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Changes in Leaf Morphology and Composition with Future Increases in CO_2 and Temperature Revisited: Wheat in Field Chambers

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Abstract Whether leaf morphology is altered by future increases in atmospheric CO_2 and temperature has been reexamined over 3 years in wheat grown in field chambers at two levels of nitrogen supply. Flag leaf fresh and dry mass, area, volume, and ratios of these parameters, as well as the contents of water, chlorophyll, nonstructural carbohydrates, and nitrogen compounds have been determined at anthesis and 14 days later. High CO_2 decreased rather than increased, as reported in the literature, leaf mass per area and leaf density, and increased water content per area and per volume and water percentage. Warmer temperatures also decreased leaf mass per area, but did not affect density or water per area or per volume, whereas they increased water percentage. Nitrogen supply did not change CO_2 and temperature effects on leaf morphology. Nonstructural carbohydrates increased and nitrogen compounds decreased in elevated CO_2, and the sum of these compounds decreased with warmer temperatures. These changes in composition did not account for modifications of leaf morphology. We conclude that increases in atmospheric CO_2 and temperature after leaf initiation can decrease leaf mass per area, and elevated CO_2 can also decrease leaf density, due to decreases in leaf structural compounds. The functional significance of these changes is probably a decrease in photosynthetic capacity per unit leaf area.

Keywords Elevated CO_2 · Elevated temperature · Leaf mass per area · Leaf morphology · Leaf composition · Carbohydrates · Nitrogen · Photosynthesis · Water content · Wheat

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Introduction

There are many aspects of plant physiology that are influenced by rising CO_2, but some would argue that there is only convincing evidence that Rubisco activity and stomatal aperture are directly affected (Long and others 2004). Elevated CO_2 modifies C_3 photosynthesis directly by increasing the substrate for the carboxylation reaction catalyzed by Rubisco and decreasing the competing oxygenation reaction (Long 1991). In the long term, elevated CO_2 decreases Rubisco amount, in association with either decreased enzyme transcripts (Drake and others 1997; Moore and others 1999) due to repressed gene expression by sugars (Sheen 1990; Krapp and others 1993) or low nitrogen contents (Riviere-Rolland and others 1996; Nakano and others 1997; Farage and others 1998; Geiger and others 1999; Pérez and others 2005). Rising CO_2 concentrations caused stomatal closure through an as yet unknown mechanism. Gas exchange studies by Mott (1988) concluded that stomata respond to intercellular rather than external atmospheric CO_2 concentrations. However, it is not clear whether stomata and photosynthesis acclimate to elevated CO_2 in parallel or independently (Morison 1998). Together with the previous two processes, another way in which elevated CO_2 could modify the rate of carboxylation per unit leaf area is through changes in leaf morphology,

which can include the number of mesophyll cells per area (Eguchi and others 2004), mesophyll thickness (Thomas and Harvey 1983; Radoglou and Jarvis 1992; Sims and others 1998), and leaf mass per area (Peterson and others 1999). A change in leaf mass per area modifies the light that can be intercepted per unit dry mass. With thicker leaves having greater mass per area, the number of chloroplasts and the amount of enzymes of CO_2 assimilation increase, and so does photosynthetic capacity, although at a cost of lower light interception per unit leaf biomass, especially at low irradiances (Evans and Pooter 2001). Several studies on the relationship between photosynthetic capacity and anatomy of leaves have concluded that photosynthesis decreases with dry mass per area (Reich and others 1997; Roderick and others 1999b; Yin 2002). In many plant species, variations in dry mass per area are the cause of the differences in photosynthetic characteristics (for instance nitrogen per unit area) of leaves (Frak and others 2001).

Data in the literature indicated that leaf mass per unit area generally increases with elevated CO_2 (Curtis 1996; Luo and others 1998; Sims and others 1998; Peterson and others 1999; Roderick and others 1999a; Yin 2002; Ishizaki and others 2003). This was associated with increases in leaf thickness (Sims and others 1998) and to changes in mesophyll area and number and size of mesophyll cells per leaf area (Eguchi and others 2004). The increase in mass per area is often associated with decreases in elevated CO_2 of Rubisco amount and activity (Long and others 2004; Pérez and others 2005) and photosynthetic capacity on a leaf area basis (Sims and others 1998; Martínez-Carrasco and others 2005), as well as with changes in leaf composition such as increased carbohydrate contents (Radoglou and Jarvis 1992; Moore and others 1999; Poorter and others 1997; Pérez and others 2005) and decreases in nitrogen per unit leaf mass (Luo and others 1998; Yin 2002; Ishizaki and others 2003) and area (Peterson and others 1999).

