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Physiology

Effects of pre-industrial, current and future [CO₂] in traditional and modern wheat genotypesSalvador Aljazairi^{a,*}, Claudia Arias^a, Elena Sánchez^a, Gladys Lino^a, Salvador Nogués^a^a Unitat de Fisiologia Vegetal, Departament de Biologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, Barcelona, Spain

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ABSTRACT

Wheat is one of the most important cereal food crops in the world today. The productivity and quality of this crop is greatly affected by environmental conditions during grain filling. In this study, we have analyzed two genotypes of durum wheat, Blanqueta and Sula (traditional and a modern wheat respectively) in pre-industrial, current and future [CO₂]. Plant growth and physiological parameters were analyzed during anthesis and grain filling in order to study the capacity of these plants to create new sinks and their role during the process of the acclimation of photosynthesis. It was observed that plants underwent photosynthetic acclimation at pre-industrial and future [CO₂] (up and down-regulation respectively). However, the modern genotype averts the process of down-regulation by creating a new carbon sink (i.e. the spike). Here, we have shown the essential role that the spike plays as a new sink in order to avert the down-regulation of photosynthesis at future [CO₂]. Moreover, we have demonstrated that at future [CO₂] the growth response will depend on the ability of plants to develop new sinks or expand existing ones.

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Introduction

Global atmospheric [CO₂] and other greenhouse gases are increasing due to human activities. Through data gathered from ice core studies, it has been possible to construct climate models from the Paleolithic era, from which it has been possible to characterize the composition of the atmosphere over the last 250,000 years and the changing levels of CO₂. These models have shown that [CO₂] were 30–50% lower than currently (between 180 and 260 μmol mol^{−1}) and that atmospheric [CO₂] had remained stable in the period from 150 to 1200 years ago standing at around 260 μmol mol^{−1} (Jouzel et al., 1993; Cowling and Sage, 1998). Since

the Industrial Revolution, increases in atmospheric [CO₂] have been produced at an alarming rate and currently, [CO₂] stand at around 398 μmol mol^{−1} (NOAA-ESRL, 2014). Increases in atmospheric [CO₂] are expected to continue into the future due to the burning of fossil fuels and biomass (Pagani et al., 1999; Pearson and Palmer, 2000) and by the end of this century, according to predictions using multi-model averages, atmospheric [CO₂] will have reached 985 ± 95 ppm (IPCC, 2013). This change in the composition of greenhouse gases is producing effects on the climate around the world and for that reason, it is of the utmost importance to study how plants have adapted from pre-industrial to current CO₂ levels. Knowledge of these adaptations may help us to better understand how plants will respond to future increases in CO₂ levels (Prentice et al., 2001; Sage and Coleman, 2001; Nogués and Azcón-Bieto, 2013).

Specifically, photosynthesis in C₃ plants is usually affected by changes in [CO₂]. Moreover, there is a wide variation of responses to these changes in different species such as the acclimation of photosynthesis to different atmospheric [CO₂] after a long period of exposure (Aranjuelo et al., 2009a,b, 2011a,b; Pardo et al., 2009). Acclimation is the physiological adjustment carried out by plants in response to a given level of CO₂, where photosynthesis can either decrease (down-regulation) in response to high [CO₂] or increase (up-regulation) in response to low [CO₂] through adjustments made to the photosynthetic machinery (Sage, 1994; Anderson et al., 2001; Nogués and Azcón-Bieto, 2013).

Abbreviations: A_{max}, light and CO₂-saturated net assimilation rate; A_{sat}, light-saturated net assimilation rate; cm, centimeter; F_v/F_m, maximum quantum yield of PSII; F_v/F_m, efficiency of the capture of excitation energy by open PSII reaction centers; g_s, stomatal conductance; HI, Harvest Index; ITE, instantaneous transpiration of efficiency; J_{max}, rate of photosynthetic electron transport; Ns, number of spikelets per spike; PSII, Photosystem II; Φ_{PSII}, relative quantum yield of PSII; q_p, photochemical quenching; q_n, non-photochemical quenching coefficient; NPQ, non-photochemical quenching; L, leaf; R, root; R_n, dark respiration; S, spike; SL, spike length; SN, spike number; St, stem; StL, stem length; StN, stem number; TFA, total flag area; TLA, total leaf area; TSA, total spike area; TStA, total stem area; V_c, maximum carboxylation velocity of Rubisco.

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Many studies suggest that the influence of low CO₂ during pre-industrial periods may have affected plants at many different levels ranging from the physiological effects on plants to changes in how ecosystems functioned, and may even have had an influence in the development of agriculture (Ward et al., 2000; Gerhart and Ward, 2010). Some studies have even shown increases in photosynthesis (up-regulation) in plants subjected to pre-industrial [CO₂] (Sage and Reid, 1992; Sage, 1994; Cowling and Sage, 1998; Anderson et al., 2001).

Furthermore, it has been observed in many other studies how plants increase photosynthetic rates in response to future [CO₂] in short-term experiments. However, in long-term experiments at future [CO₂], it has been shown that plants respond through a process of acclimation of photosynthesis with decreases in photosynthetic rates (down-regulation) (Long et al., 2004; Leakey et al., 2004). One of the parameters that can affect photosynthetic down-regulation is the modification of the source-sink ratio (Urban, 2003). Many studies suggest that down-regulation is the consequence of insufficient plant sink capacity (Thomas and Strain, 1991; Aranjuelo et al., 2009a,b; Sanz-Sáez et al., 2010). When plants which are exposed to future [CO₂] have limitations in terms of increasing the C sink strength, these plants decrease their photosynthetic rates to balance the C source with its sink. The ability of a plant to develop new sinks (e.g. new vegetative or reproductive structures and/or enhanced respiratory rates) or to expand the storage capacity or growth rate of existing sinks condition photosynthesis and lead to down-regulation. For instance, if plants increase carbohydrate production associated with future [CO₂], they exceed the capacity to make new sinks available and net photosynthetic rates may decline in order to balance the source activity with the sink capacity (Thomas and Strain, 1991).

Studies at pre-industrial [CO₂] allow for the characterization of the effects of limited CO₂ on physiological growth and reproductive processes (Gerhart and Ward, 2010). However, to date not many studies have been carried out using traditional genotypes and it is becoming increasingly necessary to identify, understand and quantify the mechanisms associated with crop responses to future [CO₂] (Aranjuelo et al., 2013).

