Elevated CO_2 and temperature differentially affect photosynthesis and resource allocation in flag and penultimate leaves of wheat

P. PÉREZ, G. ZITA**, R. MORCUENDE, and R. MARTÍNEZ-CARRASCO*

*Instituto de Recursos Naturales y Agrobiología de Salamanca, CSIC, Apartado 257, E-37071 Salamanca, Spain**

Abstract

Differences in acclimation to elevated growth CO_2 (700 μmol mol^{-1}, EC) and elevated temperature (ambient +4 ºC, ET) in successive leaves of wheat were investigated in field chambers. At a common measurement CO_2, EC increased photosynthesis and the quantum yield of electron transport (Φ) early on in the growth of penultimate leaves, and later decreased them. In contrast, EC did not change photosynthesis, and increased Φ at later growth stages in the flag leaf. Contents of chlorophyll (Chl), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO), and total soluble protein were initially higher and subsequently lower in penultimate than flag leaves. EC decreased RuBPCO protein content relative to soluble protein and Chl contents throughout the development of penultimate leaves. On the other hand, EC initially increased the RuBPCO:Chl and Chl *a/b* ratios, but later decreased them in flag leaves. In the flag leaves but not in the penultimate leaves, ET initially decreased initial and specific RuBPCO activities at ambient CO_2 (AC) and increased them at EC. Late in leaf growth, ET decreased Chl contents under AC in both kinds of leaves, and had no effect or a positive one under EC. Thus the differences between the two kinds of leaves were due to resource availability, and to EC-increased allocation of resources to photon harvesting in the penultimate leaves, but to increased allocation to carboxylation early on in growth, and to light harvesting subsequently, in the flag leaves.

Additional key words: acclimation; ribulose-1,5-bisphosphate carboxylase/oxygenase; temperature; *Triticum aestivum*.

Introduction

Many studies of the responses of photosynthesis to elevated air CO_2 concentration (EC) have focused on the youngest, actively photosynthesising leaves. We are aware of two studies of leaves in different positions within the canopy of wheat (Osborne *et al.* 1998, Adam *et al*. 2000). In these studies, EC caused no decrease in the carboxylation capacity of flag leaves, but during grain development—and not before anthesis—it led to a decrease in net photosynthetic rate (P_N) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) content, together with an increase in content of light-harvesting proteins, in the lower shaded leaves. However, the interactive effects of EC and elevated temperature (ET) on leaves in different positions were not addressed in these studies. We found previously a downward acclimation of P_N in flag leaves under EC as early as at ear emergence; with ET (ambient +4 °C) and a high N supply, this acclimation decreased (Martínez-Carrasco *et al*. 2005). Nie *et al*. (1995) observed an early decrease of RuBPCO content in wheat leaves under EC, followed by a more marked and later decrease in contents of this and other proteins and chlorophyll (Chl), which could reflect an acceleration of senescence. Leaf constituents exhibit changes with age, with increases followed by decreases in contents of proteins, Chl, and other compounds; soluble proteins reach a maximum before Chl and insoluble or

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*Corresponding author; fax: +34923219609, e-mail: rafaelmc@usal.es

***Present address*: Departamento de Ciencias Agrícolas, Universidad Nacional Autónoma de México, Cautitlan-Izcalli, 54712 Mexico.

Abbreviations: AC – ambient CO_2 concentration; AT – ambient temperature; EC – enhanced CO_2 concentration; DA – days after anthesis; ET – enhanced temperature; F_m' – maximum fluorescence yield in the light-adapted state; F_t – light-adapted steady-state fluorescence yield; P_N – net photosynthetic rate; PS2 – photosystem 2; RuBP – ribulose-1,5-bisphosphate; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase; Φ – quantum yield of electron transport; Φ_{max} – maximum quantum yield of electron transport in light-adapted leaves.

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membrane proteins (Martín del Molino *et al*. 1995). Since prolonged growth at EC alters the contents of these constituents (Stitt and Krapp 1999), the question arises as to whether their balance and pattern of change with time is affected, and whether variations occur among leaves in a stem.

During development, a transition from sink to source for assimilates occurs in leaves. In addition to the irradiance, sink demand may be different for source leaves in different positions on a stem. In warm environments, where temperatures rapidly increase in spring and accelerate plant development, flag leaf growth in wheat takes place while a strong demand for assimilates is posed by the rapidly elongating last stem internode and, later on, by the ear and grains. Moreover, Rogers *et al*. (1998) have suggested that acclimation to EC is caused by a limitation in sink development when N content is low, rather than being a direct effect of the N supply on photosynthesis.

