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Highlights

Environmental and Experimental Botany xxx (2014) xxx–xxx

The effects of depleted, current and elevated growth [CO_2] in wheat are modulated by water availability

Salvador Aljazairi*, Salvador Nogués

Unitat de Fisiologia Vegetal, Departament de Biologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Diagonal, 645 Barcelona, Spain

- We have shown a photosynthetic acclimation of plants exposed to 260 and 700 ppm of CO_2.
- Photosynthetic acclimation was caused by changes in Rubisco protein and N content.
- Absence of effects on biomass production at depleted and elevated [CO_2].
- Photosynthetic acclimation is modulated by water availability.

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The effects of depleted, current and elevated growth [CO_2] in wheat are modulated by water availability

Salvador Aljazairi*, Salvador Nogués

Unitat de Fisiologia Vegetal, Departament de Biologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Diagonal, 645 Barcelona, Spain

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ABSTRACT

Drought is the main constraint on wheat yield in Mediterranean conditions. The photosynthesis, chlorophyll fluorescence and plant growth parameters of durum wheat (*Triticum turgidum*, L. var. *durum*) were compared at three [CO_2] (i.e., depleted 260 ppm, current 400 ppm and elevated 700 ppm) in plants subjected to two water regimes (i.e., well-watered WW, and mild water stress by drought or water deficit WS), during pre-anthesis, post-anthesis and the end of grain filling. We showed that [CO_2] effects on plants are modulated by water availability. Plants at depleted [CO_2] showed photosynthetic acclimation (i.e., up-regulation) and reduced plant biomass and Harvest Index, but depleted [CO_2] combined with WS has a more negative impact on plants with decreases in C assimilation and biomass. Plants at elevated [CO_2] had decreased plant growth and photosynthesis in response to a down-regulation mechanism resulting from a decrease in Rubisco and N content, but plants exposed to a combination of elevated [CO_2] and WS were the most negatively affected (e.g., on plant biomass).

1. Introduction

Wheat is one of the most important and extensively cultivated cereal food crops in the Mediterranean region and in the world. The productivity and quality of this crop is greatly affected by environmental conditions, with water-deficit being the main abiotic constraint on productivity (Araus et al., 2002). Wheat is grown mostly in rain-fed conditions, under which it can be subjected to water deficit and subsequent growth limitation (Oweis et al., 2000; Lopes et al., 2004).

The effect of drought on yield is mediated to a considerable extent by changes in photosynthetic activity and stomatal conductance. Changes in precipitation associated with continued emissions of CO_2 will bring changes in land suitability and crop yields (IPCC, 2013). These negative impacts are greater for wheat than for any other crop (IFPRI, 2007, 2013). Water deficit reduces photosynthesis by decreasing leaf area and photosynthetic rate (McCree, 1986). There have been many reports that water deficit leads to a general depletion of total soluble sugars and starch in leaves, for example, Hanson and Hitz (1982) and Huber et al. (1984) have concluded that water deficit has a larger effect on carbon assimilation than on translocation and use of photosynthate.

The increases in atmospheric CO_2 concentration from ca. 260 ppm before the beginning of the industrial revolution (i.e., 250 years ago) to the current level ca. 398 ppm (NOAA-ESRL, 2014) have affected long-term net assimilation rates (Araus and Buxó, 1993; Araus et al., 2002). Future scenarios are predicting a further steady increase in atmospheric CO_2 concentrations, due to the burning of fossil fuels and biomass (Pagani et al., 1999; Pearson and Palmer, 2000). By the end of this century, according to predictions using multi-model averages, atmospheric [CO_2] will have reached 985 ± 95 ppm (IPCC, 2013), with consequent increases in temperature (4 and 5 degrees) and drought periods. While photosynthesis in C_3 plants is usually affected by changes in [CO_2], there is a wide variation of responses in different species such as the acclimation of photosynthesis to different atmospheric CO_2 concentrations after a long period of exposure (Aranjuelo et al., 2009a, 2011; Pardo et al., 2009). Many studies suggest that the influence of low [CO_2] during pre-industrial periods may have affected plants at many different levels, ranging from the physiological effects on plants to changes in ecosystem functioning

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Abbreviations: A_{max}, light- and CO_2-saturated net assimilation rate; A_{sat}, light-saturated net assimilation rate; F_v/F_m, maximum quantum yield of PSII; F'_v/F'_m, efficiency of the capture of excitation energy by open PSII reaction centres; g_s, stomatal conductance; HI, Harvest Index; J_{max}, rate of photosynthetic electron transport;; PPFD, photosynthetic active photon flux density; PSII, Photosystem II; Φ_{PSII}, relative quantum yield of PSII; q_p, photochemical quenching; R_n, dark respiration; $V_{c,max}$, maximum carboxylation velocity of Rubisco; WS, mild water-stress by drought or water deficit; WW, well-watered.

* Corresponding author. Tel.: +34 93 4033714; fax: +34 93 4112842.
E-mail address: sal337@hotmail.com (S. Aljazairi).

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(Gerhart and Ward, 2010). Some studies have even shown an increase in photosynthesis in plants subjected to pre-industrial [CO_2] (Sage and Reid, 1992; Sage, 1994; Cowling and Sage, 1998; Anderson et al., 2001). Furthermore, many other studies have shown how plants respond to future CO_2 in short-term experiments (days or weeks) by increasing photosynthetic rates. However, responses to long-term experiments (i.e., weeks, months or years) show a process of acclimation of photosynthesis at projected future CO_2 levels (Long et al., 2004; Leakey et al., 2004). Acclimation is a physiological adjustment carried out by plants in response to a given [CO_2]. Photosynthesis acclimation can undergo up-regulation with depleted [CO_2] or down-regulation with elevated [CO_2] or through adjustments to the photosynthetic machinery (Sage, 1994; Anderson et al., 2001; Nogués and Azcón-Bieto, 2013). Free air CO_2 enrichment (FACE) experiments have provided ample evidence that photosynthetic capacity acclimates to elevated [CO_2] in C_3 plants, and the scale of down regulation varies with genetic and environmental factors (Nowak et al., 2004; Ainsworth and Long, 2005; Leakey et al., 2009). Photosynthetic acclimation is explained by different processes. Mechanisms to explain within-species variation often focus on changes in the balance between carbohydrate production (source) and the capacity to use and store carbohydrates (sink). For example, if increased carbohydrate production associated with future [CO_2] exceeds the capacity to use or store it, net photosynthetic rates may decline in order to balance the source activity with the sink capacity (Thomas and Strain, 1991; Aranjuelo et al., 2013; Aljazairi et al., 2014b). Other basic mechanism leading to photosynthesis acclimation are changing the Rubisco content caused by decreases in leaf N content in the case of down-regulation (Ellsworth et al., 2004; Aranjuelo et al., 2005, 2007; Kant et al., 2011; Aljazairi et al., 2014a), increases in Rubisco and leaf N content in the case of up-regulation (Gesch et al., 2000; Anderson et al., 2001) or reallocation of N within the plant (Nakano et al., 1997). Extensive evidence that nitrogen limits the growth response of plants at future [CO_2] has been demonstrated in many experiments conducted in controlled environmental chambers and under field conditions in FACE experiments (Ainsworth and Long, 2005; Rogers et al., 2006; Gutierrez et al., 2013; Bloom et al., 2010, 2014). Results from FACE studies have provide clear evidence for the link between N supply and acclimation (Ainsworth et al., 2003), and have shown that PNUE is improved in C_3 plants grown at future [CO_2] (Leakey et al., 2009).

As mentioned before, changes in the composition of [CO_2] in the atmosphere are affecting the climate and the water cycle around the world. For that reason, it is essential to study how plants have adapted from depleted to current [CO_2] and how this has been modulated by water availability. These adaptations may help to understand how plants will respond to future increases in [CO_2] and water deficit (Sage and Coleman, 2001; Nogués and Azcón-Bieto, 2013). The study of the effects of CO_2 on plants is also fundamental in understanding plant evolution in response to changes in water availability over time. Plants control their stomata to regulate the amount of water transpiration, which is modulated by [CO_2] in the environment. CO_2 and water experiments allows a better understanding of stomatal responses to elevated and depleted [CO_2], and the ability to accurately measure CO_2 responses of stomatal conductance, canopy evapotranspiration, and soil moisture is an important component of climate change studies (Leakey et al., 2009). The response of plants to [CO_2] is modulated by water deficit.