Changes in leaf morphology and composition with growth in elevated CO_2 depend, in turn, on nitrogen supply. Thus, CO_2 enrichment increased leaf thickness with high but not with low nitrogen (Sims and others 1998), and the decrease in leaf nitrogen content in high CO_2 was enhanced by temperature and light intensity and was mitigated by nitrogen application (Yin 2002). Elevated CO_2 decreased photosynthetic capacity and mesophyll area per unit leaf area with low but not with high nitrogen (Eguchi and others 2004). In spite of the described relationships between mass per area, photosynthesis, and nitrogen, the increase in leaf mass per area caused by elevated CO_2 could decrease photosynthesis by a mechanism independent of nitrogen concentration per unit area and based on morphology. The nature of this mechanism is not completely clear and may be due to decreases in nitrogen allocation to photosynthetic components, greater biomass allocation to structural rather than photosynthetic components, greater internal shading, or higher limitation to internal diffusion (Peterson and others 1999). As pointed out by Roderick and others (1999c), the expression of leaf composition on a weight basis is problematic, because water content and the proportion of air in the tissue can vary. These authors found that the mass of nitrogen per unit liquid mass was relatively constant, and the area:volume ratio of leaves was proportional to leaf liquid content. In turn, the mass of carbon per unit dry mass was relatively constant. Because nitrogen was a constant fraction of the liquid mass and carbon a constant fraction of the dry mass, the nitrogen:carbon ratio was positively related to liquid content. Measurements of leaf density or volume and of liquid content are not frequent in the research on the effects of CO_2 enrichment (Roderick and others 1999a) and should be a priority in future investigations.

Some preliminary (unpublished) observations in our wheat field experiments described elsewhere (Del Pozo and others 2005; Martinez-Carrasco and others 2005; Pérez and others 2005) depart from the commonly observed effects of CO_2 enrichment on leaf anatomy, which prompted us to reassess this topic. The aim of this work was to know whether elevated CO_2, either alone or in combination with warmer temperatures and nitrogen supply, decreases rather than increases leaf dry mass per unit area, and to understand the causes of this modification and possible implications for photosynthetic acclimation to climate change. Over 3 years, flag leaf changes in mass, area, volume, and their ratios in response to doubling air CO_2 concentrations, increased temperatures, and higher nitrogen supply have been assessed in wheat field crops under temperature gradient chambers. Leaf water, chlorophyll, nitrogen compounds, and nonstructural carbohydrates were also determined to understand the possible reasons for changes in leaf mass per area and leaf density in response to the factors under study.

Materials and Methods

Spring wheat (*Triticum aestivum* L. cv. Gazul) was sown at a rate of 200 kg ha^{-1} and 0.13-m row spacing on 29 January 2004, 10 February 2005, and 24 January 2006. Every year, 60 kg ha^{-1} each of P and K (as P_2O_5 and K_2O, respectively) and, in 2004, also 32 kg ha^{-1} N (as NH_4NO_3) were added before sowing. An application of nitrogen fertilizer [$Ca(NO_3)_2$] as an aqueous solution was made by hand at the two different amounts indicated below, on 21 April 2004, 11 April 2005, and 27 March 2006. Ten days after sowing, herbicides (clortoluron + diflufenican, 2.3 L ha^{-1}) were added; insecticides were applied as required. The crop was watered weekly with a drip irrigation system

providing the amount of water required to equal the long-term (20-year) average rainfall for each particular month (April, 49.7 mm; May, 57.8 mm; and June, 34.3 mm). The soil was a clay-sand in the farm of the Institute of Natural Resources and Agrobiology, CSIC, in Salamanca (40°95′ N, 5°5′ W, 800 m a.s.l.). Climate corresponds to a Mediterranean type. The long-term (20-year) average for the minimum temperature in the coldest month (January) is 0.0°C and the maximum temperature of the warmest month (July) is 27.2°C. Mean annual rainfall is 506 mm.