Leaves of cereals emerge under full irradiance and are progressively shaded by organs higher up in the leaf canopy (Osborne *et al*. 1998). In the low irradiance down in the canopy, leaf P_N is limited by the capacity of regeneration of ribulose-1,5-bisphosphate (RuBP) and electron transport. Although acclimation to EC may decrease RuBPCO activity, an increase in CO_2 concentration under such conditions will increase the efficiency of P_N because photorespiration is decreased (Long 1991, Osborne *et al*. 1997). In addition to this, the response of leaves to low irradiance involves changes in photosynthetic capacity, with decreases in the Chl *a/b* ratio that are consistent with an increase in the light-harvesting antenna, and decreases in cytochrome b_6f and RuBPCO contents and ATPase activity (Bailey *et al*. 2001). This shade acclimation could vary with acclimation to EC, which increases the light-harvesting complex (Osborne *et al*. 1998).

When carbon fixation is inhibited, photosynthetic electron transport is down regulated to match the decline in electron demand; at the same time, non-photochemical quenching increases (Golding and Johnson 2003). Seasonal decreases in photochemistry and increases in photoinhibition under EC have been found (Hymus *et al*. 1999). CO_2 enrichment can either increase or decrease the photochemistry, depending on whether a down-regulation of photosynthetic capacity occurs; with N deficiency, there is an acclimatory decrease in RuBPCO that decreases the electron transport destined to photosynthetic carbon reduction (Hymus *et al*. 2001). We have observed that the quantum yield of photosystem 2 (PS2) electron transport (Φ) decreases in EC in wheat; at later stages of growth, ET decreased the maximal photochemical efficiency with low N, but not with high N (Martínez-Carrasco *et al*. 2005). Moreover, plants grown at EC maintain the maximal photochemical efficiency of PS2 up to significantly higher temperatures than in leaves at ambient CO_2 (AC), so it has been concluded that growth in EC protects against high-temperature lesions (Taub *et al*. 2000).

Under CO_2 enrichment, carboxylation becomes more efficient and, in theory, for maximum photosynthesis rates per unit N, a decrease in RuBPCO relative to RuBP-regeneration would be expected (Sage 1994). There is some evidence for re-balancing, but this is small and frequently absent (Sage 1994, Sage *et al*. 1995, Nakano *et al*. 1997, Mitchell *et al*. 2000), although N from the decreased RuBPCO in anti-sense rice plants induces an increase in contents of other photosynthetic components for any given content of leaf N (Makino *et al*. 1997). The question of whether acclimation to EC reduces the excess RuBPCO investment thus remains open.

The aim of this work was to learn whether the effects on photosynthesis of growth EC and ET vary between leaves of different age and position on wheat stems. In particular, we investigated possible differences between the leaves in the response of the balance between carboxylation and electron transport to EC and ET. With this purpose, we analysed the content of Chl, all of which is bound to thylakoid proteins (Markwell *et al*. 1979) and may therefore reflect the size of the photochemical apparatus, contents of RuBPCO and soluble proteins, and the activity of RuBPCO during growth of the penultimate and flag leaves. To investigate the effects of EC and ET on Φ and P_N of light-adapted leaves, rapid irradiance-response curves of Chl fluorescence, and carbon fixation under saturation irradiance and AC or EC were determined.

Materials and methods

After harvesting an alfalfa crop on a clay soil, spring wheat (*Triticum aestivum* L. cv. Alcázar) was sown at a rate of 190 kg ha^{-1} and 0.13 m row spacing, on 26 February. Before sowing, N (as NH_4NO_3), P, and K fertilizers (28, 48, and 28 kg ha^{-1}, respectively) were added. An additional application of 108 kg ha^{-1} N was made on 27 March. Pests were prevented with herbicides and insecticides. The crop was watered weekly with a drip irrigation system supplying the average rain water in the area during the period of the experiment (198 mm between February and June). The experimental site was located in the IRNASA farm in Salamanca (41ºN, 800 m above sea level), Spain.

Two temperature-gradient chambers were mounted on the crop on 2 April, rather than at sowing, to select crop areas with a uniform plant cover. The chambers followed the design of Rawson *et al*. (1995). They were 9.0 m long, 2.2 m wide, and 1.7 m high at the ridge and consisted of three longitudinal modules separated by polycarbonate septa. These chambers were described in Pérez *et al*. (2005). One chamber was kept at AC (360 μmol mol^{-1}), and another at EC, *i.e*. twice this

concentration (700 μmol mol^{-1}). An inlet module in a chamber closely tracked the AT fluctuations, while the temperature was increased by 4 ºC in the opposite module. Samplings in the four combinations of CO_2 and temperature were repeated in three consecutive sections within each of the module halves (six repeated measurements). Leaves were sampled at full emergence (penultimate leaves, Chl fluorescence), 2 (flag leaves), 12–14, 25–28, and 39–41 (penultimate leaves) d after leaf emergence, corresponding to –22, –9 to –7, 3–5, and 17–19 d from the start of anthesis (DA), respectively (anthesis took place on 25 and 28 May for plants at EC and AC, respectively).