Wheat is one of the most important cereal food crops in the modern world. Modern wheat genotypes were improved through plant breeding in the last century where the greatest increases in capacity were seen in reproductive organs and Harvest Index (HI) (grain production). In contrast, traditional genotypes have more vegetative production and a lower HI. Also, productivity and quality vary considerably as a result of environmental conditions during grain filling. And in addition to this, climate changes associated with the continued emission of CO₂ will bring about changes in land suitability and crop yields (IPCC, 2008, 2013). In particular, these negative impacts are predicted to be greater for wheat than for any other crop (IFPRI, 2008, 2013).

As was pointed out before, improvement of the quality of the grain and the HI of wheat are key objectives in crop improvement programs. Two types of sources contribute photoassimilates to the process of grain filling in wheat. These are current photoassimilates which are transferred directly from green tissues to the grain and photoassimilates which are redistributed from reserve pools in vegetative tissues (i.e. leaves, stems and roots). Further, the use of photoassimilates depends on the different genotypes and environmental factors such as increased CO₂. However, the mechanisms that control the partitioning of photoassimilates between the grain and reserve pools and the allocation to different types of reserve pools is not yet well understood (Schnyder, 1993).

With regard to the role of nitrogen in plants it accounts for less than 1% of dry biomass in plants, it is an essential element for life and that which most often limits plant growth in many terrestrial ecosystems (Vitousek, 1994). Extensive evidence that

nitrogen limits the growth response of plants at future [CO₂] has been demonstrated in many experiments conducted in controlled environmental chambers and under field conditions in free air CO₂ enrichment (FACE) experiments (Ainsworth and Long, 2005; Rogers et al., 2006; Bloom et al., 2014).

The primary objective of our study was to characterize the behavior of two genotypes of durum wheat (traditional and modern) and the variation of responses exhibited in terms of assimilation, growth and reproduction at pre-industrial (260 μmol mol⁻¹), current (400 μmol mol⁻¹) and future predicted [CO₂] (700 μmol mol⁻¹). This may help us to understand how plant species adapted in the past to pre-industrial [CO₂] and may be important in determining the potential of plants to evolve in response to rising [CO₂]. In addition to the primary objective, this study aimed to improve our understanding of the processes of up and down-regulation of photosynthesis in these plants during the grain filling stage.

Materials and methods

Plant material

Two durum wheat genotypes (*Triticum turgidum* ssp. *durum* Desf. var. Sula and var. Blanqueta) were used in this experiment, both of which are cultivated in Spain. Blanqueta is a land race that was widely grown in Sicily and the west of Spain in the first half of the last century. Nowadays, it is grown in small areas mainly to satisfy local consumers who appreciate the sensorial properties of its products. It is characterized by its tall stature, high tillering capacity, medium-late heading and maturity, moderate productivity, and good adaptability to environments characterized by scarce water and nutrient resources. Sula (released in 1994) is a modern and commercially grown genotype in Spain. It is characterized by its short stature, early heading and maturity and high yield potential.

Seeds of the two wheat genotypes were germinated in Petri dishes on wet Whatman paper. After 84 h, seedlings were transferred to 4-l pots (one plant per pot) filled with quartz sand of 1 mm grain size.

Experimental design

Plants were grown in three fully controllable plant-growth chambers (Convion 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 (PPDF) of about 400 ± 30 μmol m⁻² s⁻¹ during a 16 h light period (day) and then 8 h dark period (night). Plants were watered with Hoagland complete nutrient solution (Arnon and Hoagland, 1939) and alternated with distilled water every other time in order to avoid salt accumulation over the whole life cycle. Pots were kept at 100% of water field capacity and were refilled depending on the needs of the plants and the Zadock phenological stage. Humidity, temperature and [CO₂] 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 min and compared every two weeks with separate sensors (HMP75: humidity and temperature, and GMP222: 0–2000 μmol mol⁻¹ carbon dioxide. Vaisala MI70 Helsinki, Finland) in order to maintain a complete record of environmental parameters.

The plants were grown in three plant-growth chambers under three different [CO₂] (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 $[\text{CO}_2]$ (ca. $700 \pm 18 \mu\text{mol mol}^{-1}$) 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 $[\text{CO}_2]$ (ca. $400 \pm 20 \mu\text{mol mol}^{-1}$). Finally, the same number of plants was located in the third plant-growth chamber, which was maintained at pre-industrial $[\text{CO}_2]$ (ca. $260 \pm 28 \mu\text{mol mol}^{-1}$). Air in this chamber was maintained at pre-industrial $[\text{CO}_2]$ by using a pump to send the air inside the chamber through a 1-l 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.

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

Gas exchange and chlorophyll fluorescence measurements

An infrared gas analyzer (LI-6400 system, LI-COR Inc., Lincoln, NB, USA) supplied with a Leaf Chamber Fluorometer (LI-6400-40) was used to perform simultaneous measurements of gas exchange and chlorophyll fluorescence. $A-C_i$ curves with chlorophyll fluorescence determinations were conducted in fully expanded flag leaves from each treatment of CO_2 and for each genotype, Sula and Blanqueta. The $A-C_i$ curves were repeated in four different plants per treatment and genotype, and were measured from 0 to $2000 \mu\text{mol mol}^{-1}$ of CO_2 . The curves were made at $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetic active photon flux density and at a temperature of 25°C . CO_2 assimilation rate (A), CO_2 assimilation at light saturated net (A_{sat}), the maximum photosynthetic rates at CO_2 saturated net (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 ($V_{\text{c,max}}$), the rate of photosynthetic electron transport based on NADPH requirement (J_{max}) and the rate of day respiration (R_d) were made by fitting a maximum likelihood regression below and above the inflexion of the $A-C_i$ response using the method by McMurtrie and Wang (1993).

Modulated chlorophyll fluorescence measurements were determined in the flag leaf after 30 min of dark adaptation. These 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 centers (F_v'/F_m'), the maximum quantum yield of PSII (F_v/F_m), photochemical quenching (q_p), non-photochemical quenching coefficient (q_N) and non-photochemical quenching (NPQ) (Nogués and Baker, 2000).

Instantaneous transpiration of efficiency (ITE) was calculated for samples as Assimilation/stomatal conductance ($\text{ITE} = A/g_s$).

Leaf nitrogen content and nitrogen use efficiency

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 percentage of C and N contents 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 N content (g)/dry weight (g).

Rubisco and protein determination

Total soluble protein content (TSPC) was determined using the Bradford method (Bradford, 1976). 100 mg of frozen leaf was

ground with PBS solution and was centrifuged at $13,000 \times g$ number for 5 min. An aliquot of each extract was used to measure soluble protein by spectrometry, with reference to a standard line which was calculated with BSA (Bovine Serum Albumine). Another aliquot of the same extract was used for protein separation using an acrylamide gel SDS-PAGE. Gel images were scanned and analyzed using the Motic Images Plus 2.0 program. The concentration of Rubisco Large (L) and Small (S) subunit was measured against a Rubisco standard protein (Bio-Rad).