After seedling emergence, six temperature gradient chambers (Aranjuelo and others 2005; Pérez and others 2005), based on those described by Rawson and others (1995), were mounted over the crop in different field sites each year, at a distance of about 15 m between chambers. The chambers were 9 m long, 2.2 m wide, and 1.7 m high at the ridge. Chambers had transparent polycarbonate walls and polyethylene sheet roofing and comprised three consecutive modules (each 3 m long) separated by horizontally 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 close to those in the outside air and the final outlet module at 2°C higher temperatures; the central module was left as a spacer (Fig. 1). Three chambers were kept at ambient CO_2 (370 μmol mol^{-1}), whereas in the other three atmospheric CO_2 was increased to 700 μmol mol^{-1} (elevated CO_2) during the light hours. CO_2 was not elevated during the night because little or no effect on dark respiration has been reported (Jahnke and Krewitt 2002; Davey and others 2004; Bernacchi and others 2005; Dermody and others 2006). To raise CO_2 levels in the air, the signal of an infrared gas analyzer (SBA-4, 2; PP Systems, Hitchin, Herts, UK) monitoring the CO_2 concentration at the outlet module of each elevated CO_2 chamber was fed into a proportional, integral, derivative (PID) controller (TTM 005/TTM009 series, PID Eng&Tech, Madrid, Spain) regulating a solenoid valve, which injected pure CO_2 at the two inlet fans. Ventilated temperature and humidity sensors and air probes for CO_2 analysis connected to another infrared gas analyzer (LCA2, ADC, Hoddesdon, Herts, UK) were placed at the center of each module, 20 cm above the crop. The data were recorded every 30 s by a computer using analog-to-digital converters (Aranjuelo and others 2005; Pérez and others 2005) and hourly averages were obtained (Fig. 2). Two levels of nitrogen supply (low and high) were established in the 3 years. In 2004 this was done by adding 32 kg ha^{-1} (low nitrogen) before sowing to one longitudinal half of the chambers and 140 kg ha^{-1} (high nitrogen, 32 kg ha^{-1} before sowing + 108 kg ha^{-1} on 21 April) to the other half. In the following years, 15 kg ha^{-1} (low nitrogen) and 140 kg ha^{-1} (high nitrogen) were added to each longitudinal half of the chambers on 11 April 2005 and 27 March 2006.

Figure 2 shows the chambers' mean values of temperature and humidity compared to open air and the CO_2 concentration in the chambers in the month prior to leaf sampling and in the three experimental years. Temperatures in the light hours in 2004, 2005, and 2006 were, respectively, 2.5, 2.3 and 2.1°C higher in the warm outlet than the cool inlet modules of the chambers. Differences in temperatures between the inlet module and outside air during the light hours were −0.6, 1.2, and −1.5°C in 2004, 2005, and 2006, respectively; the 2005 value may have been due to a failure in the outside temperature sensor. Compared to the warm chamber modules, air humidity in the cool modules was higher in the night and similar in the day. Chamber humidity in 2005 and 2006 was higher than that of the outside air during the light hours and in 2006 it was also higher during the night.

Leaf Measurements

On dates close to anthesis—31 May 2004 (3 days after anthesis, daa), 25 May 2005 (1 daa), and 18 May 2006 (1 daa)—and 14 days later, two subsamples of flag leaves, each consisting of two leaves, from each CO_2, temperature, and nitrogen combination in all chambers were taken about 4 h after the start of the photoperiod. The leaves

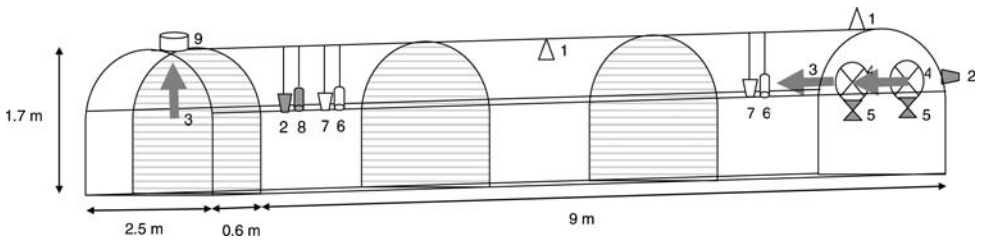

Fig. 1 Schematic drawing of the temperature gradient chamber. 1 Light sensors; 2 Temperature probes for fan and heater control; 3 Air flow; 4 Inlet fans; 5 CO_2 injector; 6 CO_2 sensors; 7 Temperature/humidity sensors; 8 CO_2 probe for injector control; 9 Outlet fan

