measured at ear emergence and 8-15 days after anthesis. Only significant interactions are shown. Mean values for two years. sed, standard error of the difference between means; ns, not significant.

		Initial Rubisco activity $\mu\text{mol m}^{-2} \text{s}^{-1}$	Total Rubisco activity $\mu\text{mol m}^{-2} \text{s}^{-1}$	Rubisco activation %	$k_{\text{cat}}, \text{s}^{-1}$
CO_2 (C)	Amb. CO_2	45.73	58.95	78.64	1.47
	Elev. CO_2	27.95	39.23	74.88	1.35
	sed	4.56	4.50	1.94	0.075
Temperature (T)	T	39.11	51.69	77.04	1.31
	T+4	34.57	46.49	76.48	1.51
	sed	1.62	ns	ns	0.077
Nitrogen (N)	Low N	33.02	41.54	80.05	1.41
	High N	40.66	56.64	73.48	1.40
	sed	1.62	3.15	2.91	0.077
Date (D)	Ear emergence	46.19	63.24	73.7	1.35
	After anthesis	27.5	34.95	79.82	1.47
	sed	1.37	4.02	ns	0.101
$\text{CO}_2 \cdot \text{Temp}$ (C·T)	Amb $\text{CO}_2 \cdot \text{T}$	50.45			1.45
	Amb $\text{CO}_2 \cdot \text{T}+4$	41.02			1.48
	Elev. $\text{CO}_2 \cdot \text{T}$	27.77			1.17
	Elev. $\text{CO}_2 \cdot \text{T}+4$	28.13			1.53
	sed	3.995			0.108

Table 4. Soluble and Rubisco protein contents, and Rubisco as a percentage of soluble protein in flag leaves of wheat grown in the field under temperature gradient chambers with ambient ($370 \mu\text{mol mol}^{-1}$) or elevated ($700 \mu\text{mol mol}^{-1}$) CO_2 , ambient (T) or ambient + 4 °C (T+4) temperatures, and high or low nitrogen supply. Leaves were measured at ear emergence and 8-15 days after anthesis. Only significant interactions are shown. Mean values for two years. sed, standard error of the difference between means; ns, not significant.

		Soluble protein g m^{-2}	Rubisco protein $\mu\text{mol m}^{-2}$	Rubisco % soluble protein
CO_2 (C)	Amb CO_2	6.08	5.25	36.81
	Elev. CO_2	4.79	3.90	33.06
	sed	0.26	0.70	1.23
Temperature (T)	T	6.09	5.12	34.13
	T+4	4.79	4.03	35.74
	sed	0.64	0.43	ns
Nitrogen (N)	Low N	4.65	3.84	33.40
	High N	6.22	5.31	36.48
	sed	0.64	0.43	ns
Date (D)	Ear emergence	7.37	6.07	33.53
	After anthesis	3.50	3.08	36.34
	sed	0.69	0.70	1.43
CO_2 · Date (C.D)	Amb. CO_2 · Ear emer.			33.36
	Amb. CO_2 · After anth.			40.26
	Elev. CO_2 · Ear emer.			33.70
	Elev. CO_2 · After anth.			32.42
	sed			1.93

Figure legend

Fig. 1. Photosynthesis-intercellular CO₂ concentration response curves in flag leaves of wheat grown in the field under temperature gradient chambers with ambient (370 $\mu\text{mol mol}^{-1}$) or elevated (700 $\mu\text{mol mol}^{-1}$) CO₂, ambient or ambient + 4 °C temperatures, and high or low nitrogen supply. The lines represent rates of Rubisco-limited (solid lines) and RuBP regeneration-limited (broken lines) carboxylation, fitted using the functions of Farquhar et al. (1980). Mean values for two sampling dates (ear emergence and 8-15 days after anthesis) and for two years. Each point is the mean of 16 samples. Arrows represent intercellular CO₂ concentrations corresponding to measurement air CO₂ levels of 370 and 700 $\mu\text{mol mol}^{-1}$.

Fig. 1