Once you have Acrobat Reader open on your computer, click on the [Comment](#) tab at the right of the toolbar:

This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the [Annotations](#) section, pictured opposite. We've picked out some of these tools below:

1. [Replace \(Ins\)](#) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
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- Type the replacement text into the blue box that appears.

standard framework for the analysis of microeconomic behavior. Nevertheless, it also led to the development of strategic form games. The number of competitors in the industry is that the structure of the game. The main components of the model, at the micro level, are exogenous variables and parameters. Important works on entry by Chamberlain (henceforth) we open the 'black box'.

2. [Strikethrough \(Del\)](#) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the [Strikethrough \(Del\)](#) icon in the Annotations section.

there is no room for extra profits as mark-ups are zero and the number of firms (set) values are not determined by the market. Blanchard and Kiyotaki (1987), in a perfect competition in general equilibrium model of aggregate demand and supply, the classical framework assuming monopoly power between an exogenous number of firms.

3. [Add note to text](#) Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the [Add note to text](#) icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

dynamic responses of mark-ups consistent with the VAR evidence.

sation by Markov. The number of competitors and the impact of the structure of the sector on the demand.

4. [Add sticky note](#) Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the [Add sticky note](#) icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

standard and supply shocks. Most of the standard framework for the analysis of microeconomic behavior. The number of competitors and the impact of the structure of the sector on the demand.

5. **Attach File** Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

How to use it

- Click on the **Attach File** icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
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END

6. **Drawing Markups** Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks. Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.

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- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

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Keywords

ABSTRACT

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The rapid increase in the concentration of atmospheric CO₂ due to the continued emission of this gas through anthropogenic activities is the main factor driving global climate change. Climate change models predict significant impacts on agriculture, with a decrease in crop yields by 2050 (IPCC 2007, 2013). In particular, climate change is expected to have a slightly larger negative impact on wheat than on other crops, as *e.g.* potato, maize and rice (IFPRI 2007, 2013).

The concentration of atmospheric CO₂ remained stable at 260 ppm for thousands of years prior to the Industrial Revolution, however, since then, CO₂ has steadily been accumulating in the atmosphere (Pagani *et al.* 1999; Pearson & Palmer 2000). Currently, CO₂ concentrations ([CO₂]) stand at around 397 ppm (NOAA-ESRL 2014), and atmospheric concentrations continue to rise year after year. Averages of multi-model predictions indicate that by the end of this century atmospheric CO₂ will have reached 985 ± 95 ppm (IPCC 2013). This is expected to result in increases in temperatures (up to 5 °C) and more frequent and prolonged periods of drought. Therefore, in order to provide answers as to how plants will adapt to future [CO₂], it is essential to understand how they have adapted from past to current CO₂ levels (Prentice *et al.* 2001; Sage & Coleman 2001).

In C₃ plants, photosynthesis is usually affected by changes in [CO₂]. Plants undergo a wide variety of changes in response to increases in [CO₂] and can acclimatise to different atmospheric

[CO₂] after prolonged periods of exposure (Aranjuelo *et al.* 2009b, 2011a; Pardo *et al.* 2009). Photosynthetic acclimation can be defined as the physiological adjustment of plants to a given [CO₂] where plants undergo increases in photosynthesis at pre-industrial [CO₂] (up-regulation) and decreases in photosynthesis at future [CO₂] (down-regulation) through adjustments to the photosynthetic machinery (Nogués & Azcón-Bieto 2013). These responses can also vary between species or varieties that have physiological or phenological differences, as is true in the case of modern varieties that were selected during the Green Revolution in order to obtain a higher harvest index (HI).

Many studies have shown that photosynthesis is up-regulated in plants subjected to pre-industrial CO₂ (Sage & Reid 1992; Sage 1994; Cowling & Sage 1998; Anderson *et al.* 2001). Studies of the effects of pre-industrial [CO₂] on plants are also fundamental in order to understand plant evolution in response to changes in CO₂ resource availability over time (Ward & Strain 1997; Ward *et al.* 2000; Gerhart & Ward 2010). Previous studies carried out in this area suggest that the influence of low [CO₂] during pre-industrial eras affected plants at many levels, ranging from physiological effects (*i.e.* photosynthetic acclimation) to changes in the functioning of ecosystems (*i.e.* the evolution of C₃ species; Dippert *et al.* 1995; Ward *et al.* 2000), and even played a major role in the emergence of agriculture (Gerhart & Ward 2010).

In short-term experiments (*i.e.* during only a short part of the plant life cycle), plants have shown increases in photosynthetic

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rate in response to future [CO₂]. However, in experiments with prolonged exposure to future [CO₂] (over a long period or the entire plant life cycle), in contrast, down-regulation of photosynthesis has been observed (Leakey *et al.* 2004; Long *et al.* 2004). One of the parameters that can affect photosynthetic down-regulation is modification of the source–sink ratio (Urban 2003). For example, if increases in carbohydrate production (source) associated with elevated [CO₂] exceed the capacity of the plant to produce new sinks, net photosynthesis rates may decline in order to balance the source activity with the sink capacity (Thomas & Strain 1991).

In terms of grain filling in wheat, two types of C source contribute to this process: (i) current photoassimilates are transferred directly to the grain from green tissues (mainly flag leaves and spikes), and (ii) photoassimilates are redistributed from reserve pools to vegetative tissues (leaves, stems and roots; Aranjuelo *et al.* 2011a). Moreover, the use of photoassimilates is influenced by the genotype and growth conditions, such as stress and/or increases in [CO₂] (Tambussi *et al.* 2007; Aranjuelo *et al.* 2009a,b).

Additionally, nitrogen frequently controls or limits plant growth in many terrestrial ecosystems (Vitousek 1994) and is tightly coupled with the leaf C cycle (Fisher *et al.* 2010). The relationship between C and N inputs and metabolism are further complicated by the dynamic exchanges between plant organs and the effects of the environment. Therefore, the appropriate balance of C and N between sink and source strengths will be an essential objective for maximising the response of cereals to growth under different C and N availability conditions (Aranjuelo *et al.* 2013). With this aim in mind, through the use of stable isotopes, the allocation and partitioning of C and N throughout the plant and between organs can be traced and studied. For instance, it was observed that plants at pre-industrial [CO₂] have stimulated the allocation of C towards leaves and shoots because of increasing C demand (Gerhart & Ward 2010). However, at future [CO₂] plants can invest more C in roots due to the increased C source availability (Ghashghaie & Tcherkez 2013). Another example is the higher requirement for N during grain filling in wheat (Fuentes-Mendizábal *et al.* 2012). N is remobilised from different parts of the plant (stored before grain filling) or new N is taken up in the same period (Dupont & Altenbach 2003). N content in plants is modulated by CO₂ and it is lower at future than at current or pre-industrial [CO₂] (Mitsutoshi *et al.* 2005). Explanations for this decline in N content under elevated [CO₂] include different processes: (i) dilution as a result of higher plant growth, (ii) down-regulation and lower protein content in the form of Rubisco induced by increases in carbohydrate, (iii) limitations in the N available to plants due to C enrichment of the rhizosphere, and (iv) inhibition of nitrate assimilation from the soil due to elevated [CO₂] (Bloom *et al.* 2014). Understanding the mechanisms controlling whole wheat plant N and C isotope composition will further advance our knowledge of the acquisition and allocation of N and C in plants under different climate scenarios (Farrar & Jones 2000; Fisher *et al.* 2010).