Atmospheric CO_2 reductions can enhance stress intensity, for example, in the case of drought stress, because depleted [CO_2] tends to open stomata and exacerbates limitations associated with drought (Sage and Cowling, 1999; Sage and Coleman, 2001). However, elevated [CO_2] plants have lower stomatal conductance, which should ameliorate the water deficits and even in some cases increase plant growth significantly in dry conditions (Morgan et al., 2004, 2011; Perry et al., 2013). Also, other studies have reported that elevated [CO_2] can compensate for water deficit-induced reduction in growth (Aranjuelo et al., 2009a). Elevated [CO_2] also increases water use efficiency (WUE) in many plants under dry conditions, thus reducing their overall demand for water (Ainsworth and Long, 2005). Most studies on the impact of climate change on plants have been conducted with high [CO_2] and water deficit (West et al., 2005; Erice et al., 2007; Aranjuelo et al., 2009b); however, the interaction between depleted, current and elevated CO_2 and water deficit has received little attention and studies on photosynthesis and chlorophyll fluorescence are generally scarce and are lacking for cereals (Lopes et al., 2004).

The overall aim of this study was to explore the combined effects of [CO_2] and water availability on wheat physiology (i.e., growth, photosynthesis and chlorophyll fluorescence). This study tested the following hypotheses. Firstly, long term exposition of depleted and elevated [CO_2] will lead to photosynthetic acclimation on wheat. Secondly, given that plants increase the biomass when [CO_2] is higher and water deficit induce reduction on biomass, it was hypothesized that the combinatory effect of both lead to a large decreases on biomass in all the CO_2 treatments. Thirdly, given the higher potential of plants to control stomatal closure at elevated [CO_2], it was hypothesized that plants at depleted [CO_2] would suffer more water deficit. Finally, a better understanding of up- and down-regulations of photosynthesis in these plants during grain filling was also studied.

2. Material and methods

2.1. Plant material

Durum wheat (*Triticum turgidum* var. Sula) was used in this experiment. Sula (released in 1994) is a modern commercial genotype grown in Spain. It is characterised by its short stature, early heading and maturity and high yield potential. Seeds of wheat were germinated in petri dishes on wet Whatman paper. After 84 h, seedlings were transferred to 4-litre pots (one plant per pot) filled with quartz sand of 1 mm grain size.

2.2. Experimental design

Plants were grown in three fully controlled plant-growth chambers (Conviron E15, Controlled Environments Ltd., Winnipeg, Canada) at a temperature of 22/18 °C (day/night) and 60% relative humidity. Plants were supplied with a photosynthetic photon flux density (PPFD) of 400 ± 30 μmol m^{-2} s^{-1} during the 16 h light period (day) and 8 h dark period (night). Plants were watered with Hoagland complete nutrient solution (Arnon and Hoagland, 1939) alternating with distilled water in order to avoid salt accumulation over the whole life cycle. Humidity, temperature and [CO_2] in the air within the chambers were monitored continuously by a sensor (CMP3243 Controlled Environments Ltd., Winnipeg, Canada) over the period of the experiment at intervals of every 5 minutes by a sensor and compared every two weeks with a separate sensor (HMP75 humidity and temperature;

Table 1
Growing conditions (i.e. [CO_2], relative humidity and temperature) in the three controlled growth chambers.

Chamber	CO_2 ppm	Relative humidity (%)	Temperature (°C)
Depleted CO_2	259.4 ± 13.6	61.2 ± 0.7	22.8 ± 0.3
Current CO_2	409.3 ± 2.5	60.7 ± 0.2	23.1 ± 0.2
Elevated CO_2	731.7 ± 16.9	62.2 ± 1.1	22.8 ± 0.2

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and GMP222; 0–2000 μmol mol^{-1} carbon dioxide; Vaisala MI70Helsinki, Finland) in order to capture a complete record of environmental parameters (Table 1).

The plants were grown in three plant-growth chambers under three different [CO_2] (i.e., 700, 400 and 260 μmol mol^{-1}) for the entire life cycle (from September to January) at the Experimental Field Service of Barcelona University, Barcelona, Spain. Forty-eight plants were placed in the first plant-growth chamber, which was maintained at future [CO_2] (ca. 731.7 ± 16.9 μmol mol^{-1}) by injecting CO_2 into the chamber from an external bottle (Carburos Metálicos SA. Barcelona, Spain). Another forty-eight plants were placed in the second plant-growth chamber, which was maintained at current [CO_2] (ca. 409.3 ± 2.5 μmol mol^{-1}). Finally, the same number of plants was located in the third plant-growth chamber, which was maintained at pre-industrial [CO_2] (ca. 259.4 ± 13.6 μmol mol^{-1}). Air in this chamber was maintained at pre-industrial [CO_2] by using a pump to send the air inside of the chamber through a 1-litre column filled with soda lime (Soda lime with indicator QP Panreac Química SA, Barcelona, Spain). The soda lime was changed every two weeks. Plants were rotated inside the chamber each week and between chambers every three weeks in order to avoid chamber influences in the treatments.

Mild water deficit treatment was applied to half of the plants in each plant-growing chamber. Control plants (WW) were kept with 100% water content of soil pot capacity. Plants with mild water stress by drought or water deficit (WS) were kept at 60% water content of soil pot capacity. The soil pot capacity is the amount of water content held in the soil after excess water has drained away from the pot. Each pot was weighed every four days and each pot was refilled with water. Plant water status was evaluated by measuring the leaf relative water content (RWC, Weatherley, 1950).

In this experiment, plants were measured during three measuring periods (Pre-anthesis, T0; grain filling, T1; and end of grain filling, T2).

2.3. Gas exchange and chlorophyll fluorescence measurements

An infrared gas analyser (LI-6400 system, LI-COR Inc., Lincoln, NB, USA) supplied with a Leaf Chamber Fluorometer (LI6400-40) was used to perform simultaneous measurements of gas exchange and chlorophyll fluorescence. A–Ci curves with chlorophyll fluorescence determinations were conducted in fully expanded flag leaves from each of the CO_2 and water treatments. The A–Ci curves were repeated in four different plants per treatment, and were measured from 0 to 2000 μmol mol^{-1} of CO_2. The curves were made at 1200 μmol m^{-2} s^{-1} of PPFD and at a temperature of 25 °C. The CO_2 assimilation rate (A), CO_2 assimilation rate at light saturation (A_{sat}), the maximum photosynthetic rates at CO_2 saturation (A_{max}), and stomatal conductance (g_s) were estimated using equations developed by Von Caemmerer and Farquar (1981). Estimations of the maximum carboxylation velocity of Rubisco ($V_{c,max}$), the rate of photosynthetic electron transport based on NADPH requirements (J_{max}) and the rate of day respiration (Rd) were made by fitting a maximum likelihood regression below and above the inflexion of the A–Ci response using the method of McMurtrie and Wang (1993).

Modulated chlorophyll fluorescence measurements allowed for the estimation of the relative quantum yield of photosystem II (PSII), the efficiency of the capture of excitation energy by open PSII reaction centres (F'_v/F'_m), the maximum quantum yield of PSII (F_v/F_m), photochemical quenching (q_p), the non-photochemical quenching coefficient (q_N) and non-photochemical quenching (NPQ) determined in the flag leaf after 30 min of dark adaptation (Nogués and Baker, 2000).

3. SPAD and chlorophyll content

Chlorophyll and carotenoid contents (mg g^{-1} fresh leaves) were determined by weighing 100 mg of fresh leaves and were immediately frozen in liquid nitrogen and stored at −80 °C to preserve pigment content until they could be analysed (Kurasová et al., 2003). Then pigments were extracted using the acetone method (Arnon, 1949). Extracts were analysed using a spectrophotometer and pigment estimations of total, a, and b chlorophylls and carotenoids were made according to the equations of Lichtenthaler (1987).

4. Leaf carbon and nitrogen content, nitrogen use efficiency (NUE) and photosynthetic nitrogen use efficiency (PNUE)

Leaves used for gas exchange were collected and dried at 65 °C until constant weight, and ground to a powder. Powder samples were assessed for the C and N content using an Elemental Analyzer Flash 112 (Carbo Erba, Milan) at the Scientific Technical Services of Barcelona University, Barcelona, Spain. Nitrogen use efficiency (NUE) was calculated for samples as NUE = Total dry weight (g)/N content (g). Photosynthetic nitrogen use efficiency (PNUE) was calculated for leaf samples as PNUE = A_{sat}/N content.

4.1. Rubisco and protein determination

Total soluble protein content (TSPC) was determined using the Bradford method (Bradford, 1976). One hundred mg of frozen leaf tissue was ground with PBS solution and centrifuged at 13,000 rpm for 5 min. An aliquot of each extract was used to measure soluble protein by spectrometry, with reference to a standard line that was calculated with BSA (Bovine Serum Albumin). Another aliquot of the same extract was used for protein separation using SDS-PAGE. Gel images were scanned and analysed using the Motic Images Plus 2.0 program. The concentration of Rubisco Large (L) and Small (S) subunits was measured against a Rubisco standard protein (Bio-Rad Laboratories, Inc., Berkeley, California).