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 and during the three measuring periods (i.e. pre-anthesis, T0; grain filling, T1; and the end of grain filling, T2). Plant material was dried in an oven at 80°C for over 48 h to obtain the dry weight. The areas of flag leaves (TFA), other leaves (TLA), spikes (TSA) and stems (TStA) were determined using a scanner (Hewlett-Packard scanJet model IIcx, San Diego, USA) and images were measured with the software, Image (University of Sheffield, 2003). The number of spikes (SN), spikelets per spike (NsS) and stems (StN), length of spike and stem (StL), and Zadok phenological stage were also measured.

Data analysis

The effects of CO_2 on plant development in both species were tested by two-factor (CO_2 treatment and durum wheat genotype) analyses of variance (ANOVA). The statistical analysis was conducted with the SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA). The means \pm standard errors (SE) were calculated for each parameter. When a particular test was significant we compared the means using a Duncan multiple comparison test. The results were accepted as significant at $P < 0.05$.

Results

Growth parameters

Analyses of growth parameters showed that Blanqueta had more vegetative biomass (i.e. flag, stems, roots, leaves) but less reproductive biomass (spikes) than Sula. We found significant differences between the two genotypes in terms of the number of spikelets per spike (NsS) and spike biomass (SN), stem number (StN), stem length (StL), biomass (St) and total stem area (TStA), leaf biomass (L) and total leaf area (TLA) and root biomass (R) (Fig. 1; Table S1). No large differences were observed in other biomass parameters between genotypes, i.e. spike length (SL) or flag weight (F) (Table S1). Blanqueta showed a lower HI (0.26; 0.24; 0.31) than Sula (0.56; 0.54; 0.55) in future, current and pre-industrial $[\text{CO}_2]$ conditions respectively (data not shown) with significant differences between genotypes ($P = 0.001$) but not between CO_2 treatments ($P = 0.191$).

The CO_2 treatments also had an effect on plants and significant differences were found in StL, S, L and R biomass, TSA, TLA and TStA (Table S1). However, at future $[\text{CO}_2]$ those differences were clearer in Sula after the grain filling period in terms of SN, SL, S and R weight. In Blanqueta, the effects of $[\text{CO}_2]$ were not as great as those observed in Sula in terms of S, L, St and R weight, TSA, TLA and TStA. On the other hand, Blanqueta showed more down-regulation of photosynthesis at future $[\text{CO}_2]$ than did Sula and at current $[\text{CO}_2]$, the effects on biomass were not significant. A similar effect was found in Sula plants grown at pre-industrial $[\text{CO}_2]$ (Fig. 1; Table S1).

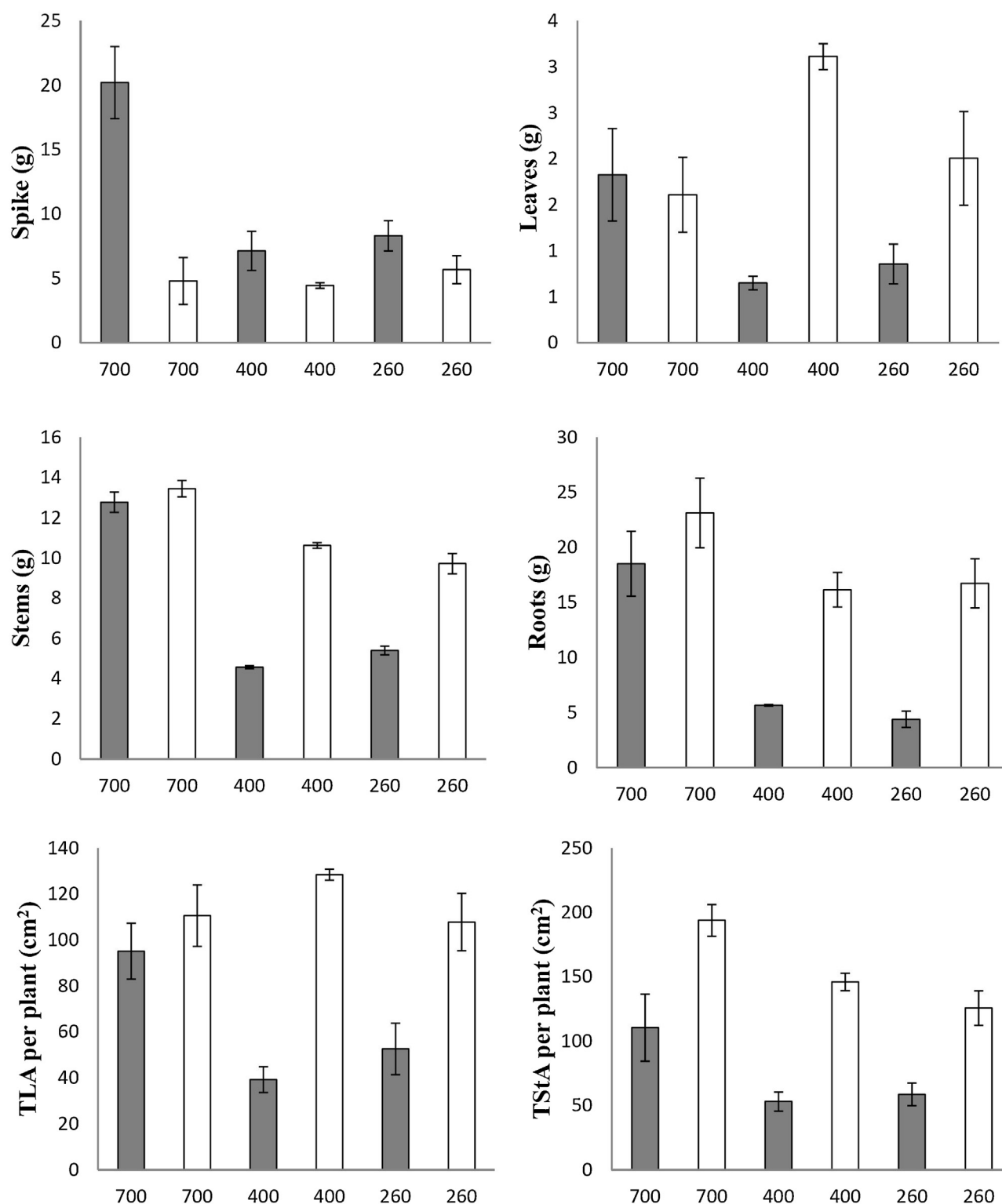


Fig. 1. Total spike biomass per plant (g), total leaf biomass per plant (g), total stem biomass per plant (g), total root biomass per plant (g), total leaf area per plant TLA (cm²) and total stem area per plant TStA (cm²) in durum wheat genotypes Sula (gray bars) and Blanqueta (white bars) under three CO₂ growth conditions (future 700 μmol mol⁻¹, current 400 μmol mol⁻¹ and pre-industrial 260 μmol mol⁻¹) during the end of grain filling, T2. Statistical analyses are presented in Table S1. Data are means ± SE, n = 4.