The ¹³CO₂ isotope labelling technique was used in this paper to study recently fixed C in wheat organs and the respiratory metabolism. This allowed calculation of the contribution of stored C *versus* current photoassimilates to the production of CO₂ through respiration (Schnyder *et al.* 2003; Nogués *et al.*

2004, 2014). Labelling with ¹³CO₂ permitted us to calculate the proportion of ‘new’ (*i.e.* recently fixed) C in total organic matter (TOM) and respired CO₂ (Nogués *et al.* 2004).

Similarly, the ¹⁵NH₄-¹⁵NO₃ isotope labelling technique is used to understand the N cycle in plants under ambient conditions (Robinson 2001). ¹⁵N can act as a powerful tool to assess whether processes in the N cycle are influenced by the increasing concentration of atmospheric CO₂. The IPCC (2013) predict that with climate change, there will be a reduction in N availability (N limitation) with increasing CO₂. A large part of the uncertainty in models predicting climate change feedbacks lies in the role of the N cycle in modulating the exchange of CO₂ between plants, the ecosystem and the atmosphere (Hungate *et al.* 2003). More importantly, the pattern of changes in plant δ¹⁵N in response to future [CO₂] could guide future studies in identification of the exact processes in the N cycle that respond to future climate change (BassiriRad *et al.* 2003).

The main objective of this study was to characterise the C and N allocation and its implications in terms of biomass, photosynthesis and reserves in traditional and modern wheat genotypes grown in pre-industrial, current and future CO₂ environments. To date, the mechanism conditioning C and N allocation responses to pre-industrial [CO₂] in wheat has not been sufficiently documented, and studying this may help us to understand the behaviour of plants in future climate change scenarios. In order to better understand C and N partitioning among the organs of these plants exposed to pre-industrial and future [CO₂], double labelling with ¹³CO₂ and ¹⁵NH₄-¹⁵NO₃ was conducted.

MATERIAL AND METHODS

Plant material

Wheat seeds were germinated in Petri dishes. After 4 days, seedlings were transferred to 4-l pots (one plant per pot) filled with quartz sand of 1-mm grain size. Plants were grown in three fully controllable plant growth chambers (Conviron E15; Controlled Environments Ltd, Winnipeg, MB, 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 ca. 400 μmol·m⁻²·s⁻¹ for a 16-h light period (day) and the remaining 8 h in darkness (night). Plants were watered with Hoagland complete nutrient solution. Each plant growth chamber was maintained at a different CO₂ level (*i.e.* 700, 400 and 260 ppm).

Two durum wheat genotypes (*Triticum turgidum* var. Sula and var. Blanqueta) were used in the 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, but which is now grown in small areas mainly to satisfy local consumers who appreciate the properties of this variety. It is characterised by its tall stature, high tillering capacity, medium to late heading and maturity, moderate productivity and good adaptability to environments characterised by scarce water and nutrient resources. Sula (released in 1994) is a modern and commercially grown genotype. It is characterised by its short stature, early heading and maturity and high yield potential. It is grown in Andalucía, Catalonia and Extremadura in Spain.

Experimental design

The humidity, temperature and [CO₂] in the chamber air were continuously monitored at 5-min intervals with a combined sensor (CMP3243; Controlled Environments Ltd.) and compared every 2 weeks with separate sensors (HMP75: humidity and temperature, and GMP222: for 0–2000 ppm CO₂; Vaisala MI70; Vaisala, Helsinki, Finland) in order to maintain a good characterisation of environmental parameters.

Plants were grown during the whole life cycle (from September to January) under three different levels of CO₂ (700, 400 and 260 ppm) at the Experimental Fields Service at the University of Barcelona, Barcelona, Spain. Forty-eight plants were placed in the first plant growth chamber and were maintained at a high (future) [CO₂] (ca. 700 ± 18 ppm) during the whole life cycle. In order to raise the CO₂ level in the chamber, commercial CO₂ (99.5% pure CO₂, without H₂O, O₂, N₂, CO or hydrocarbons) was used (Carburos Metálicos S.A. Barcelona, Spain). An infrared gas analyser (IRGA) connected to the chamber continuously monitored CO₂ levels. When the level of CO₂ dropped below 700 ppm, commercial CO₂ was injected into the chamber, thus maintaining CO₂ concentration at 700 ppm. The air in the three plant growth chambers (future, current and pre-industrial CO₂) was collected using 10-ml vacutainers and analysed using gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS). Mixing of the commercial CO₂ (δ¹³C ca. −38.2‰) with the ambient air (δ¹³C ca. −12.5‰) resulted in a δ¹³C of CO₂ of ca. −22.6 ± 0.9‰ inside the plant growth chamber. Another 48 plants were placed in the second plant growth chamber and were maintained at current [CO₂] (ca. 400 ± 20 ppm) during the whole life cycle, with a δ¹³C of CO₂ of ca. −11.2 ± 0.6‰. Finally, the same number of plants was placed in the third plant growth chamber and maintained at pre-industrial [CO₂] (ca. 260 ± 28 ppm) during the whole life cycle, with a δ¹³C of CO₂ of ca. −10.8 ± 0.5‰. In this chamber, CO₂ was removed using a pump that sent the air inside the chamber through a 1-l column filled with soda lime (soda lime with indicator QP; Panreac Quimica SA, Barcelona, Spain). The soda lime was changed every 2 weeks. The CO₂ levels of these two chambers (400 and 260 ppm) were also continuously monitored with an

IRGA. Plants were rotated in the plant growth chamber each week to avoid chamber influences in the treatments.

Isotope labelling procedures with ¹³C and ¹⁵N

Simultaneous C and N labelling was conducted in the plants and at different CO₂ levels. Double labelling with ¹³C and ¹⁵N was carried out over 3 days during the anthesis period in order to ensure that both genotypes were labelled with the same amount of ¹³C and ¹⁵N in the different plant growth chambers. All plants assimilated the same amount of labelled CO₂ (ca. 3000 mmol C·m^{−2}) during ca. 3 days, but the assimilation time varied between the different CO₂ treatments and was calculated according to their net assimilation rates (Table 1; Nogués *et al.* 2014).