***P*$_N$**: An infrared gas analyser (*LCA-2*, *ADC*, Hoddesdon, Herts., UK) with differential operation in an open system was used for gas exchange measurements. Air flow rate was adjusted to 83 cm^3 s^{-1} with a mass flow regulator (*ASUM*, *ADC*), and the leaves were individually enclosed in a leaf chamber having an 11 cm^2 window [*PLC(N)-2*, *ADC*] to which a new quantum sensor and an additional humidity sensor had been added (codes *LCH-030/S y LCH-032/s*, respectively, *ADC*) allowing simultaneous measurement of inlet and outlet air vapour pressures. Measurements were performed on clear days between 2 and 8 h after dawn, with a 1 300±140 μmol m^{-2} s^{-1} quantum flux density, obtained by maintaining the leaf chamber facing the sun. Ambient air taken from 3 m above the soil at a place separated from the operator, or air from a gas cylinder containing 700 μmol(CO_2) mol^{-1} was humidified by passing through a bubbler and the vapour pressure deficit was adjusted to 1.7 kPa by passing through columns filled with silica gel. Leaf area within the chamber was calculated by multiplying the chamber length by the average leaf width at the two ends of the chamber. Gas exchange parameters were calculated according to Long and Hallgreen (1985). A replicate leaf from each treatment, with treatments in random order, was measured before the next replicate, so that differences during the day could be included in the replicate effect in the analysis of variance.

Rapid irradiance-response curves of Chl fluorescence: In addition to estimating the maximum rate of electron transport and the quantum yield under saturating irradiance (Rascher *et al*. 2000), fluorescence-irradiance response curves without previous darkening of leaves allowed the maximum quantum yield in light-adapted leaves (Φ_{max}) to be calculated. Rapid light-response curves of Φ at AC were obtained with the *PAM-2000* modulated fluorometer with the *2030-B* leaf clip (*Walz*, Effeltrich, Germany), using the red "actinic light" (peak at 655 nm) provided by the instrument. Φ was calculated as $(F_m' - F_t)/F_m'$, where F_t is the fluorescence yield in the light-adapted sample and F_m' is the maximum fluorescence yield in the light-adapted state when a saturating pulse of approximately 3 000 μmol m^{-2} s^{-1} is superimposed for 0.8 s over the prevailing radiation. Light-adapted leaves were placed in the leaf clip and covered with a black cloth to exclude sunlight, and the "actinic light" was increased in eight 20 s-steps, each separated by a saturating pulse, from less than 10 to 100 μmol m^{-2} s^{-1} (White and Critchley 1999, Rascher *et al*. 2000). Hyperbolic regressions [$y = a + b/(1 + d\,x)$] were fitted to the Φ-irradiance data for the different treatments and were compared as detailed in the section on statistical analysis (see below). Φ_{max} in light-adapted leaves is given by $a + b$, while the initial slope of the curve corresponds to $b \times d$. The number and order of repeated measurements for each treatment combination was as described for the determinations of P_N.

Leaf analyses: Samples consisting of three leaves per replicate were rapidly transferred *in situ* to liquid nitrogen and stored at –80 ºC until analysis. The fresh mass, area, and Chl contents in sub-samples of frozen leaves were determined as described (Pérez *et al*. 2005), thus allowing the results to be expressed on a leaf area basis.

Chls were extracted from a sub-sample of frozen leaves with 80 % acetone and determined according to Arnon (1949). A procedure (Pérez *et al*. 2005) based on the NADH oxidation-coupled spectrophotometric assay of Lilley and Walker (1974) was used to determine RuBPCO activity before (initial activity) and after (total activity) carbamylation of active sites; RuBPCO activation was estimated as the percent ratio of initial to total activities. The amounts of RuBPCO and total soluble protein in sub-samples of frozen leaves were measured after extraction, spectrophotometric analysis, and gel electrophoresis followed by densitometric scanning (Aranjuelo *et al*. 2005). RuBPCO specific activity was determined by dividing total activity by the number of moles of active sites, which was taken as equivalent to the moles of RuBPCO large subunit. This would include any RuBPCO sites bound to inhibitors and yield low specific activities compared to methods determining free RuBPCO sites (Sharkey *et al*. 1991).