Gas exchange and chlorophyll fluorescence analysis

Non-significant differences were found in $V_{c,max}$, J_{max} , A_{max} and A_{sat} . However, at future [CO₂], we observed that before anthesis and after the grain filling (T0 and T2), carboxylation activity was diminished as indicated by the reduction in A_{max} and A_{sat} in

both genotypes (Table 1). Furthermore, reductions in $V_{c,max}$ and J_{max} were also found. However, during T1, all of these parameters showed a large increase and values were higher at future [CO₂] than at current [CO₂]. This increase was more elevated in modern plants as the spike weight is much greater than in traditional plants. During T2, the decrease in $V_{c,max}$ and A_{sat} in Sula is greater

Table 1

Q8 Physiological parameters in durum wheat genotypes (Sula and Blanqueta) at three CO₂ growth conditions (700, 400 and 260 μmol mol⁻¹) and three measuring periods (pre-anthesis, T0; grain filling, T1; and end of grain filling, T2).

CO ₂	Genotype	Period	V _{c,max}	J _{max}	A _{sat}	A ₃₆₀	A _{max}	C _i /C _a	R _d
700 μmol mol ⁻¹	Blanqueta	T0	77.0 ± 12.2	126.1 ± 9.1	14.4 ± 1.5	16.7 ± 1.5	23.0 ± 1.3	0.592 ± 0.026	-2.0 ± 0.24
		T1	96.6 ± 3.2	220.2 ± 6.3	18.1 ± 1.6	24.2 ± 2.7	32.3 ± 1.2	0.684 ± 0.036	-2.0 ± 0.14
		T2	104 ± 9.2	215.8 ± 20.2	20.7 ± 2.0	25 ± 2.1	33.5 ± 2.9	0.674 ± 0.021	-2.2 ± 0.69
	Sula	T0	68.9 ± 5.1	166.4 ± 4.5	15.2 ± 0.8	19.6 ± 0.2	28.4 ± 0.8	0.66 ± 0.016	-3.4 ± 0.28
		T1	132.2 ± 14.6	296.1 ± 56.1	27.3 ± 2.1	32.2 ± 2.8	41.8 ± 4.0	0.731 ± 0.006	-3.2 ± 0.42
		T2	83.4 ± 3.8	232.1 ± 12.2	16.0 ± 1.0	21.4 ± 1.5	39.1 ± 1.9	0.726 ± 0.017	-2.2 ± 0.29
400 μmol mol ⁻¹	Blanqueta	T0	80.6 ± 14.2	171.9 ± 36.6	17.4 ± 3.0	21.4 ± 3.3	28.9 ± 5.2	0.485 ± 0.009	-4.7 ± 0.87
		T1	90.9 ± 4.7	223.4 ± 5.3	17.9 ± 4.1	24.8 ± 2.3	36.4 ± 2.0	0.515 ± 0.006	-3.7 ± 0.13
		T2	109.9 ± 5.7	235.7 ± 9.6	22.0 ± 0.0	28.9 ± 0.8	35.6 ± 2.4	0.62 ± 0.009	-1.8 ± 0.27
	Sula	T0	84.2 ± 15.4	144.3 ± 30.9	18.8 ± 2.4	22.5 ± 3.1	26.5 ± 3.7	0.743 ± 0.007	-3.4 ± 0.61
		T1	92.4 ± 3.3	204.1 ± 24.6	20.6 ± 1.4	25.4 ± 1.6	34.4 ± 4.0	0.56 ± 0.007	-2.0 ± 0.04
		T2	50.9 ± 14.3	107.3 ± 15.5	10.5 ± 3.7	13.2 ± 2.3	21.8 ± 4.3	0.735 ± 0.008	-0.9 ± 0.19
260 μmol mol ⁻¹	Blanqueta	T0	88.5 ± 17.9	181.5 ± 41.8	18.4 ± 4.9	22.7 ± 5.9	28.7 ± 4.3	0.623 ± 0.006	-2.8 ± 0.16
		T1	118.7 ± 2.3	235.5 ± 1.2	22.9 ± 2.6	27.3 ± 1.8	37.1 ± 1.7	0.55 ± 0.002	-1.9 ± 0.01
		T2	103.5 ± 8.0	196.8 ± 34.1	24.1 ± 1.4	27.6 ± 2.6	31.8 ± 2.6	0.557 ± 0.007	-1.3 ± 0.18
	Sula	T0	74.3 ± 3.6	148.2 ± 20.3	18.5 ± 0.0	22.0 ± 0.5	26.8 ± 0.7	0.742 ± 0.006	-1.3 ± 0.06
		T1	104.0 ± 3.7	197.3 ± 13.6	22.9 ± 0.5	26.6 ± 1.2	33.8 ± 2.0	0.723 ± 0.005	-1.3 ± 0.25
		T2	80.8 ± 20.4	157.2 ± 34.8	23.0 ± 4.7	26.8 ± 6.0	32.7 ± 6.7	0.742 ± 0.004	-1.4 ± 0.18
CO ₂ treatment		0.452	0.472	0.074	0.365	0.680	0.435	0.061	
Genotype		0.690	0.783	0.263	0.421	0.138	0.216	0.153	
CO ₂ treatment × genotype		0.378	0.092	0.666	0.523	0.123	0.118	0.094	

ANOVA Duncan (ns, non-significant; * < 0.05; ** < 0.01; *** < 0.001).

V_{c,max}, maximum carboxylation velocity of Rubisco; J_{max}, the rate of photosynthetic electron transport; A_{sat}, assimilation rate at light saturation; A₃₆₀, assimilation rate at C_i of 360; A_{max}, maximum assimilation rate at light and CO₂ saturation; C_i/C_a, internal CO₂/ambient CO₂ ratio; R_d, dark respiration.

than in Blanqueta (Table 1). At current [CO₂], Sula showed higher V_{c,max}, A_{sat}, A_{max} and lower J_{max} than Blanqueta. Nevertheless, during grain filling some of those parameters such as V_{c,max}, A_{sat}, A_{max} were higher at pre-industrial than current [CO₂], thereby showing up-regulation of photosynthesis. A decrease in the respiration rate was also observed when [CO₂] decreased and during grain filling, however, differences were not significant (P = 0.061). Also, no differences were found between genotypes.