4.2. Growth parameters

Plant production was estimated by weighing separately flag leaves, other leaves, spikes, stems and roots, for each of the three corresponding CO_2 treatments, two water regimes and during the three measuring periods (i.e., pre-anthesis, T0; grain filling, T1; and end of grain filling, T2). Plant material was dried in an oven at 80 °C over 48 h to obtain the dry weight. The number of spikes (SN), spikelets per spike (NsS) and stems (StN) as well as the length of spikes (SL) and stems (StL) and the Zadok phenological stages were also measured.

4.3. Data analysis

The effects of [CO_2] on plant development in wheat plants were tested by two factor (CO_2 and water treatments) analyses of variance (ANOVA). The statistical analysis was conducted with the SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA). The means ± standard errors (SE) were calculated for each parameter. When a particular test was significant we compared the means using a Duncan multiple comparison test or LSD multiple comparison test. The results were accepted as significant at $P < 0.05$.

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5. Results

5.1. [CO_2] effects on plants

Analyses of growth parameters showed that the CO_2 treatments had effects on wheat plants. We found significant differences between [CO_2] treatments in terms of flag, spike, leaf, stem, and root biomass and flag, spike and stem areas and length but not significant differences were found between [CO_2] in spike number, spike length, number of spikelets per spike, stem number or Zadock phenological stage (Fig. 1; Table S1). Plants showed increases in Harvest Index (HI) with increases in the [CO_2] (i.e., 0.154; 0.198; 0.25) in depleted, current and elevated [CO_2] respectively (data not shown), with significant differences between CO_2 treatments ($F = 9.947$; $P = 0.004$). However, at elevated [CO_2], plants showed less biomass than at current [CO_2] in terms of weight and areas of spikes, leaves, stems and roots. A similar effect was found in plants grown at depleted [CO_2].

Analyses of gas exchange parameters showed that the CO_2 treatments had effects on our plants. At elevated [CO_2], we observed that carboxylation activity was diminished as indicated by the reduction in A_{max}, $V_{c,max}$ and J_{max} (Table 2). At depleted [CO_2] lower values were found than current [CO_2], however, some of those parameters such as $V_{c,max}$ or A_{max} during grain filling were higher, showing up-regulation of photosynthesis. A decrease in the respiration rate was observed when [CO_2] decreased. However, we did not find significant differences between [CO_2] in terms of gas exchange parameters (A_{sat}, g_s, A_{max}, $V_{c,max}$ and J_{max}; Table S2).

Table 2

CO_2 effects (260, 400 and 700 ppm) on $V_{c,max}$, J_{max}, stomatal limitation and A_{sat} under the two water treatments, well-watered (WW) and mild water deficit (WS), before anthesis (T0), at the beginning of grain filling (T1) and the end of grain filling (T2). Each value represents means of 4 plants. Statistical analysis is in Table S2.

CO_2	Timing	H_2O	$V_{c,max}$	J_{max}	Stom. Lim.	A_{sat}
700	T0	WW	68.9 ± 5.1	166.4 ± 4.5	22.7 ± 3.5	15.2 ± 0.8
		WS	93.0 ± 5.9	197.9 ± 2.9	30.5 ± 4.0	16.6 ± 1.9
	T1	WW	152.5 ± 18.5	296.1 ± 56.1	15.1 ± 1.3	27.3 ± 2.1
		WS	113.6 ± 23.3	234.2 ± 71.5	21.3 ± 4.9	21.1 ± 5.7
	T2	WW	83.4 ± 3.8	232.1 ± 12.2	25.0 ± 1.3	16.0 ± 1.0
		WS	98.8 ± 14.0	220.1 ± 29.9	17.9 ± 1.3	21.2 ± 3.1
400	T0	WW	104.7 ± 19.0	201.9 ± 27.8	20.3 ± 3.3	18.0 ± 1.8
		WS	85.6 ± 18.9	213.9 ± 46.4	28.8 ± 5.6	15.7 ± 3.8
	T1	WW	157.8 ± 8.8	267.3 ± 7.0	14.3 ± 1.3	28.6 ± 0.6
		WS	137.6 ± 16.1	274.7 ± 32.8	14.0 ± 2.2	28.4 ± 2.7
	T2	WW	102.1 ± 4.6	225.8 ± 21.0	18.6 ± 1.4	22.5 ± 2.7
		WS	105.2 ± 3.2	243.2 ± 19.4	22.4 ± 3.0	21.8 ± 0.7
260	T0	WW	90.5 ± 3.8	182.3 ± 13.9	20.2 ± 1.4	18.3 ± 1.3
		WS	19.8 ± 4.8	34.8 ± 6.9	15.1 ± 5.0	4.7 ± 1.3
	T1	WW	123.4 ± 8.0	277.2 ± 28.1	20.2 ± 0.4	26.2 ± 1.9
		WS	109.8 ± 4.2	250.1 ± 10.9	20.3 ± 2.3	22.90 ± 0.7
	T2	WW	101.6 ± 6.3	229.4 ± 6.3	22.9 ± 5.1	20.9 ± 1.8
		WS	94.5 ± 12.4	208.8 ± 32.7	19.6 ± 2.0	20.0 ± 2.1

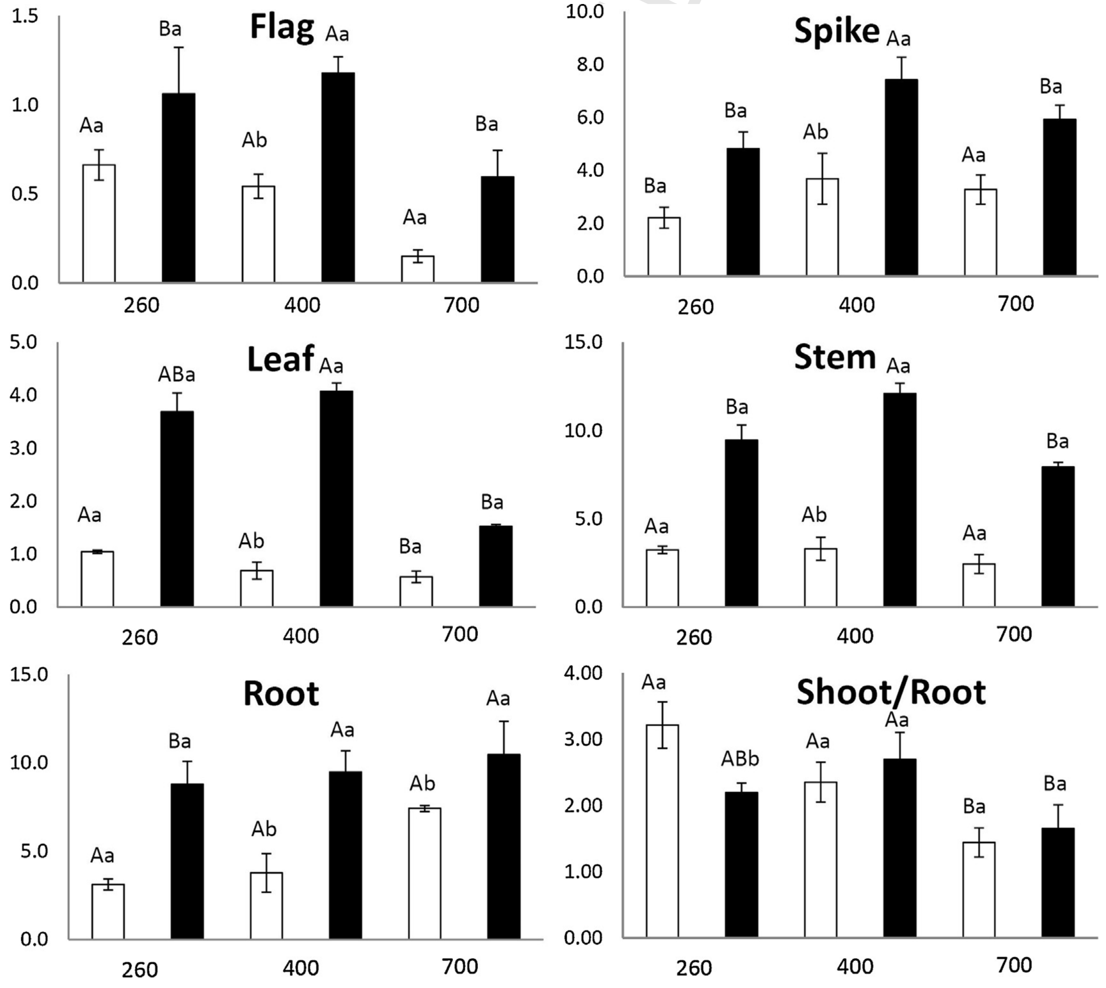

Fig. 1. CO_2 effects (260, 400 and 700 ppm) on Biomass (flag leaves, leaves, spikes, stems and roots) and the Shoot/Root ratio under well-watered (WW, close bars) and mild water deficit (WS, open bars) conditions. Uppercase letters are expressing differences between CO_2 treatments (A, B). Lowercase indicates differences between water treatments (a, b). Each value represents means of 4 plants. Means with identical letters are not significantly different ($P > 0.05$).