The ¹³C composition of air inside the three plant growth chambers was modified during the labelling period. In each of the chambers, mixing of commercial CO₂ (¹³C ca. 99.9%; Euriso-top, Saint-Aubin, France) with the ambient air (δ¹³C ca. −22.6‰, −11.2‰ and −10.8‰ in future, current and pre-industrial plant growth chambers, respectively) resulted in a δ¹³C of CO₂ of ca. 165‰. Air samples from the chambers and air respired by plants in darkness were taken before and after labelling in order to analyse the ¹³CO₂ isotopic composition using GC/C/IRMS according to Nogués *et al.* (2004).

Labelling with ¹⁵N was also applied during the same period by replacing the ¹⁴NH₄–¹⁴NO₃ in the Hoagland solution with double ¹⁵N labelled ammonium nitrate (¹⁵NH₄–¹⁵NO₃) that had a ¹⁵N excess atom fraction of 5%. After labelling, ¹⁵N was removed by washing the quartz sand with distilled water. Plants were then irrigated with normal Hoagland solution.

Carbon and N isotope composition of TOM

Samples from different parts of the plant (*i.e.* leaves, stems, roots and spikes) were collected before, 1 and 10 days after labelling, dried in an oven at 60 °C for 48 h and ground to a fine powder. Then, 1 mg was weighed in tin capsules and C and N isotope composition determined using an elemental analyser (Flash EA 112; Carlo Erba, Milan, Italy) coupled to an

Table 1. CO₂ effects (700, 400 and 260 ppm) on physiological parameters in the flag leaf of durum wheat Sula and Blanqueta genotypes.

CO ₂ treatment	Genotype	V _{c,max}	J _{max}	R _d	g _s	A _{sat}	A ₃₆₀	A _{max}	C/C _a
700	Blanqueta	77.0 ± 12.2	126.1 ± 9.1	−0.6 ± 0.5	14.0 ± 1.3	14.4 ± 1.5	16.7 ± 1.5	23.0 ± 1.3	0.59 ± 0.01
700	Sula	68.9 ± 5.1	166.4 ± 4.5	−1 ± 0.5	22.7 ± 3.5	15.2 ± 0.8	19.6 ± 0.2	28.4 ± 0.8	0.66 ± 0.009
400	Blanqueta	80.6 ± 14.2	171.9 ± 36.6	−0.3 ± 0.8	19.2 ± 1.9	17.4 ± 3.0	21.4 ± 3.3	28.9 ± 5.2	0.48 ± 0.006
400	Sula	84.2 ± 15.4	144.3 ± 30.9	−1.7 ± 0.9	15.9 ± 1.7	18.8 ± 2.4	22.5 ± 3.1	26.5 ± 3.7	0.74 ± 0.008
260	Blanqueta	88.5 ± 17.9	181.5 ± 41.8	−0.7 ± 0.3	19.1 ± 0.5	18.4 ± 4.9	22.7 ± 5.9	28.7 ± 4.3	0.62 ± 0.006
260	Sula	74.3 ± 3.6	148.2 ± 20.3	−1.8 ± 2.3	15.0 ± 1.0	18.5 ± 0.0	22.0 ± 0.5	26.8 ± 0.7	0.74 ± 0.006
CO ₂ treatment		n.s.	n.s.	***	*	n.s.	n.s.	n.s.	n.s.
Genotype		n.s.	n.s.	**	n.s.	n.s.	n.s.	n.s.	n.s.
CO ₂ treatment*genotype		n.s.	n.s.	*	*	n.s.	n.s.	n.s.	n.s.

V_{c,max} = maximum carboxylation velocity of Rubisco; J_{max} = rate of photosynthetic electron transport; R_d = rate of daytime respiration; g_s = stomatal conductance; A_{sat} = assimilation rate at saturation of light; A₃₆₀ = assimilation rate at 360 ppm C_i of CO₂; A_{max} = maximum assimilation rate; C/C_a = internal CO₂ concentration/ambient CO₂ ratio.

Data are means ± SE, n = 4.

ANOVA Tukey-b (n.s., non-significant; *P < 0.05; **P < 0.01; ***P < 0.001).

isotope ratio mass spectrometer (Delta C with ConFlo III; Thermo Finnigan, Bremen, Germany).

Results of C isotope ratio analyses are reported as $\delta^{13}\text{C}$ in per mil (‰) and referenced against the international standard V-PDB (Vienna Pee Dee Belemnite) according to the following equation:

$$\delta^{13}\text{C} (\text{‰}) = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000 \quad (1)$$

where R is the $^{13}\text{C}/^{12}\text{C}$ ratio.

Carbon isotope discrimination ($\Delta^{13}\text{C}$) was calculated for unlabelled plants from δ_a and δ_p (Farquhar *et al.* 1989) as in the following equation:

$$\Delta^{13}\text{C} = \frac{\delta_a - \delta_p}{\delta_p + 1} \quad (2)$$

where a and p refer to $\delta^{13}\text{C}$ of air CO₂ and plant material, respectively.

Nitrogen results were also expressed in $\delta^{15}\text{N}$ notation (‰), using the international secondary standards with known $^{15}\text{N}/^{14}\text{N}$ ratio (IAEA N₁ and IAEA N₂ ammonium sulphate and IAEA NO₃ potassium nitrate) with reference to the international primary standard air N₂, which has a $\delta^{15}\text{N}$ value of 0‰ (Werner & Brand 2001):

$$\delta^{15}\text{N} (\text{‰}) = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000 \quad (3)$$

where R is the $^{15}\text{N}/^{14}\text{N}$ ratio.

Labelled samples were expressed in atom fraction (%) ^{15}N as recommended in the international guidelines for stable isotope ratio (Coplen 2011).

$$\text{Atom fraction (\%)} = \frac{\delta + 1000}{\delta + 1000 + \frac{1000}{R_{\text{standard}}}} \quad (4)$$

where δ is the isotopic signature of ^{15}N samples. R_{standard} is the international standard reference.

The N isotope discrimination ($\Delta^{15}\text{N}$) of TOM for unlabelled plants was calculated from δ_s and δ_p (Farquhar *et al.* 1989) as:

$$\Delta^{15}\text{N} = \frac{\delta_s - \delta_p}{\delta_p + 1} \quad (5)$$

where s and p refer to $\delta^{15}\text{N}$ of solution and plant, respectively.