In relation to the acclimatory effects induced in PSII at future and pre-industrial [CO₂], these were studied by means of chlorophyll fluorescence measurements. Future [CO₂] caused the increase in the F_v/F_m, NPQ and q_N and the decrease in the Φ_{PSII}, the F_v'/F_m' and the q_p at current [CO₂]. On the other hand, at pre-industrial [CO₂], with respect to current [CO₂] decreases in F_v/F_m, Φ_{PSII} and q_p, and increases in NPQ and q_N were observed (Fig. 2). There were no significant differences between modern and traditional genotypes in terms of chlorophyll fluorescence parameters at different CO₂ treatments. Only in the case of pre-industrial [CO₂] did the modern genotype have lower photochemical efficiency and higher NPQ than the traditional genotype (Fig. 2).

Instantaneous transpiration efficiency (ITE)

Although no significant differences in ITE between CO₂ treatments were observed, there were significant differences between genotypes (P < 0.05). Lower ITE in Sula was directly proportional to lower levels of CO₂ suggesting that the ability to scale this response may be relatively straightforward. On the other hand, stomatal conductance (g_s) decreased when [CO₂] increased in the environment. Differences in ITE in Sula were 44, 18 and 22% less than in Blanqueta in 260, 400 and 700 μmol mol⁻¹ respectively. The ITE of Blanqueta was greater at pre-industrial than at current [CO₂] (Fig. 3).

Leaf nitrogen content and nitrogen-use efficiency

At future [CO₂], plants have less N concentration in leaves (32 and 37% for Blanqueta and Sula respectively) than at current [CO₂], whereas at pre-industrial [CO₂] plants were seen to have a

higher N concentration (40 and 50% for Blanqueta and Sula respectively). NUE was lower in plants maintained at 400 μmol mol⁻¹ than in plants at 700 μmol mol⁻¹ but was greater in plants at 260 μmol mol⁻¹. In addition, Sula had lower levels of NUE than Blanqueta at all [CO₂]. Furthermore, these differences were much greater at pre-industrial and current [CO₂] than at future [CO₂]. Specifically, NUE in Sula at 260 and 400 μmol mol⁻¹ was 76% lower than Blanqueta whereas at future [CO₂] it was only 7% lower (Fig. 4).

Rubisco and protein determinations

Two patterns of leaf protein changes were observed in this study. On the one hand, protein concentration levels were lower in plants at future [CO₂] than at current [CO₂] (22% for Sula and 31% for Blanqueta) whereas levels were greater at pre-industrial [CO₂] than at current [CO₂] (20% for Sula and 29% for Blanqueta). On the other hand, levels of protein concentrations were lower in Sula than Blanqueta across all CO₂ treatments (Table S2A).

Both genotypes showed greater concentrations of Rubisco at pre-industrial [CO₂], however, only in the case of Sula were concentrations of Rubisco lower at future [CO₂]. The biggest differences between CO₂ treatments were observed in the small subunit of the protein (Table S2B).

Discussion

The agronomic and physiologic parameters of two genotypes of durum wheat, Sula (modern) and Blanqueta (traditional) were characterized in order to study the response of wheat in pre-industrial, current and future [CO₂].

At the beginning of the experiment, it was seen that at future [CO₂] there was an increase in C assimilation and growth parameters of plants, however, at pre-industrial [CO₂] a decrease in all these parameters was observed. Nevertheless, after a few weeks, we found that plants underwent an acclimation process at different [CO₂]. Acclimation is the physiological adjustment carried out by plants where photosynthesis can decrease with elevated