We found significant differences between [CO_2] in terms of chlorophyll fluorescence only in F_v/F_m, however, we observed decreases in F_v/F_m and F'_v/F'_m when the [CO_2] increased. We carried out multiple comparison LSD post-hoc tests for parameters where differences were indicated between CO_2 treatments and we found that the 260 ppm treatment had significant differences with 400 ppm and 700 ppm but no differences were found between 700 ppm and 400 ppm. Elevated [CO_2] did not cause a large effect in chlorophyll fluorescence parameters, however F_v/F_m, Φ_{PSII}, F'_v/F'_m and q_P were lower than at current [CO_2]. The *NPQ* and q_N values increased with increasing [CO_2]. On the other hand, at depleted [CO_2], decreases in F_v/F_m, Φ_{PSII} and q_p and increases in NPQ and q_N were observed before anthesis (Fig. 2).

Table 3
CO_2 effects (260, 400 and 700 ppm) on relative water content (%), SPAD and the Rubisco large (Rbc L) and small (Rbc S) subunits under the well-watered (WW) and mild water deficit (WS) conditions. Each value of RWC and SPAD represents means of 4 plants.

Parameter	Water	260	400	700
RWC (%)	WW	89 ± 2	92 ± 1	81 ± 5
	WS	76 ± 1	71 ± 8	69 ± 14
SPAD	WW	46.9 ± 1.6	53.7 ± 0.3	48.1 ± 0.8
	WS	47.3 ± 1.2	51.9 ± 0.7	50.3 ± 0.4
Rubisco large subunit	WW	203.5	100	75.5
	WS	108.0	100	71.9
Rubisco small subunit	WW	148.2	100	61.5
	WS	88.2	100	50.3

Chlorophyll was measured with a SPAD and the pigment contents (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) were measured with a spectrophotometer. With the SPAD, the highest values of chlorophyll were observed at 400 ppm and the lowest at 260 ppm (Table 3). Significant differences in spectrophotometer measurements of pigment contents were observed between all the different [CO_2]. Pigment

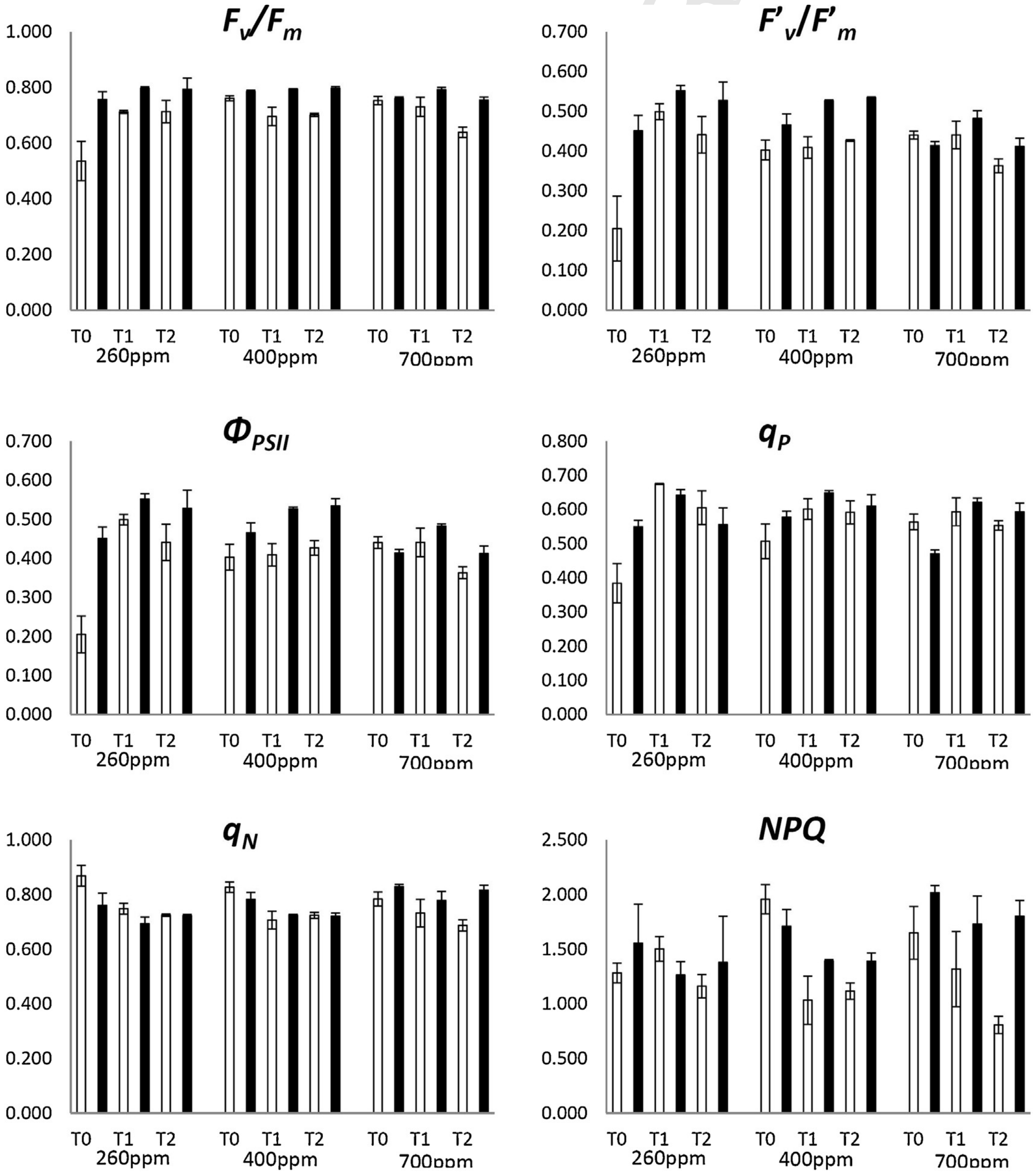

Fig. 2. CO_2 effects (260, 400 and 700 ppm) on leaf fluorescence under well-watered (WW, close bars) and mild water deficit (WS, open bars) conditions before anthesis (T0), the beginning grain filling (T1) and the end of grain filling (T2). Each value represents means of 4 plants. Statistical analysis is in Table S2.