Open system for isotopic dark respiration determinations

The $\delta^{13}\text{C}$ of the CO₂ respired after 20 min in darkness of the different plant organs (*i.e.* flag leaf, remaining leaves, spikes, stems and root) was studied in a respiration chamber as previously described (Nogués *et al.* 2004). The chamber was connected in parallel to the sample air hose of a portable gas exchange analyser (Li-Cor 6400; Li-Cor Inc., Lincoln, NE, USA). The PPFD inside the chamber was maintained at 0 $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ by covering the chamber with a black

piece of, which kept the chamber in darkness. The organ was first placed in the chamber with ambient air ($\delta^{13}\text{C}$ ca. $-10.3 \pm 0.5\text{‰}$). The chamber was then flushed with CO₂-free air and the CO₂ respired by the organ was allowed to accumulate over a period of 10 min. This was then collected using gas syringes (SGE International Pty Ltd, Australia) and stored in 10-ml vacutainers.

The air in the three growth chambers (*e.g.* pre-industrial, current and future CO₂) was also sampled using 10-ml vacutainers in order to obtain isotopic composition of the air (the source of C). The CO₂ inside the vacutainers was analysed using GC/C/IRMS.

All the GC/C/IRMS and EA/IRMS analyses were performed at the Scientific Technical Services of the University of Barcelona.

Calculation of the proportion (p) of new C and N

We assumed that 100% of C and N supplied during short-term labelling could be assimilated by the different parts of the plant, and that this C and N is allocated throughout the plant. The proportion of 'new' carbon (derived from the labelling) in CO₂ respired in darkness after illumination and the proportion of 'new' C and N in TOM was calculated as described in Nogués *et al.* (2004):

$$x = 100 \times \frac{\delta_{\text{after}} - \delta_{\text{control}}}{\delta_{\text{fixed}} - \delta_{\text{control}}} \quad (6)$$

where δ_{control} , δ_{fixed} and δ_{after} are the isotope compositions of the fraction of interest (CO₂ and TOM) of the control (not labelled), of C and N atoms fixed during labelling and of the sample after labelling, respectively. The isotope composition of fixed C and N was calculated as:

$$\delta_{\text{fixed}} = \frac{\delta_{\text{source}} - \Delta}{1 + \Delta} \quad (7)$$

where Δ is the isotope discrimination (equations 2 and 5) and δ_{source} is the isotopic composition of the source during the labelling.

Leaf C and N content

Leaves, spikes, stems and roots used for gas exchange were collected and dried at 65 °C until constant weight and ground to a powder. An aliquot of 1 mg dry powder was analysed for the C (mg C·mg⁻¹) and N content (mg N·mg⁻¹) with an elemental analyser at the Scientific Technical Services at the University of Barcelona, Spain.

Gas exchange analyses

For simultaneous measurements of gas exchange and chlorophyll fluorescence in an expanded flag leaf, the LI6400 was connected to a leaf chamber fluorometer (LI6400-40). A/C_i curves with chlorophyll fluorescence and dark respiration rates were determined. A/C_i curve determinations were conducted on totally expanded flag leaves for each CO₂ treatment and in each genotype. The A/C_i curves were repeated in four different

plants for each treatment and genotype and were measured from 0 to 2000 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂. The curves were generated at 1200 $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPFD and 25 °C. Measurements were carried out before anthesis (T0). CO₂ assimilation rates (A), CO₂ assimilation rate at light saturation (A_{sat}), maximum CO₂ assimilation rate at CO₂ saturation (A_{max}) and stomatal conductance (g_s) were estimated using equations developed by von Caemmerer & Farquhar (1981). Estimations of the maximum carboxylation velocity of Rubisco ($V_{\text{c,max}}$), the rate of photosynthetic electron transport based on NADPH requirement (J) and the rate of 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 of Nogués & Baker (2000).

Biomass parameters

Biomass parameters in durum wheat genotypes grown at three different CO₂ levels (700, 400 and 260 ppm) were analysed. Leaves were scanned with a commercial scanner (HP ScanJet 3400C; Hewlett-Packard, Palo Alto, CA, USA) and images were analysed with leaf area meter software (Comprises WINDIAS; Delta-T Devices Ltd. Cambridge, UK) to obtain the total leaf area (TLA; cm²). Harvest index (HI), reproductive biomass (RB; g), shoot dry weight (shoot DW; g), root dry weight (root DW; g) and shoot/root were also studied. HI was calculated as the ratio between grain DW and total DW.

Data analysis

The effects of CO₂ in both wheat genotypes were tested with two-factor (CO₂ treatment and durum wheat genotype) ANOVA. The statistical analysis was conducted with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The means $\pm$ SE were calculated for each parameter. When a particular test was significant, we compared the means using the Duncan multiple comparison test. The results were accepted as significant at $P < 0.05$.

RESULTS

Before labelling (T0), TOM was more ¹³C-enriched at pre-industrial than at current [CO₂] (with increases of 5.4‰ and 7.3‰ in spikes, 7.7‰ and 6.3‰ in leaves, 7.1‰ and 8.6‰ in stems, 8.7‰ and 7.4‰ in roots for Blanqueta and Sula, respectively) and more ¹³C-depleted at future [CO₂] than at current [CO₂] (with decreases of 9.7‰ and 8.2‰ in spikes, 6.4‰ and 9.5‰ in leaves, 8.6‰ and 8.6‰ in stems, 8.3‰ and 9.7‰ in roots for Blanqueta and Sula, respectively; Fig. 1, Figure S1). In general, the Blanqueta genotype was between 2.7‰ and 5.9‰ more ¹³C-enriched than Sula in all organs and CO₂ treatments, with some exceptions, e.g. root and stem at elevated CO₂. It is worth noting that plants at future CO₂ levels were grown with a $\delta^{13}\text{C}$ of ca. $-22.6 \pm 0.9\text{‰}$ inside the plant growth chamber, while the $\delta^{13}\text{C}$ of the other two plant growth chambers were ca. $-10.8 \pm 0.5\text{‰}$ and $-11.2 \pm 0.6\text{‰}$ for 260 and 400 ppm, respectively (see Material and Methods).

During labelling, the $\delta^{13}\text{C}$ of the air in the three plant growth chambers was ca. 165‰. After labelling (T1), the $\delta^{13}\text{C}$ of TOM in labelled plants was more ¹³C-enriched than the corresponding non-labelled plants in both wheat genotypes (Fig. 1), where spikes were the main C sink. In general, Blanqueta was more

¹³C-enriched than Sula in all organs and CO₂ treatments. However, plants were more ¹³C-depleted at future than at current CO₂ conditions and more ¹³C-enriched at pre-industrial than current CO₂ ($F = 23.05$, $P < 0.001$). We also observed that on the last sampling day (T2), plants were less ¹³C-enriched at higher [CO₂].