Statistical analyses: The significance of treatment effects was assessed through variance analyses using a nested design, according to Snedecor and Cochran (1967), with temperature as a stratum included in CO_2, replicates as a stratum included in temperature, and the sampling date as a further stratum included in that for replicates. Additional details about this analysis are described by Pérez *et al*. (2005).

Fitting of the Φ-irradiance curves [$y = a + b/(1 + d\,x)$] was performed by non-linear regression with the *GenStat 6.2* statistical package. The regressions for the different treatments were compared by analysis of parallelism (*GenStat 6.2*), which successively fits four models with different degrees of parallelism among the curves for these treatments. Thus, the model is first fitted with the same parameters for all treatments; then different *a* para-

meters are fitted for each of the treatments, after which separate *a* and *b* parameters are fitted and, finally, different values for all parameters are fitted to each treatment. With these four models, an accumulated analysis of variance is carried out to assess whether common or separate parameters should be fitted to the regressions for the treatments compared.

Results

***P*$_N$**: In the penultimate leaf 9 d before the beginning of anthesis, growth EC increased irradiance-saturated P_N measured with 360 μmol(CO_2) mol^{-1} at AT, and did not affect it at EC (Fig. 1). At later dates, EC decreased P_N. The effect on P_N of a 4 ºC increase in the growth temperature did not reach significance. Neither CO_2 nor temperature had significant effects on flag leaf P_N on the different measurement dates. When CO_2 assimilation was measured at EC in the second sampling, the effects of growth in EC (data not shown) were similar to those measured at 360 μmol(CO_2) mol^{-1}. Nevertheless, when measured at the respective growth CO_2, P_N was higher at growth EC than AC (data not shown).

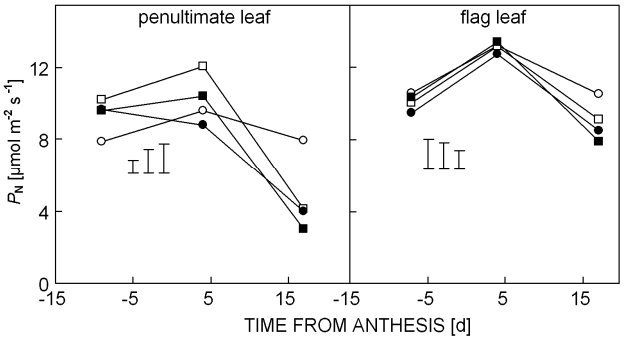

Fig. 1. Photon-saturated net photosynthetic rate (P_N) measured under 360 μmol(CO_2) mol^{-1} in leaves in different positions in the stem of wheat grown at 360 (*open symbols*) or 700 (*closed symbols*) μmol(CO_2) mol^{-1} and ambient (*circles*) or ambient + 4 ºC (*squares*) temperatures, at different dates from the start of anthesis. Plants were grown in the field under temperature gradient chambers. *Vertical bars* represent least significant differences (p<0.05) for effects of CO_2 (*left*), temperature (*middle*), and date (*right*) derived from the pooled analysis of variance for CO_2 concentrations, temperatures, and dates.

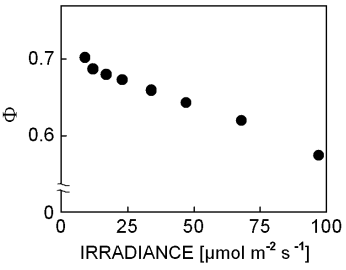

Fig. 2. Typical responses of the quantum yield of electron transport (Φ) to irradiance in rapid measurements of light-adapted wheat leaves. Irradiance was increased from <10 to 100 μmol m^{-2} s^{-1} in 20-s-steps.

Rapid Chl-irradiance response curves: Typical responses to irradiance of Φ in light-adapted leaves are shown in Fig. 2. Φ decreased in the range of irradiances from <10 to 100 μmol m^{-2} s^{-1}. A hyperbolic regression [$y = a + b/(1 + d\ x)$] showed good fitting to the Φ data. Comparison of regressions showed that the *b* and *d* parameters were common for the four treatments on each sampling date, except for a significant difference in *b* for the 3-DA penultimate leaf (Table 1). Thus, with this exception, the irradiance-response curves of Φ for the different treatments were parallel, showing that decreases in Φ with irradiance did not differ among the treatments, which is consistent with the absence in the rapid irradiance-curves of appreciable increases in non-photochemical quenching, up to irradiances higher than 600 μmol m^{-2} s^{-1} (White and Critchley 1999). In contrast, significant differences between the CO_2-temperature combinations for the *a* parameter were found (Table 1), revealing that Φ_{max} when iradiance tended to zero, was affected by the treatments. In the penultimate leaf, EC and ET increased Φ_{max} in the early growth stages, but the enhancement disappeared with the progress of development and was later reversed. In contrast, in the flag leaf EC and ET did not increase Φ_{max} at the beginning of growth, but EC increased it in later samplings. Under EC, ET had only a small positive effect on Φ_{max} at intermediate and later growth stages, while ET under AC decreased Φ_{max} in the last sampling.