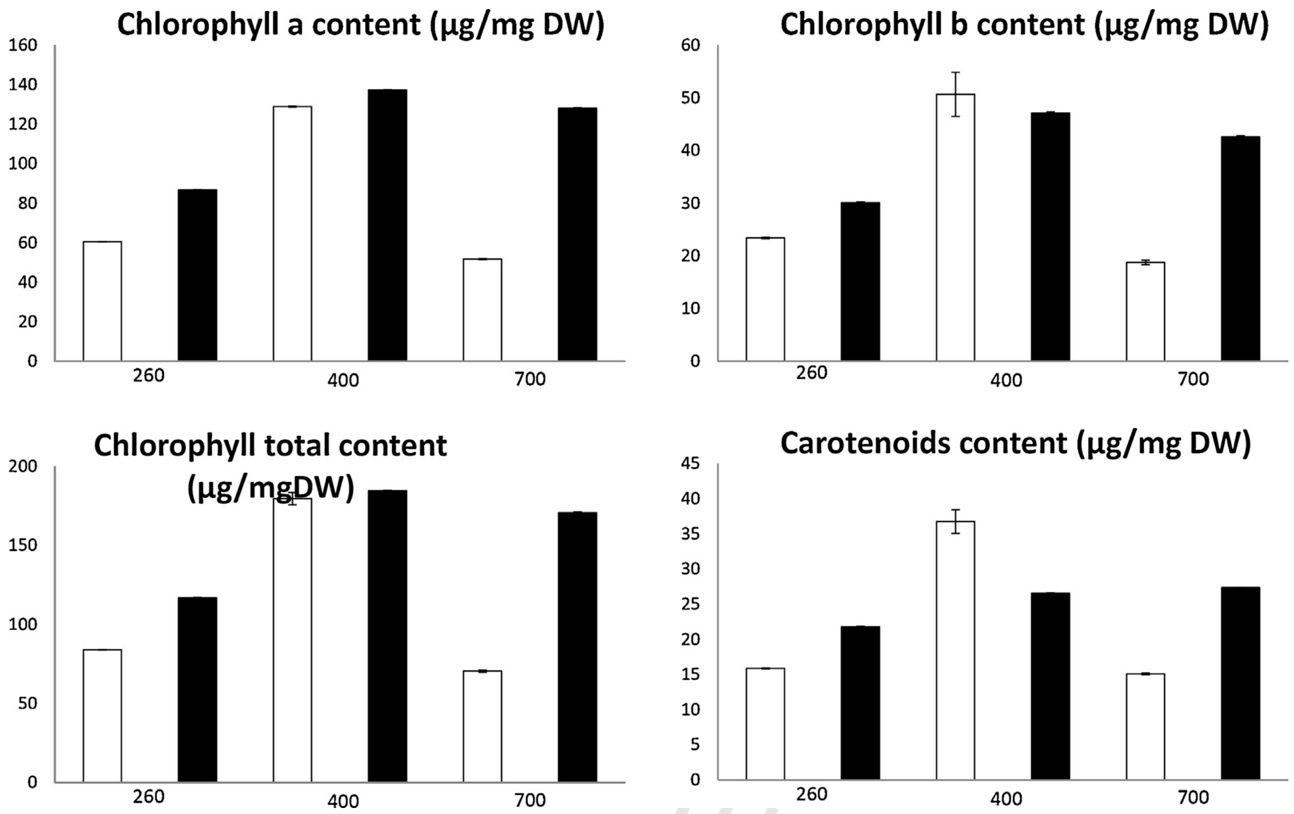

Fig. 3. CO_2 effects (260, 400 and 700 ppm) on pigment contents (Total chlorophyll, chlorophyll a, chlorophyll b and carotenoids) under well-watered (WW, close bars) and mild water deficit (WS, open bars) conditions. Each value represents means of 4 plants. Statistical analysis is in Table S2.

contents were lower at depleted [CO_2], and slightly lower at elevated [CO_2]. (Fig. 3; Table S2). The carotenoid content also showed significant differences between different [CO_2]. The highest values of carotenoids were at current [CO_2] (Fig. 3; Table S2).

Large and small subunit Rubisco concentrations (Rbc_L and Rbc_S, respectively) were measured during the experiment. Plants showed higher concentrations of Rubisco at depleted CO_2 in both subunits. Plants under elevated [CO_2] showed lower Rubisco content than the current [CO_2] (Table 2). On the other hand, leaf total soluble protein content (TSPC) decreased in plants when the [CO_2] increased, with this trend in TSPC change occurring across the three [CO_2]. Plants at depleted [CO_2] were seen to have greater concentrations than at current [CO_2]. At elevated [CO_2] WW plants

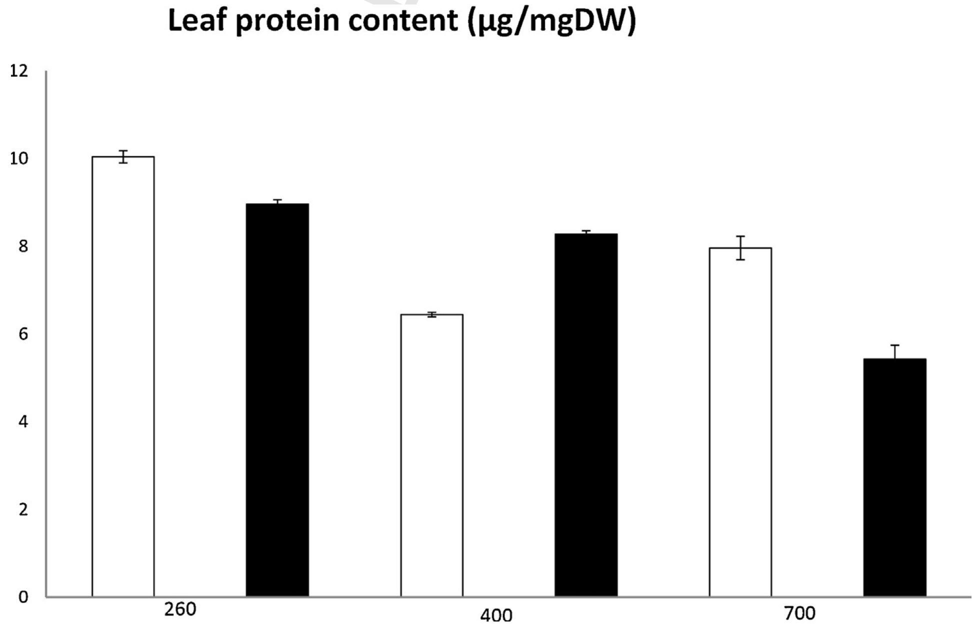

Fig. 4. CO_2 effects (260, 400 and 700 ppm) on leaf protein content under well-watered (WW, close bars) and mild water deficit (WS, open bars) conditions. Each value represents means of 4 plants. Statistical analysis is in Table S2.

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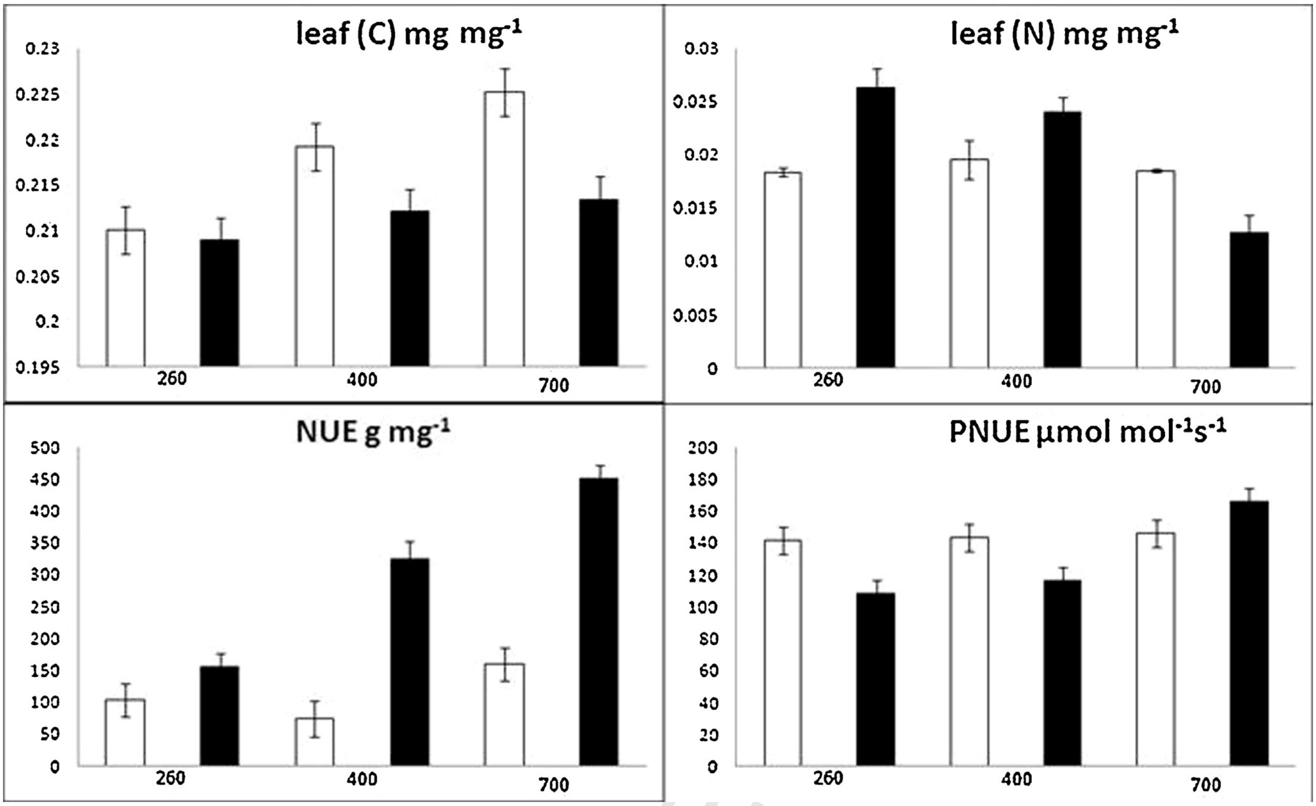

Fig. 5. CO_2 effects (260, 400 and 700 ppm) on leaf carbon and nitrogen content (mg mg^{-1}), NUE (g mg^{-1}) and PNUE (µmol mol^{-1} s^{-1}) under well-watered (WW, close bars) and mild water deficit (WS, open bars) conditions.

had lower leaf TSPC levels than at current [CO_2] and WS plants had higher leaf TSPC levels than current and lower leaf TSPC levels than depleted [CO_2] (Fig. 4).

Plants showed lower leaf C content at depleted [CO_2] and higher leaf C content at elevated [CO_2] than the current [CO_2]. However, the differences between CO_2 treatments were not significant. The leaf N content increased in plants when the [CO_2] decreased. NUE also increased with the [CO_2]. PNUE did not show large differences between different [CO_2] (Fig. 5).