The $\delta^{13}\text{C}$ of respired CO₂ ($\delta^{13}\text{CO}_{2\text{Respired}}$) in the dark in the different plant organs was analysed with a GC-C-IRMS. It was observed that, ¹³C losses through dark respiration (Fig. 2) were larger in Blanqueta than in Sula. Although respiration is an important C sink in all organs, respiration of recently fixed C was higher in spikes and stems compared to flag and other leaves. Before labelling (T0), $\delta^{13}\text{CO}_{2\text{Respired}}$ was ca. -26‰ for leaves, -26‰ for roots, -30‰ (Sula) and -34‰ (Blanqueta) for spikes, and -34‰ for stems in the 700-ppm treatment. In the other CO₂ treatments, respired CO₂ was more ¹³C-enriched. After labelling (T1), the $\delta^{13}\text{CO}_{2\text{Respired}}$ was positive in all organs, indicating that plants had assimilated labelled C, however, the largest quantities were found in spikes and stems. Moreover, the $\delta^{13}\text{CO}_{2\text{Respired}}$ values increased with decreasing [CO₂] (Fig. 2). We also observed that on the last sampling day (T2), the $\delta^{13}\text{CO}_{2\text{Respired}}$ was negative once again.

Figure 3 shows a simplified diagram of recently fixed C and respired CO₂ for wheat at 700 (Fig. 3A), 400 (Fig. 3B) and 260 (Fig. 3C) ppm. We assumed that 100% of C supplied during short-term labelling could be assimilated by the different parts of the plant, and that this C was allocated through three main processes: (i) storage in the plant tissues, (ii) translocation to other organs of the plant and (iii) losses through plant respiration.

The percentage of new C in TOM and CO₂ respired after the labelling at T1 revealed that the C stored was higher in Blanqueta than in Sula for the different wheat organs and that the spike, followed by leaves, was the organ with the highest percentage of new C; Blanqueta showed double or higher percentage of new C in pre-industrial [CO₂], up to seven times more in current [CO₂] and almost the same value at future [CO₂] than Sula. However, 10 days after labelling (T2), the percentages of new C in both varieties decreased. Plants at future [CO₂] had less new labelled C than treatments at current [CO₂], and plants at pre-industrial [CO₂] had a higher percentage of new C overall. After labelling, plants exposed to future and pre-industrial [CO₂] had higher percentages of new C in respired CO₂ than at current CO₂ treatments. Interestingly, Sula showed a higher percentage of new respired C in the different parts of the plant except in the flag (and only at future CO₂), whereas Blanqueta showed higher percentages than Sula in the other treatments. Figure 3 shows that, in both genotypes, losses of assimilated new C after the labelling through respiration were between 20–35% at 700 ppm and 10–30% at 260 ppm.

Before ¹⁵N labelling (Figure S1), significant differences in $\delta^{15}\text{N}$ were found between CO₂ treatments ($F = 9.61$, $P < 0.05$), but not between genotypes ($F = 0.527$, $P = 0.471$). At pre-industrial [CO₂], organs were more ¹⁵N-depleted than at current [CO₂] (0.7‰ and 0.5‰ in spikes, 0.4‰ and 1‰ in leaves, 0.6‰ and 1‰ in stems, 5‰ and 4‰ in roots) and more ¹⁵N-enriched at future CO₂ levels than at current [CO₂] (18‰ and 2.7‰ in spike, 21‰ and 2.2‰ in leaves, 14‰ and 4‰ in stem, 3‰ and 2‰ in roots, for Blanqueta and Sula, respectively;

Fig. 1. CO₂ effects (700, 400 and 260 ppm) on $\delta^{13}\text{C}$ (‰) and ^{15}N atomic fraction (%) values of total organic matter (TOM) in spikes, leaves, stems and roots of two different genotypes of durum wheat: Blanqueta (open bars) and Sula (close bars) before labelling (before anthesis, T0), 1 day after labelling (beginning of grain filling, T1) and 10 days after labelling (end of grain filling, T2). Labelling was made during anthesis. Statistical analysis is presented in Table S1. Data are means $\pm$ SE, n = 4.

Figure S1). After labelling with $^{15}\text{NH}_4\text{-}^{15}\text{NO}_3$, at future [CO₂], Sula had a higher $\delta^{15}\text{N}$ than Blanqueta. In the other CO₂ treatments, Blanqueta was more ^{15}N -enriched. Furthermore, at 260 ppm, between labelling and the end of grain filling, spikes and leaves were more ^{15}N -enriched, while roots and stems showed decreases in $\delta^{15}\text{N}$. In this same period, plants at pre-industrial [CO₂] had more ^{15}N than plants in current conditions, which in turn were more ^{15}N -enriched than plants at future [CO₂] (Fig. 1).

The percentage of N absorbed during the labelling ($^{15}\text{NH}_4\text{-}^{15}\text{NO}_3$) was calculated as a percentage of new N in T1 and T2. Overall, the percentage of new N found in the different plant organs and genotypes ranged from 0.6% (i.e. spike in Blanqueta at future CO₂) to 8.8% (i.e. roots in Blanqueta at pre-industrial CO₂; Table 2). Sula presented a higher percentage of new N in the spike at future CO₂ (3.6%) than Blanqueta (0.6%), whereas Blanqueta presented higher percentages of new N than Sula in

the other treatments (2.8% and 4.7% higher at current [CO₂], 3.6% and 4.8% at pre-industrial CO₂ in Sula and Blanqueta, respectively). This percentage of new N was higher in plants growing at lower [CO₂] in both genotypes. We also found that at T2, percentages were increased in spikes and leaves, since spikes are an important N sink. Percentage of new N content decreased in roots as N was redirected to other organs. In stems, the percentage of new N decreased at future [CO₂] since N was allocated to the leaf or spike where it was used for storage or the production of new proteins and metabolites. This was not the case at pre-industrial [CO₂], where the percentage of new N increased in the stem.

All genotypes showed higher leaf and spike N content (mg N·g⁻¹) at lower [CO₂] at T0 and T1. However, at T2, leaf N content was higher at current [CO₂] than in any other CO₂ treatment (Table 3). On the other hand, data was more variable in stems with the lowest N content at current [CO₂], at T0 and

Fig. 2. CO₂ effects (700, 400 and 260 ppm) on δ¹³ values (‰) of respired CO₂ (δ¹³CO₂-Respired) in spikes, leaves, stems and roots of two different genotypes of durum wheat: Blanqueta (open bars) and Sula (close bars) before labelling (before anthesis, T0), 1 day after labelling (beginning of grain filling, T1) and 10 days after labelling (end of grain filling, T2). Labelling was made during anthesis. Statistical analysis is presented in Table S1. Data are means ± SE, n = 4.

T1 but not at T2. In roots, the trend was more constant where N content decreased with higher levels of CO₂ for both Sula and Blanqueta genotypes, except for Blanqueta at T2. The relationship between δ¹⁵N and δ¹³C in TOM in Sula and Blanqueta genotypes before and after labelling and at the different CO₂ levels (700, 400 and 260 ppm) showed a positive correlation ($r^2 = 0.834$, $P < 0.05$; Fig. 4). Table 1 shows that plants underwent an acclimation process under the different CO₂ treatments, which can also in part explain C allocation. At future [CO₂], we observed that carboxylation activity was diminished by the reduction in $A_{\max}$ and A_{sat} . Furthermore, plants had a lower rate of $V_{c,\max}$ and $J_{\max}$ contributing to RuBP regeneration. $V_{c,\max}$, $J_{\max}$, A_{sat} , A_{360} and $A_{\max}$ increased after prolonged exposure to pre-industrial [CO₂], although differences between CO₂ treatments and genotypes were not significant.