RuBPCO activity: The first sampling for analysis of constituents of the penultimate leaf coincided with the first measurements of photosynthesis, 13 d after the first Chl fluorescence determinations. EC decreased initial and total RuBPCO activity throughout the development of the penultimate leaf (Fig. 3). In the flag leaf at AT, EC decreased total RuBPCO activity before anthesis and both initial and total RuBPCO activities after anthesis, while at ET it initially increased, but later did not affect this activity. CO_2 enrichment increased the activation state of RuBPCO in flag leaves in the first two samplings. EC also decreased RuBPCO specific activity at AT, but increased it at ET.

In the penultimate leaf, at the beginning of growth an increase in temperature decreased initial and total RuBPCO activities, the activation of the enzyme and, under AC, also RuBPCO specific activity. Later, ET had little effect on RuBPCO activity in the penultimate leaf. In the flag leaf, ET also decreased RuBPCO specific activity at AC and increased it at EC. This led to a decrease with temperature in total and initial RuBPCO activities in plants grown at AC, and, in the first sampling, to a slight increase of these activities at EC.

RuBPCO and soluble protein contents: In the penultimate leaf, EC decreased the RuBPCO contents (Fig. 3)

and the RuBPCO protein : soluble protein ratio (Fig. 4) throughout leaf development. In the flag leaf, EC induced an early increase in RuBPCO as a fraction of soluble protein (Fig. 4) and, when combined with AT, an increase in content of RuBPCO protein (Fig. 3), while in successive samplings EC significantly decreased the RuBPCO : soluble protein ratio and RuBPCO protein content. Soluble protein contents decreased with EC in both kinds of leaves. The increase in temperature by 4 ºC had no significant effects on RuBPCO : protein contents in both leaves.

Table 1. Parallel model analysis for the rapid irradiance-response curves of Φ in wheat leaves grown at 360 (AC) or 700 (EC) µmol(CO_2) mol^{-1} at ambient (AT) or ambient + 4 ºC (ET) temperatures. Measurements were recorded at AC. Irradiances ranged from <10 to 100 µmol m^{-2} s^{-1} in 20 s-steps. Separate parameters in the $\Phi = a + b/(1 + d \cdot \text{light})$ model for each treatment had to be fitted when a change in regression was significant. Φ_{max} ($a + b$) and initial slope ($-b \times d$) values for each treatment were derived from the parallel model analysis. DA – time from anthesis [d].

Leaf	Time [DA]		Change in regression: Common	Separate *a*	*a* and *b*	*a*, *b*, *d*
			Degrees of freedom: 2	3	3	3
Penultimate	−22	F	373.47	16.19	2.320	1.500
		Prob	<0.001	<0.001	0.076	0.216
	4	F	173.91	29.39	6.660	0.600
		Prob	<0.001	<0.001	<0.001	0.616
	17	F	258.00	13.09	2	0.430
		Prob	<0.001	<0.001	0.115	0.730
Flag	−7	F	176.15	3.56	1.390	0.620
		Prob	<0.001	0.015	0.246	0.600
	4	F	30.53	8.58	0.430	0.120
		Prob	<0.001	<0.001	0.731	0.948
	17	F	136.34	11.88	1.490	0.890
		Prob	<0.001	<0.001	0.219	0.448

		[DA]	Treatment: AC AT	AC ET	EC AT	EC ET
Penultimate	Φ_{max}	−22	0.681	0.704	0.694	0.717
		4	0.727	0.681	0.719	0.723
		17	0.740	0.729	0.714	0.699
	Initial slope	−22	−0.0014			
		4	−0.0010	−0.0009	−0.0011	−0.0017
		17	−0.0023			
Flag	Φ_{max}	−7	0.703	0.706	0.709	0.694
		4	0.693	0.690	0.708	0.717
		17	0.741	0.699	0.723	0.729
	Initial slope	−7	−0.0009			
		4	−0.0010			
		17	−0.0023			