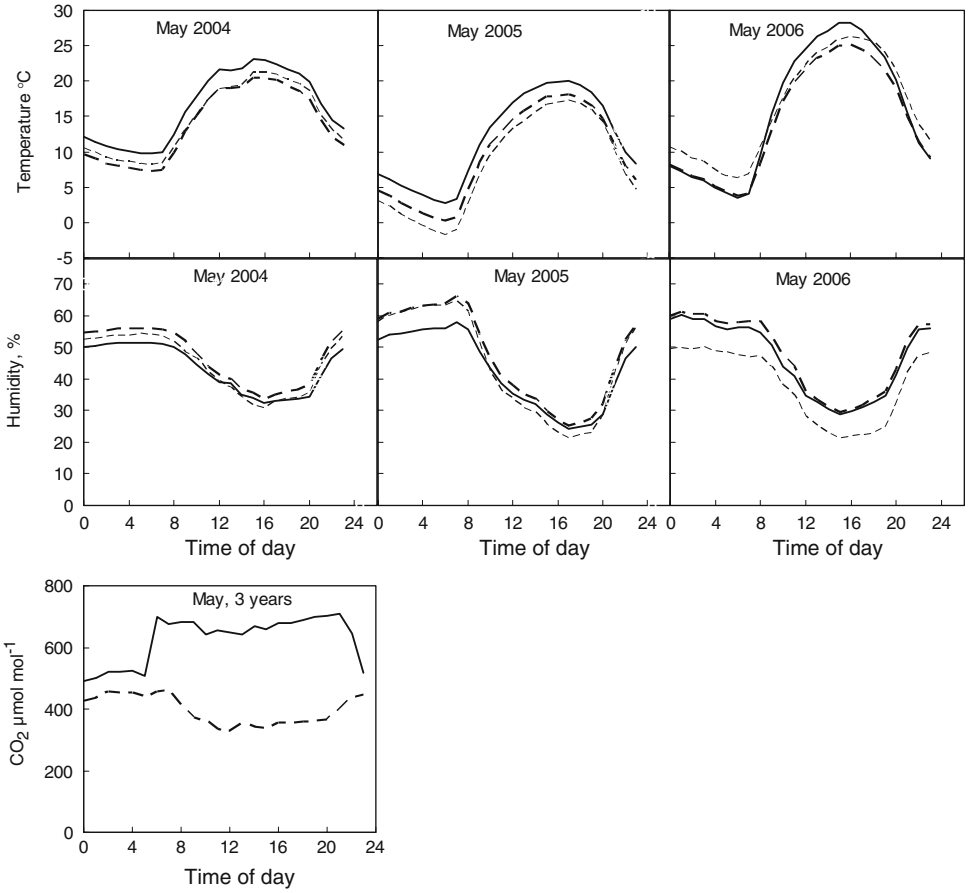

Fig. 2 Mean daily courses of temperature, humidity, and CO_2 concentration in temperature gradient chambers set at either ambient (*thick broken lines*) or warmer temperatures (*thick solid lines*), and ambient (*thick broken lines*) or 700 µmol mol^{-1} CO_2 (*thick solid lines*). Thin broken lines represent temperature or humidity in open air. The temperature and humidity records correspond to hourly averages of the month preceding measurements. An average of the 3 years is presented for CO_2 because little between year variation was found

were rapidly transported in plastic bags to a cold room (5°C) in which they were kept until processing. Measurements were then carried out in the 48 samples, which took about 2 h.