6. Water availability effects on plants

Analyses of growth parameters showed that the water treatments had effects on wheat plants. WW plants had more biomass (flag leaves, spikes, leaves, stems, and roots) than mild WS plants. We found significant differences between water treatments for the following: flag, spike, leaf, stem, and root biomass; flag, spike and stem areas; spike number, spike length, number of spikelets per spike, stem number, stem length and Zadock phenological stage (Fig. 1; Table S1). Mild WS plants showed a higher Harvest Index (HI) (0.154; 0.198; 0.25) than WW plants (0.128; 0.18; 0.134) in depleted, current and elevated [CO_2], respectively (data not shown), with significant differences between water treatments ($F = 34.7$; $P = 0.000$). Analyses of gas exchange parameters showed that the water treatments had effects on our plants. We observed that carboxylation activity was diminished as indicated by the reduction in A_{max}, in $V_{c,max}$ and J_{max}. The decrease in these parameters (i.e., $V_{c,max}$ or A_{sat}) in WW is greater than in mild WS (Table 2). However we did not find significant differences between water treatments.

Mild WS plants showed lower values than WW plants in F_v/F_m and F'_v/F'_m, but also in Φ_{PSII} and we found significant differences between water treatments in these parameters (Table S2).

According to the SPAD measurements, there were no significant differences observed in chlorophyll contents between water treatments (Table 3). Pigment contents (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) were also measured by the spectrophotometer. Significant differences on pigment contents were found between water treatments. Mild WS plants showed less chlorophyll content than WW plants (Fig. 3; Table S2). Carotenoid contents were also significantly different between water treatments (Fig. 3; Table S2).

In terms of Rubisco content in the water treatments we could see large differences between water treatments. WW plants showed higher increments of Rubisco content than mild WS plants. On the other hand, plants showed greater leaf total soluble protein content at mild WS than at WW in two out of three cases (Fig. 4).

Mild WS plants showed higher leaf C content than WW plants. However, mild WS plants had lower leaf N content than WW plants. NUE increased in WW plants with respect to mild WS plants. In addition, PNUE increased more in the two lower [CO_2] under mild WS than in the WW plants (Fig. 5).

7. [CO_2] effects on plants modulated by water availability

Analyses of growth parameters showed that the [CO_2] effects were modulated by water treatments on wheat plants. WW plants showed more down-regulation of growth at elevated [CO_2] than mild WS plants, with decreases across a range of growth parameters (Fig. 1; Table S1).

Analyses of gas exchange parameters showed that the [CO_2] effects were modulated by water treatments. At current [CO_2], WW conditions resulted in higher $V_{c,max}$, A_{sat}, A_{max} and lower J_{max} than mild WS. At depleted [CO_2], lower values were found than under current [CO_2], however, some of the parameters increased, such as $V_{c,max}$ or A_{max} during grain filling, thus indicating up-regulation of

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photosynthesis. However, we did not find any significant differences between CO_2 × water treatments.

Regarding CO_2 × water, we found significant differences in the interaction between in terms of chlorophyll fluorescence parameters (F_v/F_m, F'_v/F'_m, Φ_{PSII} and q_p). Besides, significant differences in pigment contents (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) were found between CO_2 × water treatments. The pigment contents showed effects of [CO_2] modulated by water availability, being higher in WW plants at elevated [CO_2] than at depleted [CO_2]. While the quantity of pigments was lower under the future [CO_2] than under depleted [CO_2] when combined with mild WS, the differences were not large (Fig. 3; Table S2).

Large and small subunit Rubisco concentrations (Rbc_L and Rbc_S, respectively) were measured during the experiment. Both water treatments showed higher concentrations of Rubisco at depleted CO_2 for both subunits (Rbc_L, 103% and 7% for WW and WS, respectively; Rbc_S, 48% for WW). However, WS plants showed a 12% reduction in Rbc_S concentrations under depleted [CO_2]. Plants under elevated [CO_2] showed lower Rubisco content than current [CO_2] (i.e., 25% and 28% lower for Rbc_L, and 39% and 50% lower for Rbc_S for WW and WS respectively). The biggest differences were for the Rbc_S at elevated [CO_2] and WS, and the Rbc_L at depleted [CO_2] and WW (Table 3).

Leaf TSPC decreased when the [CO_2] increased, but it was modulated by water availability. Plants at depleted [CO_2] had greater concentrations of leaf TSPC than at current [CO_2] (8.3% for WW and 55% for WS) and at elevated [CO_2] they had lower protein concentration levels than at current [CO_2] (35% for WW). However, under WS conditions the leaf protein content increased by 23% (Fig. 4).

Leaf carbon and nitrogen content, and NUE and PNUE were affected by the [CO_2] and those effects were modulated by water availability. Plants showed higher leaf C content at elevated [CO_2] (i.e., 5% and 3% under WW and WS, respectively) and lower leaf C content at depleted [CO_2] (i.e., 3% and 5% under WW and WS, respectively) than current [CO_2], but the differences were not significant. At depleted and elevated [CO_2], plants had a lower leaf N content, with the larger difference at elevated [CO_2] (48% and 6% less N for WW and WS, respectively) than at current [CO_2], whereas at depleted [CO_2], plants were seen to have also a higher N content (16% and 4% for WW and WS, respectively). NUE increased with the [CO_2] in WW plants (155%, 323% and 451% for 260, 400 and 700 ppm, respectively). In addition, WS plants had lower NUE than WW plants, and these differences were much greater at elevated [CO_2] than at depleted [CO_2]. PNUE also increase significantly at elevated [CO_2] in WW plants. However, no differences were observed in WS plants between [CO_2] (Fig. 5).

8. Discussion

The agronomic and physiological parameters of durum wheat plants (*Triticum turgidum*) were characterised in order to study the response of wheat under three CO_2 environments (i.e. depleted, current and elevated) under two water regimens (well-watered, WW, and mild water-stress by drought or water deficit, WS).

9. [CO_2] effects on plants

Plants showed photosynthetic acclimation at depleted (i.e., up-regulation) and elevated [CO_2] (i.e., down-regulation). However, neither of these acclimatory responses resulted in increases in biomass.

At depleted [CO_2], wheat showed carbon source limitations from the atmosphere (as we can see in less % C) than other CO_2 treatments, with higher allocation of biomass to green areas than roots, so plants had a higher shoot/root ratio than plants at current and elevated [CO_2], resulting in lower rates of carboxylation at depleted [CO_2] (Gerhart and Ward, 2010). Other studies have demonstrated that C_3 species have reduced biomass when grown at depleted [CO_2] (Allen et al., 1991; Dippery et al., 1995; Aranjuelo et al., 2011). This evidence suggests that, in the past, depleted atmospheric [CO_2] reduced the potential productivity of C_3 species (Gerhart and Ward, 2010). Some authors have shown that biomass of C_3 species increases when [CO_2] increases from preindustrial levels (260 ppm) to current levels (400 ppm) (e.g., 35% increases in cotton (Thomas and Strain, 1991); 24% increases in *Arabidopsis*, (Dippery et al., 1995)). Therefore, the productivity of C_3 plants in general and wheat in particular has most likely increased during the last two centuries of industrialisation due to anthropogenic CO_2 emissions. This is seen in our study where the green areas and root biomass increased 22% and 8%, respectively, from the depleted to current [CO_2].

Amthor (2001), summarising 156 experiments (in FACE, greenhouses and growth chamber studies) that analysed effects on wheat yield under elevated [CO_2], showed that the CO_2 response ranged from no effect or even negative effects in some studies to several-fold increases in others. Exposure at 700 ppm in the current study slightly decreased biomass production of the spike, stem, flag leaf and other leaves (although differences were not significant); however, there were increases in root biomass (Fig. 1 and Table S1). Aranjuelo et al. (2011) in their FACE study with durum wheat contrasting [CO_2] conditions (700 versus 370 μmol mol^{-1}), showed that elevated [CO_2] did not contribute to increased grain filling in wheat plants, and with other previous reports (Amthor, 2001; Högy et al., 2009; Uddling et al., 2008). Further, many studies indicated the variability of the responses to increased biomass at elevated [CO_2] in FACE and growth chambers. These increases are doubt to different variables as for example the crop species and environmental conditions (Nowak et al., 2004; Ainsworth and Long, 2005; Aranjuelo et al., 2009b; Leakey et al., 2009). An absence of effects on plant biomass, together with the lower spike biomass suggests that under elevated [CO_2], plants were acclimated in terms of photosynthesis and growth parameters. Grain filling may be limited by (i) translocation of photoassimilates from source to sink, ii) photosynthetic activity (iii) spike sink capacity and (iv) nitrogen source (Uddling et al., 2008; Q3 Bloom et al., 2010; Gutierrez et al., 2013). At elevated [CO_2], plants had lower shoot/root ratios than plants grown at current [CO_2]. This data suggest that plants at elevated [CO_2] invest more photoassimilates in roots than at current or depleted [CO_2]. At elevated [CO_2] plants have a greater source of C and they invest this C into increased root biomass, which gives them greater potential for exploring more soil and thereby increases their uptake of soil moisture and nutrients (Reich et al., 2006; Ghashghaie and Badeck, Q4 2013).