Chl content: The effect of EC on the Chl contents of the penultimate leaf (Fig. 5) was positive in the first sampling, while later it was reversed. In contrast, at the beginning of flag leaf growth, EC decreased Chl contents and increased the Chl *a/b* ratios, suggesting a relative decrease in the light-harvesting complex. At later dates, EC still decreased Chl contents, but the decreases in these contents over time were smaller that with AC. These effects of EC were concurrent with a slight but significant decrease in the Chl *a/b* ratio, pointing to an increase in the light-harvesting complex. The RuBPCO protein : Chl ratio decreased under EC throughout development in the penultimate leaf (Fig. 5), while in the flag leaf it initially increased, and then decreased at later stages of development. In the penultimate leaf at the end of development, and in the flag leaf in the last two samplings, ET decreased Chl contents in plants grown under AC (Fig. 5), while it had no effect, or the effect was positive, at EC.

Discussion

In measurements with AC, the penultimate and flag leaves displayed different responses of P_N and Φ_{max} to increases in CO_2 and temperature. Thus, EC and ET caused an early enhancement, followed by an inhibition, of both parameters in the penultimate leaf. In contrast, EC and ET did not induce early increases in P_N and Φ_{max} in the flag leaf, or later changes in photosynthesis, while they enhanced Φ_{max} during the intermediate and late stages of development. Varying degrees of development in both kinds of leaves at the sampling times can not account for the difference between them, first, in the responses of P_N, since in this case the stimulation found in the penultimate leaf in the first sampling (later growth stage than in the flag leaf) should be observed at intermediate stages of growth of the flag leaf. Second, the first measurement of Φ_{max} was carried out at comparable developmental stages in both leaves. The early P_N and Φ_{max} enhancements in the penultimate leaf, but not the flag leaf, resemble the increase in growth due to EC observed in young plants, which later disappears (Geiger *et al*. 1998). Similarly, the late acclimation of photosynthesis in the penultimate leaf, but not the flag leaf, is consistent with the results of Osborne *et al*. (1998), although flag leaf photosynthesis may be down-regulated by growth EC (Martínez-Carrasco *et al*. 2005).

The observed differences between penultimate and flag leaves in the response to CO_2 and temperature of irradiance-saturated P_N were not closely associated with the responses to these factors of the amount and *in vitro* activity of RuBPCO. This may be due to changes in the activation state of RuBPCO under high irradiance relative to the growth irradiance at which leaves were sampled, as observed previously (Osborne *et al*. 1998, Martínez-Carrasco *et al*. 2005). Growth EC increased enzyme activation in the *in vitro* assay in the flag, but not penultimate leaf. This increase in activation, which has been observed previously (Pérez *et al*. 2005), compensates in part the decrease in total activity and amount of RuBPCO at EC. The stronger activation of the enzyme under growth EC could be due to an increase in RuBPCO activase, although in tobacco this was observed only in plants not undergoing marked acclimation to EC (Geiger *et al*. 1999). Another difference between consecutive leaves was in the response to EC and ET of RuBPCO specific activity. Decreases in the specific activity of RuBPCO previously observed under similar environmental conditions have been attributed (Pérez *et al*. 2005) to the presence in wheat and other plants of a day-time inhibitor(s) (Keys *et al*. 1995, Parry *et al*. 1997). An accumulation of precursor metabolites could increase the contents of the RuBPCO inhibitor (Andralojc *et al*. 2002). We have previously found that EC increases, and ET decreases, the contents of metabolites, which also decrease as leaves age (Pérez *et al*. 2005). This may account for a more marked decrease in specific activity under EC at AT in the flag than penultimate leaf. It may also explain that EC combined with ET increased RuBPCO specific activity in flag leaves, but not in the penultimate ones. EC also increased content of RuBPCO protein relative to soluble protein early on in growth of the flag leaf; a similar increase in penultimate leaves prior to the first sampling cannot be excluded. At later stages,