After removing the dry leaf tips, if present, the fresh weight of each pair of leaves was determined with an electronic balance (Precisa XT 220 A, Switzerland) to four decimal figures. The projected area was then measured with a photoelectric planimeter (Li-3000 A, Li-Cor, Lincoln, NE, USA). Leaf volume was measured thereafter by placing the leaves in a 10-ml graduated test tube. The tube was filled to the mark with a graduated pipette containing 10 ml of toluene, and the pipette volume used was recorded (leaf volume = 10 ml – recorded volume). Toluene was chosen (Martinez-Carrasco and Thorne 1979) because it has lower surface tension than water and this minimizes air bubble formation. The dry mass was obtained after drying samples at 60°C for 48 h. From these measurements the fresh and dry masses per unit area and volume and the area:volume ratio were calculated. The difference between fresh and dry masses provided the percent water content [(fresh mass - dry mass) × 100/fresh mass] and the mass of liquid per leaf and per unit area and volume.

Analysis of Chlorophyll, Carbohydrates, and Nitrogen Compounds

At anthesis and 14 days later there was an additional harvest of two separate subsamples, each consisting of four flag leaves, from each treatment combination in each chamber. The subsamples were immediately frozen in situ in liquid nitrogen and then stored at -80°C until analyzed. The fresh weight, leaf area (calculated by digital image analysis), and total chlorophyll, chlorophyll a, and chlorophyll b in acetone extracts (Arnon 1949) of frozen subsamples were determined as described by Pérez and others (2005). This allowed the results to be expressed on a leaf area basis.

In subsamples (~100 mg fw) of leaves stored in liquid nitrogen, carbohydrates (glucose, fructose, sucrose, fructans, and starch) and free amino acids were extracted according to Morcuende and others (2004). Carbohydrates were analyzed with a spectrophotometric assay coupled to NADP reduction according to Morcuende and others (2004), free amino acids were determined spectrophotometrically by the ninhydrin method according to Hare (1977), nitrate was determined by the method described

by Cawse (1967), and total protein was determined colorimetrically by the method of Lowry and others (1951) with some modifications (Peterson 1977). To express the contents of these compounds on a leaf area basis, the molecular weight of hexose was used for carbohydrates and that of the anion was used for nitrate. For amino acids, a molecular weight of 138 (average for the 20 standard amino acids) was adopted. Based on the weight of the bovine serum albumin used as a standard, the protein weight was estimated. The sum of nonstructural carbohydrates and that of nitrogen compounds is presented here.

Experimental Design and Statistical Analyses

The design of the experiment was a randomized-block strip-plot design with three blocks, the two atmospheric CO_2 concentrations (one chamber each) allocated to whole plots within blocks, temperature and nitrogen as rows and columns within whole plots, and the subsamples in subplots within rows and columns. The growth stage (anthesis or 14 daa) was included in an additional stratum under the subsamples, and the experimental year was placed in a stratum containing the remaining strata. Analyses of variance of data were carried out for this design using the Genstat 6.2 statistical software. Because year of experiment was the upper stratum in the random model, differences between years are not described. Because there were only three blocks, the threshold for significance was chosen as $p < 0.09$ to avoid the possibility of a Type II error.

Results

There were few significant interactions between experimental factors, thus their main effects are described. Between anthesis and two weeks later there were no significant changes in morphologic parameters of leaves (data not shown). Elevated CO_2 did not significantly change the volume per unit leaf area (Table 1), which indicated that high CO_2 did not change leaf thickness. The fresh mass per area was also not significantly changed by high CO_2. In contrast, growth in high CO_2 decreased leaf dry mass per area and per volume while increasing water content per area and volume. Similarly, leaf water percentage was significantly higher in elevated than in ambient CO_2. All these effects of elevated CO_2 were small, ranging from 3 to 6%.

Above-ambient temperatures did not significantly modify leaf volume per unit area (Table 1). Warm temperatures did not affect the fresh mass per area or per volume, decreased the dry mass per area, but did not change leaf dry mass per volume. High temperatures did not affect water contents per area or volume, but they increased leaf water percentage. Though significant, temperature effects on leaf morphology were only small (1-6%), as was found for CO_2 effects.