An important focus of our work was to compare plant physiological responses to increases over depleted and elevated [CO_2]. Photosynthetic acclimation to CO_2 is one of the most important issues in CO_2 studies and this photosynthetic acclimation at depleted and elevated [CO_2] during long-term exposure (in our case the whole wheat lifecycle) can be compensated by the effects of CO_2 variation in plant processes (Sage and Coleman, 2001; Aljazairi et al., 2014a,b).

Many studies have shown that atmospheric CO_2 has a direct effect on photosynthesis and also on the reduction of assimilation at depleted [CO_2], at optimal temperatures and nutritional conditions C is more limiting as a substrate for the carboxylation reaction in Rubisco in C_3 plants than at current or elevated [CO_2] (Long and Drake, 1992). So, depleted [CO_2] can lead to a reduction in photosynthetic capacity (Sage, 1995). This study of short-term exposure to depleted [CO_2] showed significant decreases in assimilation and $V_{c,max}$. However, the effect of depleted [CO_2]

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can be compensated by the acclimation processes of photosynthesis. Similar to our data (Fig. 2, Table S2), increases in the maximum rate of carboxylation $V_{c,max}$, J_{max}, assimilation and also in the concentration of Rubisco in C_3 plants under long-term exposure to depleted [CO_2] have been described by other authors (Anderson et al., 2001), and this is known as photosynthetic acclimation (up-regulation). Other studies have found evidence of photosynthetic acclimation at depleted [CO_2]. Sage and Reid (1992) and Cowling and Sage (1998) found that A is limited by Pi regeneration in *Phaseolous vulgaris* plants grown at depleted [CO_2]. Allen et al. (1991) and Dippery et al.(1995) suggested that photosynthesis is also enhanced (up-regulation) by increases in partitioning of C to leaves at depleted [CO_2].

On the other hand, when photosynthetic activity was determined in plants at 400 and 700 ppm (Fig. 2, Table S2) it was found that plants grown at elevated [CO_2] had lower photosynthetic capacity than plants grown at current [CO_2] (Zhang et al., 2009; Aranjuelo et al., 2011), suggesting a photosynthetic acclimation at elevated [CO_2] caused by down-regulation. Photosynthetic acclimation has been previously described in wheat plants exposed to elevated [CO_2] (Martínez-Carrasco et al., 2005; Alonso et al., 2009; Gutierrez et al., 2009; Aranjuelo et al., 2011). The decrease in photosynthetic capacity under elevated [CO_2] has been attributed to end product inhibition, in which the demand for carbohydrates is insufficient to cope with the enhanced carbohydrate supply (Rogers and Ellsworth, 2002; Ainsworth and Long, 2005; Aranjuelo et al., 2008). Carbohydrate accumulation in leaves, grown at elevated [CO_2], has been shown to stimulate organic acid synthesis (Morcuende et al., 1998; Stitt and Krapp, 1999) and respiratory pathways, irrespective of whether it is a result of sugar-feeding or an inhibition of phloem transport.

Chlorophyll fluorescence measurements allowed for the study of acclimatory effects induced in PSII at depleted and elevated [CO_2]. At depleted [CO_2], before anthesis (T0), there was a decrease in F_v/F_m or F'_v/F'_m, however, the other two phenological periods did not show differences from current [CO_2]. At elevated [CO_2] no effects were observed in F_v/F_m and q_p, and decreases were observed in F'_v/F'_m and Φ_{PSII}, but in WW plants values were recorded that were 10% lower than under current [CO_2]. This lack of an effect on photochemical efficiency in plants has been described by other authors. For example, Hymus et al. (1999), observed a decrease, increase or no effect on the use of energy absorbed during photochemistry in trees, and Gutierrez et al. (2009) observed little effect on q_p and F'_v/F'_m as in the present study. Thus, the shift from higher to lower values at elevated versus current [CO_2] implies that restrictions in the carbon assimilation capacity under elevated [CO_2] decrease the photochemistry of the light absorbed by the PSII antenna. While q_p decreased, NPQ increased in elevated [CO_2] suggesting that on one hand plants are acclimated to elevated [CO_2] via a decrease in carbon assimilation and on the other hand, that the energy is dissipated non-photochemically thus protecting the PSII from damage (as also reported Taub et al., 2000).

As in our case, reduced values for pigment contents have been reported for many plant species growing in elevated [CO_2] (Surano et al., 1986; Polley et al., 1993; Pritchard et al., 2000). Such decreases in carotenoids contents have been linked to photodamage of the photosynthetic apparatus, with carotenoids preventing destruction of chlorophyll in high light by dissipating excess excitation energy and also potentially stabilising and photoprotecting the lipid phase of the thylakoid membranes (Havaux, 1988).

In our study we found evidence that plants showed photosynthetic up-regulation under the depleted [CO_2], because plants modulated the Rubisco content (Table 3). Rubisco content seems to be higher in plants that undergo prolonged exposure to pre-industrial levels of [CO_2] than those grown at current [CO_2] levels (Maherali et al., 2002). Gesch et al. (2000) observed increased Rubisco small subunit gene expression in *Oryza sativa* L. exposed to pre-industrial [CO_2]. Other studies have also found evidence for increases in Rubisco and photosynthetic acclimation at depleted [CO_2] (Sage and Reid, 1992; Cowling and Sage, 1998; Gerhart and Ward, 2010; Ripley et al., 2013; Pinto et al., 2014). Accordingly, our wheat plants exhibited increased TSPC, including Rubisco under depleted [CO_2]. As mentioned before, wheat growth under depleted [CO_2] leads to increased photosynthetic capacity, g_s, Rubisco content or TSPC, and also leaf N content in C_3 plants (Dippery et al., 1995; Ward et al., 1999; Anderson et al., 2001; Cunniff et al., 2010). Up-regulation of A may be related to increases in leaf N content, as well as to re-allocation of N within leaves. As Rubisco is a significant N sink, Sage and Reid (1992) predicted that large increases in leaf N would be needed for plants grown at 260 ppm to achieve assimilation equal to plants grown at 400 ppm. Similar to Anderson et al. (2001), we found significantly higher leaf N ($g\,m^{-2}$) in wheat under depleted [CO_2], which could be additional evidence for up-regulation in wheat.

The analysis of Rubisco content at elevated [CO_2] revealed that the photosynthetic down regulation was caused by a lower Rubisco content (Theobald et al., 1998; Aranjuelo et al., 2005; Gutierrez et al., 2009). In our study we observed decreases in Rbc L (between 25 and 30%) and Rbc S (between 40 and 50%; Table 4). These data corroborate that plants suffered down-regulation to elevated [CO_2]. Jifon and Wolfe (2002) suggested that enhancement of photoassimilate contents in plants exposed to elevate [CO_2] induces repression of genes coding for photosynthetic proteins, leading to a down-regulation of photosynthetic capacity. At the whole plant level this occurs when photosynthesis exceeds the capacity of sink organs to assimilate the photosynthate (Aranjuelo et al., 2009a). This might explain why the wheat plants showed lower Rubisco and TSPC contents (Table 3 and Fig. 4) at elevated [CO_2]. However, significant differences in Rubisco and TSPC were found, but the decrease in Rubisco as a fraction of TSPC was greater than other proteins suggesting that the diminished Rubisco concentration was caused by a more specific inhibition of Rubisco than other proteins in leaves exposed to elevated [CO_2] (Pérez et al., 2007; Aranjuelo et al., 2011).

10. Water availability effects on plants

Similar to other studies, mild-WS reduced and limited plant growth (i.e., mild-WS decreased shoot and root biomass between 50 and 70% compared to WW plants; Fig. 1, Table S1; Aranjuelo et al., 2007). RWC measurements revealed that differences observed in plant growth were due to water status (Table 3). Mild water deficit might reduce the water status and, consequently, affect other growth or physiological parameters.

Photosynthesis of durum wheat is affected by water availability (Tambussi et al., 2005; Terzi and Kadioglu, 2006). The photosynthetic acclimation effect is low under drought, suggesting that acclimation is modulated by water deficit. Other studies have shown the effect of drought in several C_3 plants and indicated that drought induces decreases in photosynthetic capacity of the leaves, which are accompanied by reductions in $V_{c,max}$ and J_{max}, suggesting that drought inactives or decreases Rubisco and other key Calvin cycle enzymes (Nogués and Baker, 2000).