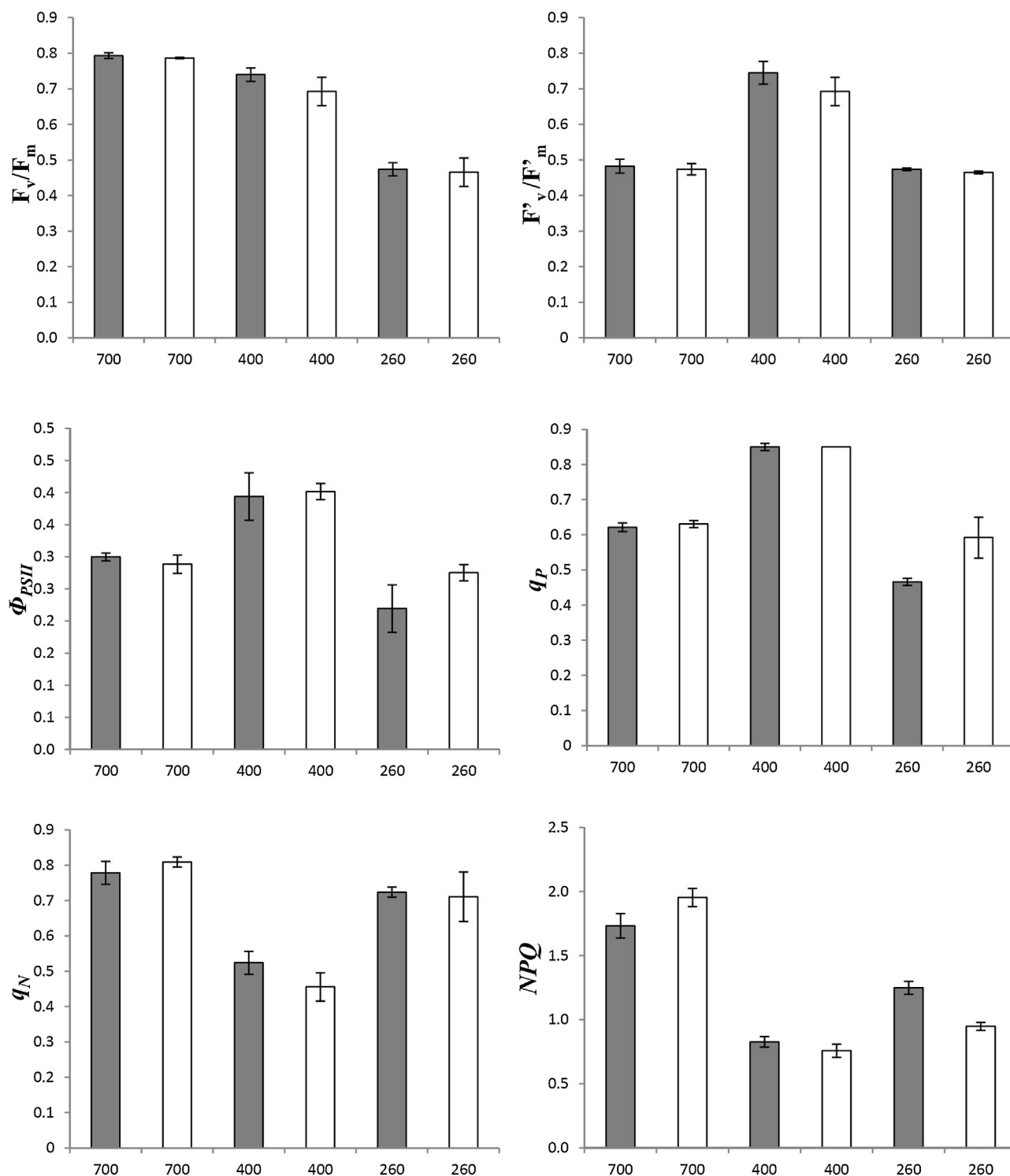


Fig. 2. Fluorescence parameters (F_v/F_m : maximal photochemical efficiency in the dark-adapted stage; Φ_{PSII} : quantum yield of Photosystem II electron transport; F_v'/F_m' : maximal photochemical efficiency in light; q_P : photochemical quenching; NPQ : non-photochemical quenching, and q_N : non-photochemical quenching coefficient) in durum wheat genotypes Sula (gray bars) and Blanqueta (white bars) under three CO₂ growth conditions (future 700 $\mu\text{mol mol}^{-1}$, current 400 $\mu\text{mol mol}^{-1}$ and pre-industrial 260 $\mu\text{mol mol}^{-1}$) during the end of grain filling, T2. Statistical analyses are presented in Table S4. Data are means \pm SE, $n=4$.

[CO₂] (down-regulation) or increase with low [CO₂] (up-regulation) (Lehmeier et al., 2005).

The two genotypes showed significant changes in the response of vegetative and reproductive biomass from pre-industrial to future atmospheric [CO₂] (Fig. 1). Traditional genotypes have more vegetative biomass, but in contrast the reproductive capacity is lower than in modern genotypes (i.e. low HI; Aranjuelo et al., 2013).

Data suggest that the Sula genotype has a large capacity to create new sinks during grain filling and can accumulate a large quantity of reserves mainly in spikes, but also in stems and/or roots during this period (Ward and Strain, 1997). Grain filling is mainly conditioned by two factors: (i) the genetic makeup of the plant (modern genotypes have been enhanced genetically so that they can invest more in reproductive parts and less in vegetative parts, Reynolds

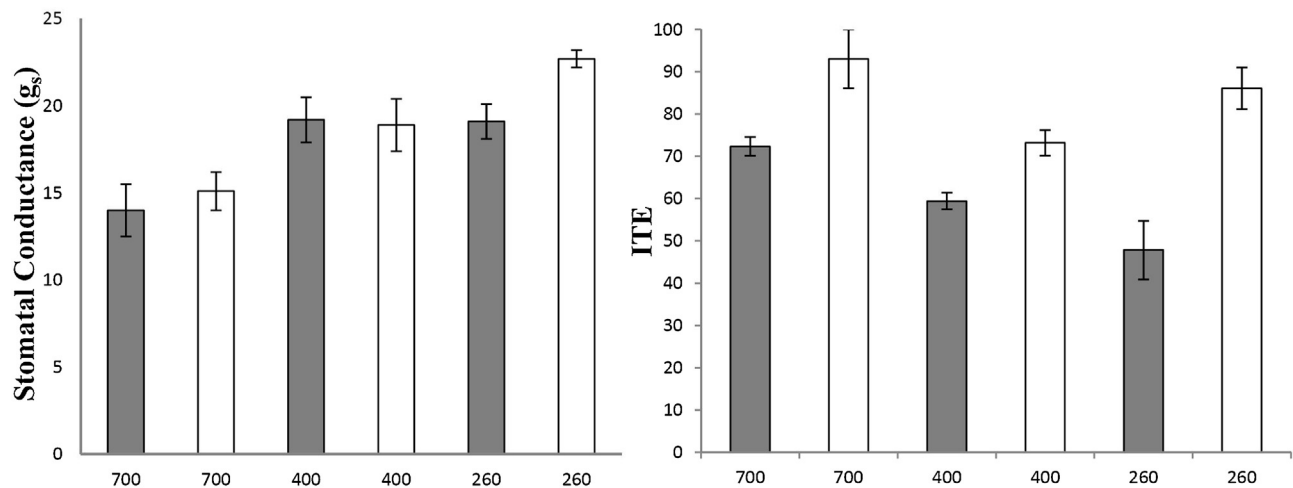


Fig. 3. CO₂ effects (700, 400 and 260 μmol mol⁻¹) on stomatal conductance (gs) and Instantaneous transpiration of efficiency (ITE) in durum wheat genotypes Sula (gray bars) and Blanqueta (white bars). Statistical analyses are presented in Table S4. Data are means ± SE, $n = 4$.

et al., 1999) and (ii) the availability of C and N to plants (Fuertes-Mendizabal et al., 2012). A priority of wheat breeding programs (e.g. in Spain since the 1940s) has been to increase yields and drought resistance. Therefore, modern crops in Mediterranean areas have been selected (i) to strengthen the reproductive organs and (ii) to avert the dry period at the end of the crop life (Royo and Briceño-Félix, 2011) i.e. to shorten the life cycle of the crop as is the case for the modern Sula genotype. In our experiment, Sula plants showed higher production of seeds and a shorter life cycle. During the end of grain filling (T2), Sula decreased photosynthetic parameters faster than Blanqueta (the traditional genotype). This is explained by the fact that Sula has a shorter life cycle and faster grain filling capacity than Blanqueta.