Leaves with a high nitrogen supply had higher fresh and dry masses, area, and volume than those with low fertilizer (Table 1). The supply of more nitrogen increased the volume per unit leaf area so that it produced thicker leaves. Nitrogen also increased fresh and dry masses per area and per volume, although the significance of differences in dry

Table 1 Morphologic parameters of flag leaves of wheat grown in field chambers in ambient (A) or elevated CO_2 (E), ambient (T) or warmer (T+) temperatures, and low (L) or high (H) nitrogen supply, in a three-year experiment

	CO_2			Temperature			Nitrogen		
	A	E	*p*	T	T+	*p*	L	H	*p*
F wt (g leaf^{-1})	0.44	0.47	ns	0.45	0.45	ns	0.37	0.54	<0.001
D wt (g leaf^{-1})	0.14	0.14	ns	0.14	0.14	ns	0.12	0.16	<0.001
Water (% F wt)	67.8	69.7	<0.001	68.3	69.2	0.003	68.5	69.0	ns
Area (cm^2 leaf^{-1})	22.4	23.5	ns	22.9	23.1	ns	19.7	26.2	<0.01
Volume (cm^3 leaf^{-1})	0.54	0.56	ns	0.55	0.55	ns	0.46	0.64	<0.01
Volume/area (cm)	0.024	0.024	ns	0.024	0.024	ns	0.0236	0.0242	0.03
F wt/area (mg cm^{-2})	19.4	19.7	ns	19.7	19.4	ns	18.8	20.3	<0.001
F wt/volume (mg cm^{-3})	0.82	0.83	ns	0.82	0.83	ns	0.81	0.84	0.007
D wt/area (mg cm^{-2})	6.26	5.96	0.02	6.25	5.97	<0.01	5.92	6.29	<0.01
D wt/volume (mg cm^{-3})	0.263	0.252	0.08	0.261	0.254	ns	0.254	0.262	0.09
Water/area (mg cm^{-2})	13.2	13.8	0.02	13.5	13.5	ns	12.9	14.0	<0.01
Water/volume (mg cm^{-3})	0.55	0.58	0.05	0.56	0.57	ns	0.55	0.58	0.004

F wt fresh mass, *D wt* dry mass, *ns* not significant

Data are main factor effects. *p* is the probability in the analysis of variance

mass per volume was low. High nitrogen increased water content per leaf area and per volume, but it had no significant effect on leaf water percentage. Nitrogen supply effects on parameter ratios were similar in size to those of CO_2 and temperature. At variance with parameter ratios, parameter values were more affected (33–46% increase) by nitrogen supply than by CO_2 or temperature.

Elevated CO_2 significantly increased (35%) the mass of nonstructural carbohydrates per leaf area at anthesis, but this effect disappeared two weeks later (Table 2; for comparison, this table also includes parameter values on a fresh weight basis). CO_2 enrichment decreased (10%), in contrast, the mass of nitrogen compounds. The total weight of analyzed compounds tended to decrease in elevated CO_2. Warmer temperatures decreased (13%) the contents of nonstructural carbohydrates and the total mass of analyzed compounds per area (Table 2). A higher nitrogen supply increased the mass of nitrogen compounds (14%) and the sum of all analyzed compounds per unit leaf area (Table 2); the increases with nitrogen in nonstructural carbohydrate contents per area did not reach statistical significance. From anthesis to 14 days later, the amount of nonstructural carbohydrates per area of leaves increased and that of nitrogen compounds decreased; the result was a net decrease (11%) in the total mass of analyzed compounds (Table 2).

Discussion

Prolonged growth in elevated CO_2 and temperature induced subtle changes in the morphology of flag leaves of wheat in three experiments in different years. At variance with previous reports (Sims and others 1998), nitrogen supply did not modify this effect of CO_2 and temperature. However, leaf morphology changed with nitrogen availability. Inspection of effects on leaf morphology of these factors reveals several differences. Thus, compared with leaves in ambient growth CO_2, leaves in elevated CO_2 experienced a decrease in dry mass per area. Because it contrasts with many preceding reports (Luo and others 1998; Sims and others 1998; Peterson and others 1999; Roderick and others 1999a; Yin 2002; Ishizaki and others 2003), this result from multiple experiments, which is consistent with our preliminary observations, is a remarkable finding. The change induced by elevated CO_2 was not in leaf thickness or volume, at variance with observations by Sims and others (1998), but in leaf density (dry weight per volume), which was decreased. The increase in liquid mass per unit area in elevated CO_2—with no major change in volume per area—suggests that the leaf density loss was associated with an increase in tissue water rather than with increases in leaf air spaces such as those observed by