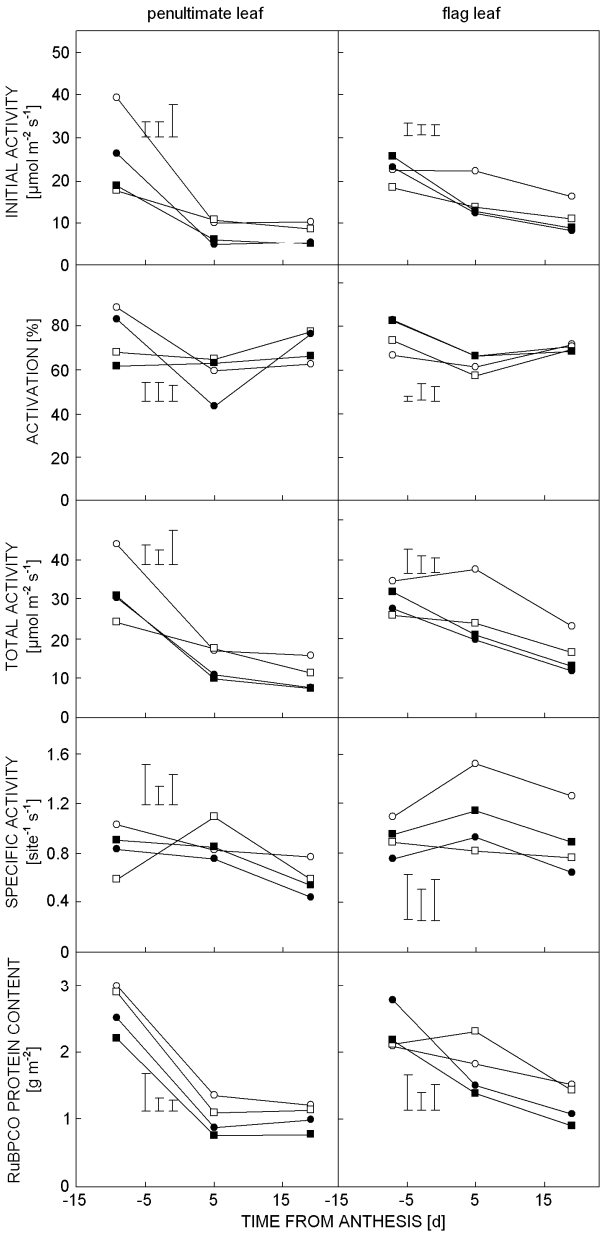

Fig. 3. *In vitro* activity, activation state, and specific activity of RuBPCO, and RuBPCO protein content at various dates from anthesis in leaves in different positions in the stem of wheat grown at 360 or 700 µmol mol^{-1} CO_2 and ambient or ambient + 4 ºC temperatures, at various dates from the start of anthesis. Symbols as in Fig. 1.

this was followed in both kinds of leaves by a down-regulation of RuBPCO activity and protein content with CO_2 enrichment, as observed in many previous studies (Nie *et al*. 1995, Drake *et al*. 1997, Stitt and Krapp 1999, Pérez *et al*. 2005), which is consistent with an earlier decrease in leaf protein under EC (Stitt and Krapp 1999).

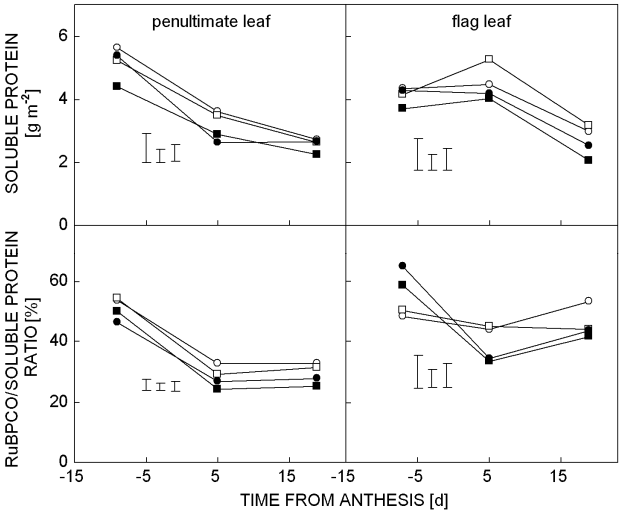

Fig. 4. Soluble protein contents and percent ratio of RuBPCO to soluble protein in leaves in different positions in the stem of wheat grown at 360 or 700 μmol mol^{-1} CO_2 and ambient or ambient + 4 ºC temperatures at different dates from the start of anthesis. Symbols as in Fig. 1.