Besides this, significant differences in pigment contents were also found between water treatments. WS plants showed lower pigment contents than WW plants at depleted and elevated [CO_2], with an overall reduction in chlorophyll content. Similar results have also been reported by other authors (Dalal and Tripathy, 2012), suggesting that chlorophyll biosynthesis is substantially down-regulated under water deficit. Down-regulation of chlorophyll content could act as a regulatory mechanism in plants to

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resist drought. Minimisation of light absorption by reduced amounts of chlorophyll would down-regulate the electron transport so as to reduce the ROS production. Decreases in the carotenoid content suggested that the photosynthetic apparatus may be predisposed to photodamage; however, carotenoid increases were observed in mild water deficit under current [CO_2]. Carotenoids prevent destruction of chlorophyll in high light by dissipating excess excitation energy and may also stabilise and photo-protect the lipid phase of the thylakoid membranes (Havaux, 1988). On the other hand, the analysis of Rubisco revealed that in our study mild water deficit treatment affected the expression of Rubisco and we observed decreases from 40 to 60% of Rbc L and Rbc S. These data corroborate that the plants suffered down expression of Rubisco content with WS stress. Drought and low water availability inhibited N fixation through their effect on biomass production (Aranjuelo et al., 2007). As in our case, the leaf N content decreased in plants kept under mild water deficit and this is related to the Rubisco content, assimilation and also the production of biomass.

11. [CO_2] effects on plants modulated by water availability

Depleted and elevated [CO_2] under drought may limit plant growth. Our study demonstrated that elevated [CO_2] and drought had a significant negative impact on biomass production.

At elevated [CO_2] the autotrophic biomass in WW and mild WS was decreased by 36% and 19%, respectively, with leaves being the most affected organs. However, the root biomass increased 10% and 5% for WW and mild WS plants, respectively, and this is in agreement with previous reports (Amthor, 2001; Uddling et al., 2008; Högy et al., 2009; Aranjuelo et al., 2011). These data suggest that plants invest more C in roots than shoots at elevated [CO_2] and this effect is modulated by water availability, with a greater effect in WW plants. An explanation for this is that plants under elevated [CO_2] do not need large autotrophic areas to obtain the C and they invest the assimilated C in roots in order to find other nutrients required under the elevated [CO_2] conditions. Further, against to our second initial hypotheses, this effect is increased with higher water availability and plant at elevated [CO_2] and WW showed lower shoot biomass than WS by acclimation processes. However, under depleted [CO_2] the plants kept or even increased their autotrophic organs and decreased roots, suggesting that plants under depleted C invest more in developing the organs that capture more C from the atmosphere. However, mild- WS limits this development. Other authors have also found that plants modify the number of stomata, leaf area, leaf thickness, biomass partitioning and other parameters to adapt to different [CO_2] (Long et al., 2004; Leakey et al., 2009; Gerhart and Ward, 2010).

As in other studies (Aranjuelo et al., 2007), drought reduced plant growth (i.e., mild WS plants decrease their shoot and root biomass between 50 and 70% compared to WW plants; Fig. 1, Table S1). Such reductions are minor at elevated than at current or depleted [CO_2] suggesting that the effect of mild water deficit is minor at elevated [CO_2] because the increased C availability gives plants greater resistance to drought and it is in accordance with our third hypothesis.

The leaf relative water content (RWC) measurements (Table 3) revealed that differences observed in plant growth were due to CO_2 and to water status. Mild water deficit might reduce the water content under elevated [CO_2].

As we mentioned before, leaves of wheat plants showed photosynthetic acclimation with increases in assimilation and there was also more C partitioning to leaves when plants were switched from current to depleted [CO_2]. Assimilation and C partitioning in leaves suffered changes under drought (Allen et al., 1991; Dippery et al., 1995) suggesting that photosynthetic acclimation is modulated by water deficit. Aranjuelo et al. (2009b) showed that the effect of drought in several C_3 plants induced decreases in $V_{c,max}$ and J_{max} at elevated [CO_2]. However, this effect is largely modulated by water availability and at elevated [CO_2] mild WS plants suffered less down-regulation than WW plants because the production and storage of carbohydrates is minor under mild WS compared to WW plants. Mild WS negatively affected the up-regulation of photosynthesis. These facts suggested that drought stress inhibited photosynthetic acclimation at elevated and depleted [CO_2].

Also, lower photosynthetic rates in WW than in WS plants before anthesis and the end of grain filling, and higher rates during grain filling, indicated that photosynthetic down-regulation is modulated by water deficit and also by the phenological period at elevated [CO_2]. Our non-stressed wheat plants showed more photosynthetic down-regulation before anthesis and during the end of the grain filling, and during grain filling had a greater ability to increase assimilation of C to then send more photoassimilates to the grain under elevated [CO_2] (Aljazairi et al., 2014 a,b).

In terms of chlorophyll fluorescence, mild WS plants showed around 10–20% lower values of F_v/F_m, F'_v/F'_m, Φ_{PSII} and q_p than WW plants (Fig. 2, Table S2). This data suggested that WS decreased the photochemical efficiency of plants. As we showed before, the capacity for carbon assimilation decreased at elevated [CO_2], but the probability of photoinhibition due to increased non-photochemical quenching, is increased. Such non-photochemical quenching would serve to protect the reaction centres from photo-inactivation and damage when the rate of excitation of PSII is in excess of the rate of photochemistry (Hymus et al., 2001).

Changes in pigment contents showed significant differences in the interaction of CO_2 × water availability, indicating that the responses of plants were modified by both factors. Plants under depleted [CO_2] showed decreased pigment contents in both water treatments, however, plants at elevated [CO_2] only showed large decreases in water deficit. These data may indicate the way plants reduce their pigment contents to protect themselves from damage due to drought and [CO_2], minimising the amount of pigment so as to reduce electron transport and ROS production (Dalal and Tripathy, 2012), this effect was higher under depleted [CO_2]. However, the pigment content reduction is maximal at elevated [CO_2] and WS indicating that both treatments together have an enhanced effect with the CO_2 and water availability modulating each other. On the other hand, decreases in pigment content suggest that the combination of mild water deficit at elevated [CO_2] put the greatest constraints on plants, but that the impact of elevated [CO_2] in WW plants was minimum. However, restrictions on C under depleted [CO_2] caused decreases in pigment content in both water treatments suggesting that C restriction at depleted [CO_2] is a more important limitation than water deficit for plants.

The analysis of Rubisco at elevated [CO_2] revealed that the photosynthetic down regulation at elevated [CO_2] was caused by a lower Rubisco content (Theobald et al., 1998; Aranjuelo et al., 2005; Gutierrez et al., 2009) and was modulated by water availability with greater decreases in mild WS plants. The lower photosynthetic rates of plants exposed to 700 ppm may be a consequence of decreases in content of both Rubisco subunits. In our study we observed decreases in Rbc L (25 and 29% for WW and WS, respectively) and Rbc S (40 and 50% for WW and WS; Table 4). These data corroborate that plants suffered down-regulation under elevated [CO_2] and with WS stress the Rubisco content was even more affected, but as we saw before, the photosynthetic acclimation was stronger in WW plants. Specifically, in WW plants there were 103% and 50% more Rbc L and Rbc S subunits, respectively, while in WS plants the Rbc L was only 10% higher and the Rbc S was 12% smaller at depleted than at current [CO_2], suggesting that the drought negatively affected the up-regulation

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processes at depleted [CO_2]. As we highlighted before, WS also modulated the response of Rubisco content to the down- and up-regulation at elevated and depleted [CO_2].

As in Drake et al. (1997), plants grown under elevated [CO_2] had increased nitrogen use efficiency, and was highest in WW plants (45% and 66% for WS and WW plants, respectively). Also, elevated [CO_2] enhanced PNUE and decreased leaf N content significantly and underlined the importance of past and future increases in CO_2 for plants grown (Anderson et al., 2001; Gutierrez et al., 2013).

In summary, we have shown a photosynthetic acclimation of plants exposed to 260 and 700 ppm [CO_2] (up-regulation and down-regulation, respectively). We also found an absence of effects on biomass production and HI in plants exposed to depleted and elevated [CO_2]. This photosynthetic acclimation is modulated by water availability. Furthermore, protein characterisation also revealed that photosynthetic acclimation was caused by an increase and a decrease in Rubisco protein content under depleted and elevated [CO_2] respectively. These changes in Rubisco content and the analyses of leaf N content, with increases at depleted [CO_2] and decreases at elevated [CO_2], suggest that under depleted [CO_2] the N content was accumulated in leaves where protein and Rubisco content were higher, and under elevated CO_2 there was reallocation of leaf N to other parts of the plants, and most likely the spikes. In this experiment, spikes of wheat did not contribute to an increase in C sink strength. Also, at elevated [CO_2] the absence of effects on biomass production, and HI, reflected the inability of plants to create new C sinks. Therefore, such plants were incapable of overcoming leaf photoassimilate accumulation, with a consequent alteration in leaf N and protein content that caused photosynthetic down-regulation. Finally, mild water deficit modulated the ability of plants to grow through its inhibitory effect on biomass production in plants at the three CO_2 levels.

Uncited reference

Lehmeier et al. (2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2014.12.002.

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