Table 2 Metabolite contents per unit leaf area (mg cm^{-2}) and unit fresh weight (in parentheses, mg g^{-1}) in flag leaves of wheat grown in field chambers in ambient (A) or elevated CO_2 (E), ambient (T) or warmer (T+) temperatures, and low (L) or high (H) nitrogen supply, at anthesis or 14 days later (14 daa), in a three-year experiment

	CO_2			Temperature			Nitrogen			Growth stage		
	A	E	*p*	T	T+	*p*	L	H	*p*	Anthesis	14 daa	*p*
TNC	Anthesis	0.54 (27.9)	0.63 (31.4)		0.63 (31.9)	0.53 (27.4)		0.58 (29.8)	0.59 (29.5)			
	14 daa	0.73 (38.1)	0.65 (33.2)		0.73 (37.8)	0.65 (33.4)		0.66 (35.3)	0.73 (35.9)			
	Mean	0.61 (32.0)	0.64 (32.1)	<0.001* (<0.001*)	0.67 (34.3)	0.58 (29.8)	0.01 (0.012)	0.61 (32.0)	0.64 (32.0)	ns (ns)	0.58 (29.3)	0.69 (37.7)	0.002 (<0.001)
TN	Anthesis	1.15 (59.6)	1.03 (51.2)		1.11 (56.0)	1.08 (54.8)		1.02 (52.6)	1.17 (58.2)				
	14 daa	0.85 (44.3)	0.78 (39.6)		0.86 (44.4)	0.77 (39.3)		0.76 (40.6)	0.88 (43.1)				
	Mean	1.03 (53.4)	0.93 (46.5)	<0.003 (<0.001)	1.01 (51.4)	0.96 (48.6)	ns (ns)	0.92 (47.8)	1.05 (52.1)	<0.001 (0.005)	1.09 (53.7)	0.82 (44.5)	<0.001 (<0.001)
TM	Anthesis	1.69 (87.5)	1.66 (82.6)		1.74 (88.0)	1.61 (82.2)		1.59 (82.5)	1.76 (87.7)				
	14 daa	1.58 (82.3)	1.44 (78.6)		1.59 (82.2)	1.43 (72.7)		1.42 (76.0)	1.60 (79.0)				
	Mean	1.65 (85.4)	1.57 (78.6)	ns (0.002)	1.68 (85.7)	1.54 (78.4)	0.002 (<0.001)	1.52 (79.9)	1.69 (84.2)	<0.001 (0.045)	1.67 (81.9)	1.51 (82.2)	<0.001 (ns)

TNC total nonstructural carbohydrates, *TN* total nitrogen compounds, *TM* total metabolites analyzed, *ns* not significant

p is the probability in the analysis of variance. *CO_2 x growth stage interaction

Masle (2000) in young wheat plants. As observed with elevated CO_2, above-ambient temperatures decrease leaf dry mass per area, consistent with recent findings in C_4 plants (Dwyer and others 2007), without significantly modifying leaf volume per area. In contrast with high CO_2, warmer temperatures did not increase the mass of liquid per unit area or volume. Probably, warmer temperatures increased leaf air spaces while high CO_2 increased leaf water. Unlike the preceding two factors, a high nitrogen supply increased leaf thickness (volume/area ratio), in agreement with Rademacher and Nelson (2001), as well as dry mass per area and density. With more nitrogen, the liquid mass per unit area and volume increased, as in elevated compared with ambient CO_2. In contrast, with more nitrogen the mass of liquid increased proportionately to leaf mass, such that water percentage was unchanged, whereas it increased in high CO_2 and temperature. Because greater leaf thickness in high nitrogen was associated with greater water mass, changes with nutrient supply in the proportion of leaf air spaces, such as the decrease found by Rademacher and Nelson (2001), are likely of little consequence for leaf thickness. Overall, leaf morphology was affected differently by the three environmental factors under study, a difference which seems to exclude that changes in anatomy caused by CO_2 and temperature are a simple consequence of the decrease in leaf nitrogen found here and elsewhere (Del Pozo and others 2007).