Currently, photosynthetic acclimation to CO₂ is one of the most important issues in CO₂ studies (Sage and Coleman, 2001) and this acclimation at pre-industrial [CO₂] (increases in photosynthesis, i.e. up-regulation) and future [CO₂] (decreases in photosynthesis, i.e. down-regulation) during long-term exposure can compensate for the effects of CO₂ variation on plant processes. Many studies have shown that photosynthesis decreases at future [CO₂] over long-term experiments through a down-regulation process (Ainsworth et al., 2003; Ainsworth and Rogers, 2007; Pardo et al., 2009; Sanz-Sáez et al., 2010) as was observed here (Table 1). At future [CO₂], Blanqueta showed a greater degree of photosynthetic

down-regulation and a lower capacity to create new sinks during grain filling. However, in the modern genotype at future [CO₂] and during grain filling there was both, an elevated source of C and a newly developed and substantial C sink in terms of the spike. Thus, Sula averted photosynthetic acclimation and increased assimilation by sending more carbohydrates to the spike (Aranjuelo et al., 2009a,b). After grain filling, assimilation decreased again by means of photosynthetic down-regulation, firstly, as a result of the plant not having the carbon sink to store its assimilation products and, secondly, because it had reached the end of its life cycle. On the other hand, at current or pre-industrial [CO₂], Sula demonstrated a lower capacity to increase the biomass of spikes and roots. Clearly, the data suggests that growth responses and photosynthetic rates at future [CO₂] will depend on the ability of plants to develop new sinks (e.g. new vegetative or reproductive structures, and/or enhanced respiratory rates) and/or expand the storage capacity or growth rate of existing sinks. Moreover, the expansion of new sinks such as spikes also depends on C availability and the genetic makeup of the plant (as previously mentioned). For that reason, during grain filling the photosynthetic rate increased much more in Sula than in Blanqueta at future [CO₂] since Sula has been shown to have the capacity to expand its sinks as previously mentioned (Table 1; Aranjuelo et al., 2009a,b; Sanz-Sáez et al., 2010). This is because, firstly, there were no effects on biomass and

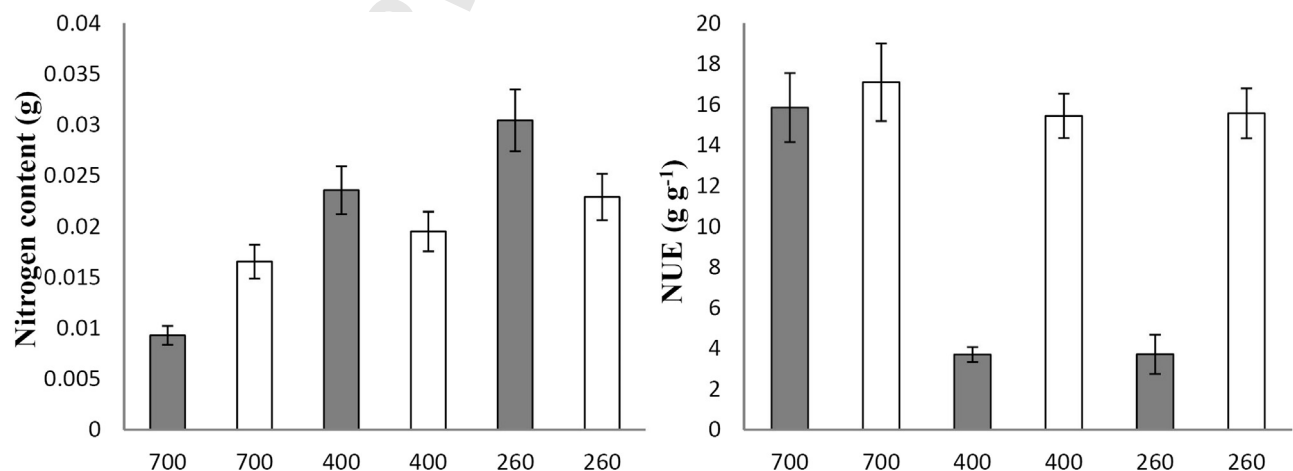


Fig. 4. CO₂ effects (700, 400 and 260 μmol mol⁻¹) on N content (g) and nitrogen use efficiency (NUE) in durum wheat plants genotypes Sula (gray bars) and Blanqueta (open bars) during grain filling. Statistical analyses are presented in Table S4. Data are means ± SE, $n = 4$.

assimilation in traditional plants and, secondly, the aversion of the down-regulation of photosynthesis in modern genotypes during grain filling at future [CO₂] suggests that the spike (as a new C sink) has an important role to play in this process.

As stated previously, our data suggested that photosynthetic down-regulation is the consequence of insufficient plant sink capacity, but is also due to a decrease in Rubisco concentration (i.e. the consequence of the decrease in C assimilation). Other authors have shown that at elevated [CO₂] plants had less Rubisco and photosynthesis was down-regulated (Sticher and Bunce, 1997; Moore et al., 1998, 1999; Urban, 2003; Pandurangam et al., 2006; Aranjuelo et al., 2011a,b; Aranjuelo et al., 2013). In our case, at future [CO₂] Sula plants had lower levels of Rubisco than at current [CO₂], but the opposite was true for Blanqueta. The traditional genotype had a large vegetative biomass, however, the spike is not a large sink, thus at future [CO₂] Blanqueta showed no decrease in Rubisco and maintained low assimilation rates during grain filling. Many studies have shown that the pre-industrial CO₂ has a direct effect on photosynthesis and also on the reduction of assimilation since C is more limiting as a substrate for the carboxylation reaction in Rubisco (Long and Drake, 1992). In C₃ plants, at optimal temperatures and nutritional conditions, pre-industrial [CO₂] can lead to a reduction in photosynthetic capacity (Sage, 1995). However, the effect of pre-industrial [CO₂] can compensate for the acclimation processes of photosynthesis. Similar to our data, Anderson et al. (2001) reported an increase in V_{C,max} at pre-industrial [CO₂] for C₃ plants and observed an up-regulation of photosynthesis in response to pre-industrial [CO₂] with an increase in assimilation and also in concentration of Rubisco (Table 1). We found evidence in our study which showed that plants were up-regulated at pre-industrial [CO₂] and that both genotypes contained large Rubisco concentrations in both subunits (small and large) as mentioned above. Specifically, Sula had lower levels of Rubisco than Blanqueta. Rubisco levels can increase more in plants that undergo prolonged exposure to pre-industrial [CO₂] than those grown at current [CO₂] (Maherali et al., 2002). Gesch et al. (2000) observed increased Rubisco small subunit gene expression in *Oryza sativa* L. exposed to pre-industrial [CO₂]. Other studies have also found evidence for photosynthetic acclimation at pre-industrial [CO₂] (Sage and Reid, 1992; Cowling and Sage, 1998). In Sula at future [CO₂], leaf respiration acted as an important C sink. However, leaf respiration rates did not demonstrate down-regulation of respiration (as previously mentioned) during grain filling. In Blanqueta at future [CO₂], acclimation responses with lower rates of leaf respiration were observed, however, it was also possible to observe that respiration acts as a large C sink at both 260 and 400 μmol mol⁻¹ with higher rates of leaf respiration than at future [CO₂] (Table 1).