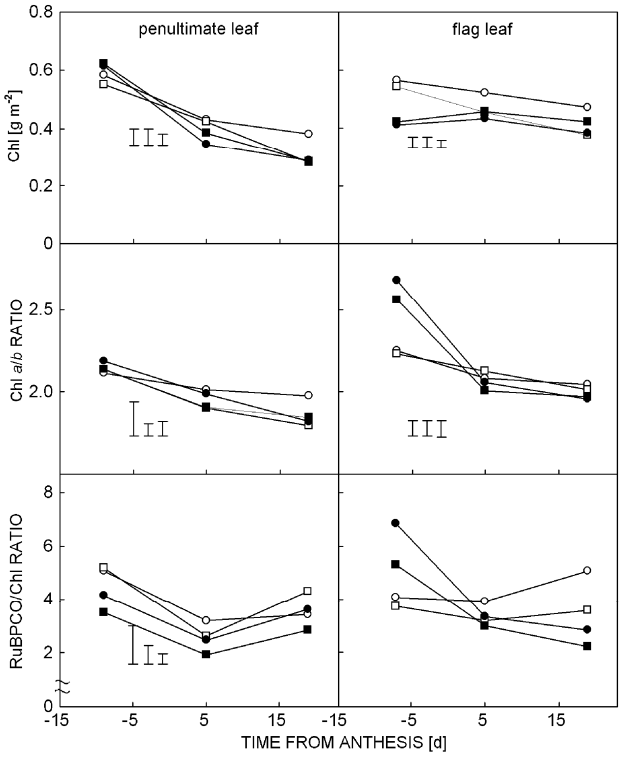

Fig. 5. Chlorophyll (Chl) content, the Chl *a/b* and RuBPCO protein : Chl ratios (m : m) in leaves in different positions in the stems of wheat grown at 360 or 700 μmol mol^{-1} CO_2 at ambient or ambient + 4 ºC temperatures at different dates from the start of anthesis. Symbols as in Fig. 1.

The increases with EC in Φ_{max} at the beginning of growth of the penultimate, but not the flag, leaf may be related to the increases in Chl contents in the former, and its decreases in the latter and, in the flag leaf, with decreases in the light-harvesting complex, as indicated by higher Chl *a/b* ratios. EC also had different effects on the photochemistry of both leaves at the later stages of growth, with decreases in Φ_{max} in penultimate leaves and increases in it in flag leaves, concurrent with decreases in Chl contents in both leaves, although with an increase in the light-harvesting antenna (decreased Chl *a/b* ratio) in the flag leaf.

Notably, an increase in temperature by 4 ºC decreased Chl contents at later stages of growth in both kinds of leaves at AC, though not at EC; the response of Φ_{max} in the flag leaf was similar. This interaction is consistent with the observation by Taub *et al*. (2000) of a protection by EC of lesions in photochemistry caused by high temperatures. Several mechanisms increasing thermostability have been reported, such as the synthesis of zeaxanthin (Havaux 1998), or the increase in contents of saturated fatty acids (Alfonso *et al*. 2001). Significantly, EC decreases lipid unsaturation (Huang *et al*. 1999).

The effects on Φ_{max} and Chl contents, together with the changes in P_N, RuBPCO content, and the RuBPCO : Chl ratios suggest that EC increases the allocation of resources from carboxylation to light-harvesting in penultimate leaves. In flag leaves, the effects of EC are indicative of an increase in the allocation of resources to carboxylation early on in leaf growth, followed later by a shift towards higher photon harvesting and lower carboxylation. The observed alterations in the balance between RuBPCO and electron transport caused by growth EC are not consistent with some reports (Sage *et al*. 1995, Nakano *et al*. 1997), but are in agreement with those of Makino *et al*. (1997), Osborne *et al*. (1998), and Mitchell *et al*. (2000). The functional significance of these effects may be that under EC RuBPCO may be less necessary (Makino *et al*. 1997), so that greater allocation of resources to excitation energy capture would represent an optimisation of resource use. However, EC actually decreased Φ_{max} later in the growth of penultimate leaves, in which an increased balance of light reactions to carboxylation would seem more advantageous because these leaves gradually become shaded. On the other hand, the biochemical models of leaf photosynthesis (Farquhar *et al*. 1980, Humphries and Long 1995) predict that EC should allow higher rates of photon-limited photosynthesis to be maintained even with substantial losses in the potential rate of electron transport. Thus, the response of the shaded penultimate leaf to CO_2 enrichment would be adaptive.

Higher contents of protein (Fig. 4) and Chl (Fig. 5) in the first sampling, and lower contents in the successive

ones were found in the penultimate leaf (delayed first sampling) as compared with the flag leaf. This leads to the conclusion that a different availability of resources for leaf growth and function during development would be the cause of the contrasting responses to EC between the two kinds of leaves, both in P_N and Φ_{max}. EC increases the N content in young N-sufficient plants, but not in older plants (Geiger *et al*. 1998). In field crops, N uptake frequently lags behind growth (Olesen *et al*. 2002), so that N and protein contents decrease with time and also in successive leaves at comparable stages of leaf development. Competition with stem elongation and ear growth in the warm Mediterranean environment of our experiments (see Introduction) may also contribute to lower resource contents early on in the growth of flag leaves than in penultimate leaves.

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