In our experiments the decrease in leaf dry mass per area caused by elevated CO_2 occurred in spite of an increase in nonstructural carbohydrates, consistent with many preceding studies (Radoglou and Jarvis 1992; Nie and others 1995; Moore and others 1999; Pérez and others 2005). This increase disappeared after anthesis, in agreement with previous reports of loss during grain filling of the high CO_2 enhancement of nonstructural carbohydrate contents found at anthesis (Nie and others 1995). The 0.3-mg cm^{-2} (Table 1) decrease in leaf dry mass per area in elevated CO_2 should overcompensate for the accumulation of carbohydrates (Table 2). This implies that the mass of some other compound(s) must have decreased more than the carbohydrates increased. Nitrogen compounds decreased in elevated CO_2 (0.1 mg cm^{-2}, Table 2), as previously reported (Luo and others 1998; Yin 2002; Ishizaki and others 2003; Peterson and others 1999), but this decrease by itself was insufficient to account for the loss of leaf dry mass per area. The contents of chlorophyll ($0.072–4 \times 10^{-3}$ mg cm^{-2}) and the changes to them (about 0.003 mg cm^{-2}, data not shown) as well as the contents of other metabolites, such as phosphorylated intermediates of carbohydrate metabolism or organic acids, are much smaller than the compounds here analyzed (Morcuende and others 1998). This suggests that long-term growth in elevated CO_2 decreases some structural compounds of leaves. In contrast, an increase of the mass of these compounds in elevated CO_2 has been found in cotton by Wong (1990) and in young wheat plants by Masle (2000). As the latter author points out, this high-CO_2 effect on final leaf anatomy may be largely determined in the leaf primordium, leading one to expect no or little mass increase in leaves initiated before exposure to elevated CO_2. In our experiments, CO_2 enrichment was delayed relative to germination and probably commenced with all leaves initiated (Masle 2000). It may be that an early, CO_2-enhanced leaf structural carbon deposition masks the opposing CO_2 effect at later growth stages.

The decreases in nonstructural carbohydrates and nitrogen compounds of leaves (0.14 mg cm^{-2}, Table 2) in warm temperatures can account for only half the decrease in dry weight per area (0.28 mg cm^{-2}, Table 1), pointing again to losses in structural compounds. This contrasts with observations that temperature increases cell wall contents per unit leaf area in *Lolium perenne* L. (Groot and others 2003). Nor can the increase of nitrogen supply in the mass of analyzed compounds (0.17 mg cm^{-2}, Table 2) account for the rise in dry weight per area (0.37 mg cm^{-2}, Table 1), indicating that there must have been an increase in structural compounds, in agreement with reports for poplar (Luo and others 2006; Pitre and others 2007).

A decrease in dry mass per area of leaves is believed to afford a benefit for photosynthesis (Reich and others 1997; Roderick and others 1999b; Yin 2002) because light interception is improved (Evans and Pooter 2001). This decrease under high CO_2 and temperatures found in our experiments could represent, therefore, an adaptive advantage. However, Luo and others (1994) suggested that an increase in leaf dry matter per area under elevated CO_2 is beneficial, because it could contribute to an increase in the nitrogen contents per area, more than a half of which is in the photosynthetic apparatus (Hikosaka and Terashima 1996) and it keeps a positive relationship with carbon assimilation (Hirose 1984). An increase in leaf dry mass per area has actually been shown to benefit growth in elevated but not ambient CO_2 (Ishizaki and others 2003). In the high light of our experiments, the loss in dry mass per area can compound photosynthetic downregulation in elevated CO_2 (Long and others 2004; Pérez and others 2005) and above-ambient temperatures (Dwyer and others 2007).

We conclude that in addition to photosynthesis and stomatal aperture, leaf morphology of wheat is also changed by high-growth CO_2. In contrast to earlier work, our repeated results have shown that future increases in atmospheric CO_2 and temperature will decrease leaf dry mass per unit area and the former also leaf density, although the CO_2 effect may be masked by opposing effects on leaf primordia. Morphologic modifications of leaves in high CO_2 and temperature are due to lower amounts of structural

compounds. The functional significance of these changes is probably a decrease in photosynthetic capacity per unit leaf area.

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