We could also observe the effect of [CO₂] treatments in chlorophyll fluorescence measurements, where it was shown that PSII activity is less affected at future than at pre-industrial [CO₂]. Our data showed that plants decreased photosynthetic carbon metabolism through down-regulation, thus decreasing demand for electron transport to PSII and increasing non-photochemical quenching (Fig. 2). Damage to PSII did not occur at future [CO₂] (F_v/F_m is higher), therefore, at future [CO₂] decreases due to acclimation in carbon assimilation and Rubisco content (as mentioned above) may prevent increases in electron transport to the photorespiratory carbon oxidase cycle (Taub et al., 2000). However, at pre-industrial [CO₂], decreases in F_v/F_m can indicate greater damage in PSII since there is an increase in electron transport to photorespiratory processes which may result in ROS formation. However, the diminishment in Φ_{PSII} and q_p at future and pre-industrial [CO₂] suggests that the reduction of electron transport to PSII could have contributed to photosynthetic acclimation. Higher dissipation by non-photochemical quenching and q_N was observed at future and pre-industrial [CO₂] indicating that limitations in

carbon assimilation caused a decrease photochemical quenching. The highest rates of NPQ and q_N were observed at future [CO₂] suggesting that the energy which reaches the leaf was more non-photochemically dissipated and would serve to protect the reaction centers from photo-inactivation and damage when the rate of excitation of PSII is in excess of the rate of photochemistry. This in turn would also help protect PSII (Hymus et al., 2001). Also, q_N indicates that photoprotective energy dissipation in the xanthophyll cycle occurs as heat emission. Plants at future [CO₂] had higher q_N than plants at pre-industrial [CO₂]. Thus, there was greater protection of PSII at future [CO₂]. At pre-industrial [CO₂], the values of q_N (and also NPQ) decreased because the energy dissipated as heat in the xanthophyll cycle was lower and there was a greater production of ROS (Maxwell and Johnson, 2000; Muller et al., 2001; Demming-Adams, 2003). At pre-industrial CO₂, the dissipation of energy as heat was not very efficient (with lower values of NPQ and q_N than at future [CO₂]) and furthermore, F_v/F_m and Φ_{PSII} decreased which suggested that plants suffered more damage in reaction centers at pre-industrial than at future [CO₂].

In terms of the potential of plants and leaves to avoid stress, this can be indicated by ITE (Farquhar and Sharkey, 1982). The exchange of water vapor and CO₂ is controlled mainly by the stomatal aperture. This, as well as conductance of the leaf depend on irradiance, temperature, air humidity and internal [CO₂] (Kutsch et al., 2001). Plant ITE and stomatal aperture is strongly dependent on atmospheric CO₂, this being lower at pre-industrial [CO₂] (Cowling and Sykes, 1999). Furthermore, Polley et al. (1993, 1995) observed that at pre-industrial [CO₂] *Triticum aestivum* had reduced ITE values. In contrast however, for the same plant species ITE was higher at future [CO₂] (Aranjuelo et al., 2011a,b). Experimental data indicate that ITE increases from pre-industrial to future [CO₂] (Polley et al., 1995; Gerhart and Ward, 2010). Moreover, plants can regulate stomatal conductance (g_s) in accordance with the [CO₂] in the environment. Plants subjected to pre-industrial [CO₂] tended to open more stomata than plants at future [CO₂] (increased stomatal closure and so conductance was lower) (Fig. 3). These results indicate that plants growing at pre-industrial [CO₂] need to keep the stomata open in order to assimilate more [CO₂]. Thus, plants have a lower ITE at pre-industrial than at future [CO₂]. On the other hand, Blanqueta increased its ITE at pre-industrial [CO₂] since plants were able to increase their assimilation rates.

The leaf is a major storage organ for nitrogen. At future [CO₂] nitrogen leaf content declined (32 and 17% for Blanqueta and Sula respectively) such that NUE increased (Fig. 4). Rubisco accounts for more than 50% of total soluble protein and over 25% of the total nitrogen of leaves (Makino et al., 1984; Hawkesford and Barraclough, 2011). Therefore, plants at future [CO₂] had less content in terms of Rubisco and proteins, and for the same reason, plants had less N content in leaves (Gutierrez et al., 2013). At pre-industrial [CO₂], plants were seen to have more N concentration (40 and 50% for Blanqueta and Sula, respectively) and lower NUE (Polley et al., 1995; Fig. 4). These data suggest that traditional plants have a higher NUE than modern genotypes since the vegetative part of the plant is a large N sink. However, in future conditions, modern genotypes have a higher NUE since the spike acts as a large sink, thereby averting the acclimation of photosynthesis and increasing assimilation, biomass and NUE.

Conclusions

In this paper, we have shown the effects of pre-industrial and future [CO₂] after long-term exposure on two durum wheat genotypes (traditional and modern). Plants underwent acclimation processes under long-term exposure at future [CO₂] resulting in a reduction in photosynthesis (down-regulation). The lower capacity

of the traditional genotype to increase the size of new sinks during grain filling resulted in a lower photosynthetic rate than that of the modern genotype. Sula plants during grain filling did not show photosynthetic down-regulation because they developed a new sink (i.e. spikes), and could therefore increase the assimilation rate. Our modern genotype showed that photosynthesis had a higher capacity to adapt at future $[CO_2]$. This could be explained by the fact that it has been bred to increase the spike capacity and HI, whereas the traditional genotype has not selected for this. The pre-industrial $[CO_2]$ treatment decreased growth and biomass production and/or leaf area, however these effects decreased with time, showing an eventual and clear up-regulation of photosynthesis. As such, acclimation processes have been shown to induce the adaptation of the regulation of Rubisco content (increasing at $260 \mu\text{mol mol}^{-1}$ and decreasing at $700 \mu\text{mol mol}^{-1}$) and leaf N content (higher at pre-industrial $[CO_2]$ and lower at future $[CO_2]$).

Future increases in atmospheric $[CO_2]$ may have positive effects on plants since they may increase growth and assimilation rates. However, these increases are greater in modern genotypes which have more carbon sinks. Furthermore, plants at future $[CO_2]$ have shown an increase in ITE, NUE and the protection of PSII as a result of increases in NPQ and q_N .

Therefore, in this study we have clearly shown that, while on the one hand there was photosynthetic acclimation of plants at pre-industrial and future $[CO_2]$, on the other hand, there was also variation between genotypes in terms of the response. This was demonstrated where modern plants acclimated more to future $[CO_2]$ (although acclimation was averted during grain filling) and Blanqueta acclimated more to pre-industrial $[CO_2]$. This implies that significant photosynthetic adjustments might have taken place in the past and will certainly take place in the future.

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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.jplph.2014.07.019>.

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