In terms of biomass parameters, significant differences were found between CO₂ treatments and genotypes. It was observed that CO₂ treatments had a larger effect on the biomass of Sula than Blanqueta (Table 4), although there were no significant differences in TLA between CO₂ treatments ($F = 1.872$,

$P > 0.05$) and shoot biomass between genotypes ($F = 2.715$, $P > 0.05$). Shoot DW and root DW were lower at lower CO₂ levels. Sula showed higher TLA, spike biomass, shoot and root DW at future [CO₂] ($F = 27.264$, $P < 0.001$). Higher values in the spike biomass, shoot DW and TLA, and lower values in root DW were found at pre-industrial [CO₂] compared with current [CO₂]. Finally, Blanqueta had more vegetative and less reproductive biomass than Sula.

DISCUSSION

In our study, the partitioning and allocation of C and N in two wheat genotypes Sula (modern) and Blanqueta (traditional) in three different CO₂ treatments were characterized by means of ¹³C and ¹⁵N labelling.

Photosynthetic acclimation at different CO₂ concentrations

Plant photosynthesis showed acclimation to the different CO₂ levels. Currently, photosynthetic acclimation to different

Fig. 3. (A) CO₂ effects at 700 ppm on plant C allocation in spikes, flag leaf, leaves, stems and roots in two genotypes, Sula (left) and Blanqueta (right), 1 day after labelling (beginning of grain filling, T1) and 10 days after labelling (end of grain filling, T2). Labelling was carried out during anthesis with ¹³CO₂. Rectangles: % stored C after labelling. Cloud shapes: % respired C after labelling. Sula has small plants and roots, but large spikes. Blanqueta has large shoots and roots but small spikes. Flag leaf total organic matter (TOM) is similar to leaf TOM. (B) CO₂ effects at 400 ppm on plant C allocation in spikes, flag leaf, leaves, stems and roots in two genotypes, Sula (left) and Blanqueta (right), 1 day after labelling (beginning of grain filling, T1) and 10 days after labelling (end of grain filling, T2). (C) CO₂ effects at 260 ppm on plant C allocation in spikes, flag leaf, leaves, stems and roots in two genotypes, Sula (left) and Blanqueta (right), 1 day after labelling (beginning of grain filling, T1) and 10 days after labelling (end of grain filling, T2).

CO₂ levels is one of the key issues in CO₂ research, and it has been demonstrated that acclimation to pre-industrial and future CO₂ during long-term exposure can compensate for

the effects of CO₂ variation in plant processes (Sage & Coleman 2001). Plants used in this study, which were maintained at optimal water and nutritional conditions, showed an

Table 2. CO₂ effects (700, 400 and 260 ppm) on the percentage of new N in spikes, leaves, stems and roots in durum wheat Blanqueta and Sula genotypes 1 day (beginning grain filling, T1) and 10 days (end of grain filling, T2) after labelling. Labelling was carried out during the anthesis period.

% New N	Organ	700 T1	700 T2	400 T1	400 T2	260 T1	260 T2
Genotype							
Blanqueta	Spike	0.6 ± 0.2	1.4 ± 0.6	4.7 ± 0.8	7.3 ± 0.2	4.8 ± 0.3	5.2 ± 0.2
Sula	Spike	3.6 ± 0.5	2.6 ± 0.1	2.8 ± 0.6	4.0 ± 0.7	3.6 ± 0.6	6.8 ± 1.0
Blanqueta	Leaf	0.7 ± 0.4	0.7 ± 0.3	5.4 ± 0.3	6.9 ± 0.5	6.5 ± 0.5	6.1 ± 1.0
Sula	Leaf	3.7 ± 0.2	1.1 ± 0.1	2.0 ± 0.4	1.9 ± 0.6	3.5 ± 0.8	4.7 ± 1.0
Blanqueta	Stem	1.4 ± 0.2	0.9 ± 0.3	5.5 ± 0.8	2.6 ± 0.4	4.9 ± 1.3	6.3 ± 0.3
Sula	Stem	5.7 ± 0.4	2.0 ± 0.2	2.5 ± 0.2	1.1 ± 0.1	4.4 ± 0.8	4.8 ± 1.7
Blanqueta	Root	2.0 ± 0.2	0.9 ± 0.2	6.6 ± 0.7	4.2 ± 1.3	8.8 ± 2.3	5.5 ± 1.2
Sula	Root	5.7 ± 0.3	1.6 ± 0.2	3.1 ± 0.2	1.7 ± 0.0	5.5 ± 0.3	5.5 ± 1.7

Data are means ± SE, n = 4.

Table 3. CO₂ effects (700, 400 and 260 ppm) on N content (mg·g⁻¹) in leaf, spike, stem and root of durum wheat Blanqueta and Sula genotypes before labeling (before anthesis, T0), 1 day after labeling (beginning of grain filling, T1) and 10 days after labeling (end of grain filling, T2).

CO ₂ treatment	700	400	260	700	400	260	700	400	260	700	400	260	700	400	260	700	400	260
Genotype	Blanqueta			Sula			Blanqueta			Sula			Blanqueta			Sula		
Timing	T0			T1			T2			T2			T2			T2		
Organ	Leaf			Leaf			Leaf			Leaf			Leaf			Leaf		
N content	1.6	1.9	2.5	1.3	2.4	3.5	1.7	2.5	3.5	2.0	2.4	3.6	1.6	3.2	2.4	1.4	2.7	2.0
±SE	0.3	0.3	0.13	0.2	0.2	0.2	0.1	0.3	0.2	0.1	0.5	0.3	0.4	0.2	0.8	0.2	0.2	0.5
Organ	Spike			Spike			Spike			Spike			Spike			Spike		
N content	1.9	2.1	2.0	1.2	1.5	1.6	1.4	2.3	2.6	0.8	1.7	2.1	1.6	2.2	2.3	1.1	2.2	3.4
±SE	0.1	0.1	0.0	0.1	0.0	0.0	0.2	0.1	0.1	0.0	0.1	0.2	0.4	0.0	0.4	0.1	0.3	0.4
Organ	Stem			Stem			Stem			Stem			Stem			Stem		
N content	1.5	0.8	1.2	0.9	0.5	0.8	1.4	1.4	1.5	1.2	0.5	1.2	1.2	2.6	1.8	0.7	1.5	1.8
±SE	0.4	0.0	0.1	0.2	0.0	0.2	0.1	0.3	0.3	0.3	0.0	0.2	0.0	0.1	0.2	0.1	0.8	0.2
Organ	Root			Root			Root			Root			Root			Root		
N content	2.0	1.1	1.9	0.9	1.6	2.1	2.1	2.2	3.8	1.3	1.5	2.8	2.1	1.7	2.0	1.4	1.9	2.5
±SE	0.2	0.0	0.2	0.3	0.3	0.3	0.2	0.3	0.4	0.2	0.1	0.3	0.3	0.3	0.3	0.2	0.3	0.4

Data are means ± SE, n = 4.

Fig. 4. δ¹³C and δ¹⁵N correlation in TOM of the leaf in wheat plants and three CO₂ treatments (700, 400 and 260 ppm), before labelling (before anthesis, T0), 1 day after labelling (beginning of grain filling, T1) and 10 days after labelling (end of grain filling, T2). Labelling was carried out during 3 days in anthesis. Linear regression is significant ($r^2 = 0.834$; $P < 0.05$).

Table 4. CO₂ effects (700, 400 and 260 ppm) on biomass parameters in durum wheat Sula and Blanqueta genotypes at the end of grain filling (T2).

CO ₂ treatment	Genotype	TLA	RB	DW shoot	DW root	Shoot/root	HI
700	Blanqueta	110.64 ± 33.40	3.2 ± 0.82	4.06 ± 1.75	4.63 ± 1.63	0.83 ± 0.08	0.26 ± 0.06
700	Sula	95.18 ± 24.15	13.47 ± 1.32	7.17 ± 1.52	3.70 ± 0.59	1.94 ± 0.29	0.56 ± 0.02
400	Blanqueta	128.46 ± 2.38	2.94 ± 0.14	3.75 ± 0.16	3.23 ± 0.31	1.17 ± 0.07	0.24 ± 0.01
400	Sula	39.30 ± 5.61	4.75 ± 1.01	2.57 ± 0.39	1.13 ± 0.01	2.27 ± 0.36	0.54 ± 0.04
260	Blanqueta	107.83 ± 32.48	3.78 ± 0.72	3.64 ± 0.50	3.35 ± 0.65	1.11 ± 0.05	0.31 ± 0.02
260	Sula	52.63 ± 11.20	5.71 ± 0.78	3.05 ± 0.49	0.88 ± 0.15	3.62 ± 0.62	0.55 ± 0.02
CO ₂ treatment		n.s.	*	*	***	*	n.s.
Genotype		***	***	n.s.	***	***	***
CO ₂ treatment*genotype		**	***	***	***	*	n.s.

TLA = total leaf area (cm²); RB = reproductive biomass (g); DW Shoot = shoot dry weight (g); DW Root = root dry weight (g); Shoot/Root; HI = harvest index.

Data are means ± SE, n = 4.

ANOVA Tukey-b (n.s., non-significant, **P* < 0.05; ***P* < 0.01; ****P* < 0.001).

up-regulation of photosynthesis with an increase and/or maintenance of physiological parameters such as $V_{c,max}$, J_{max} and A_{sat} in response to pre-industrial [CO₂] (Sage & Reid 1992; Cowling & Sage 1998; Anderson *et al.* 2001). On the other hand, many studies have shown that photosynthesis also acclimates to future [CO₂] over long-term experiments through down-regulation (Ainsworth *et al.* 2003; Urban 2003; Long *et al.* 2004; Aranjuelo *et al.* 2009b; Pardo *et al.* 2009) and, consequently, photosynthetic capacity decreases. Here, it was found that $V_{c,max}$, J_{max} , A_{sat} and A_{360} (Table 1) were lower at future [CO₂] than at current levels, clearly indicating a down-regulation of photosynthesis. This implies that significant photosynthetic adjustments (together with increases in N, Rubisco and protein content at pre-industrial [CO₂] and decreases at future [CO₂]) may have taken place in the past (up-regulation) and will certainly be likely to occur in the future (down-regulation), thereby changing the allocation and balance of C and N in plants.

Different plant responses are conditioned by the genetics of the different genotypes (Reynolds *et al.* 1999) and the availability of C and N pools (Fuertes-Mendizabal *et al.* 2010). In our study, traditional genotypes produced more vegetative and less reproductive biomass than did modern genotypes. Plant growth and C and N distribution were also modulated by the different CO₂ treatments. Sula plants showed a large capacity to increase biomass of the spike and root at future [CO₂], but less capacity at current or pre-industrial [CO₂] (Aranjuelo *et al.* 2013). However, in the case of the traditional genotype, vegetative shoots (*i.e.* leaves and stems) were the main C and N sinks. On the other hand, this growth response was associated with increased C allocation from assimilation areas to the spike, especially when C was limited, thereby resulting in an increase in the shoot/root ratio. Lehmeier *et al.* (2005) found changes in shoot/root ratios at future and pre-industrial [CO₂]. Optimal C allocation favours those parts of the plant that incur the most severe growth limitations due to a lack of resources (McConnaughay & Coleman 1999). In our case, the shoot/root ratio increased in the Sula genotype with decreasing [CO₂], from future to pre-industrial [CO₂] (Table 4). Plants at pre-industrial [CO₂] may have stimulated C allocation to shoot organs for grain filling (Anderson *et al.* 2010). Blanqueta showed a decrease in shoot/root ratio from future to current [CO₂]; however, this effect was not seen at pre-

industrial [CO₂]. This could be explained by the fact that (i) the C requirement of the reproductive shoot area is lower in the traditional genotype than in the modern genotype, and/or (ii) traditional plants have a bigger vegetative shoot area than Sula (plants were also up-regulated). For this reason, it may be possible that Blanqueta can invest more C in the root at pre-industrial [CO₂].

Dynamics of C and N

New ¹³C and ¹⁵N was allocated to all the organs of the plant in both genotypes in the different CO₂ treatments. The largest C sinks were the spike and respiration, since a large percentage of ¹³C was quickly respired in the first 24 h after labelling (Tcherkez *et al.* 2003; Nogués *et al.* 2004). In our case, 10 days after labelling, plants had lost most ¹³C through respiration, and a large proportion of the remaining ¹³C had been allocated to the spike. Plants at future [CO₂] were less enriched in ¹³C than at current or pre-industrial conditions because, on the one hand, they were photosynthetically down-regulated and the rate of assimilation was lower, and on the other hand, plants at pre-industrial [CO₂] discriminated less against ¹³C because the availability of CO₂ was lower than in plants grown at higher levels of CO₂ (Farquhar *et al.* 1989). Thus, ¹³C in TOM of plant organs increased proportionally with lower CO₂ concentrations in the environment.

The percentage of new C in TOM after the labelling confirmed that <20% of C in the different organs, genotypes and treatments was derived from recently assimilated C (Fig. 3; Nogués *et al.* 2004, 2014; Aranjuelo *et al.* 2009a). The majority of photoassimilates formed in leaves might be destined for the following processes: (i) respiration, (ii) storage and (iii) export to other tissues (Nogués *et al.* 2004). However, the photoassimilates in the spike are mainly destined for respiration and storage in the grain. In our case, the traditional genotype fixed more new C than did the modern genotype, however traditional plants also respired more the new C and had less new C fixed in the different organs than did modern plants 10 days after of labelling (T2). This suggests that in modern plants, the spike was the largest C sink, whereas this was respiration in the traditional genotype. ¹³C increased in leaves and stems at T2, but decreased in the spike due to respiration and in roots through translocation and respiration. This suggests that at pre-industrial [CO₂], photoassimilates are stored in stems

and/or leaves as plants cannot increase the capacity of the spike as a new sink.

Respired CO₂ by Blanqueta and Sula (exposed to the three levels of CO₂) was enriched in ¹³C at T1 (Fig. 2), implying that some of the C assimilated during labelling was immediately respired (Nogués *et al.* 2014). However, the fact that respired CO₂ had low levels of ¹³C enrichment implies that a large part of the respired C originated in C stored in the organs prior to labelling (Fig. 1; Aranjuelo *et al.* 2009a). C losses through dark respiration were high in the spike and less so in the flag or leaves since these allocated photoassimilate to grain filling (Evans *et al.* 1975; Schnyder 1993). Ten days after labelling (T2), ^δ¹³CO₂-Respired values were similar to those of ^δ¹³CO₂-Respired before labelling, suggesting that leaves and other organs had used almost all of the labelled C substrate. The ^δ¹³C values of TOM at T1 suggest that plant organs used the labelled C, and the ^δ¹³C of TOM at T2 shows that some ¹³C still remained in the plants. After anthesis and during grain filling, the spike had a high demand for photoassimilates, which are supplied by spike photosynthesis and by C translocation from flag leaves, other leaves and stem internodes (Gebbing & Schnyder 1999; Tambussi *et al.* 2007; Aranjuelo *et al.* 2009a, 2011b). Our data showed that exposure to future [CO₂] modified the ¹³C enrichment of respired CO₂, as the CO₂ used for establishing the 700 ppm atmosphere had an ca. 10‰ more negative ^δ¹³C value, which is also reflected in the respired CO₂ of the plants grown at 700 ppm, which was lower than at current and pre-industrial [CO₂] prior to labelling (Fig. 2). However, after labelling, plants exposed to pre-industrial [CO₂] had higher values of ^δ¹³C (in TOM and respired CO₂) and needed more time to lose the enriched ¹³C in TOM after labelling because in pre-industrial environments plants discriminate less and are more enriched in ¹³C (Fig. 1) than at current [CO₂].

Labelled ¹⁵N was applied with the ¹⁵NH₄-¹⁵NO₃ of the solution. Spikes and roots were the main N sinks. Plants were less enriched in ¹⁵N at future [CO₂] than at pre-industrial [CO₂] (Fig. 1) as photosynthesis was down-regulated and N demand was lower. This suggests that acclimation and CO₂ treatments also affected the allocation and distribution of N. We found significantly higher levels of leaf N in both genotypes at pre-industrial CO₂. Anderson *et al.* (2001) showed that the up-regulation of assimilation might be related to increases in leaf N content as well as to the re-allocation of N within leaves. Moreover, increases in leaf N and Rubisco would be needed for plants grown at 260 ppm CO₂ to achieve a similar level of photosynthetic activity as plants grown at 400 ppm (Sage & Reid 1992). Cernusak *et al.* (2011) showed that N and C content decreased in plants in response to future [CO₂], as was seen in our study in leaves, spikes and roots for both genotypes, which would provide additional evidence for down-regulation in plants (Figure S2).

After labelling and at future [CO₂], both genotypes had lower ¹⁵N than at current or pre-industrial [CO₂] (Fig. 1). However, Blanqueta was more ¹⁵N-depleted than Sula at future [CO₂], whereas in the other CO₂ treatments the opposite was true (with Sula being more ^δ¹⁵N-depleted). Also, we found different ^δ¹⁵N levels between organs. These data suggest that changes in organ ^δ¹⁵N (*i.e.* leaves, stems, roots or spikes) can be attributed to internal processes related to the assimilation and loss of ¹⁵N (as, *e.g.* translocation between organs or root exudates) in the plant and may depend on the genotype and

environmental conditions (BassiriRad *et al.* 2003). Many studies have attempted to explain why there are differences in the ^δ¹⁵N signal between roots and shoots, and between different CO₂ levels: (i) changes in the fractionation processes within the plant–mycorrhizal system and/or changes in nitrate assimilation enzymes, which discriminate heavily against ¹⁵N (BassiriRad *et al.* 2003); (ii) the influence of C availability and soil moisture on microbial activity, thereby enriching plant-available N (Dijkstra & Cheng 2008); (iii) the correlation between the influence of plant transpiration in the N acquisition from the soil (Cernusak *et al.* 2009); and (iv) isotopic fractionations along metabolic reactions (Tcherkez 2010; Gauthier *et al.* 2013). Furthermore, there is evidence that plant assimilation of nitrate can vary at different CO₂ levels (Bloom *et al.* 2014). In our experiment, we assume a reduction of nitrate assimilation in the shoot under future [CO₂], in agreement with previous work (Robinson 2001; Kruse *et al.* 2002). With regard to the percentage of new N, the same pattern was found in plants at future [CO₂] (*i.e.* at future [CO₂] Sula had a higher percentage of new N than Blanqueta). This suggests that the modern plant (with large C sinks) at future CO₂ conditions will be better adapted to assimilate more N than traditional plants. However, at current and pre-industrial [CO₂], traditional plants had higher percentages of new N, suggesting that Blanqueta is better adapted to the assimilation of N at these levels of CO₂.

Furthermore, the correlation between ^δ¹⁵N and ^δ¹³C ($r^2 = 0.834$, $P < 0.05$; Fig. 4) confirms that the distribution of labelled C and N was different between treatments and genotypes, suggesting that the partitioning and allocation of C and N was affected both by the genetics of the different genotypes of wheat and the CO₂ growth conditions. This allocation is reflected in Figure S2, where the N/C ratio decreases as the overall CO₂ increases in leaves, suggesting that at future [CO₂], leaves are more N- and C-limited. This is in accordance with Cernusak *et al.* (2011).

CONCLUSIONS

The effects of future and pre-industrial [CO₂], after exposure for a whole growing season, on two genotypes of durum wheat (traditional and modern) were studied. Our data showed, in accordance with photosynthetic parameters, a reduction in net photosynthesis rates and $V_{c,max}$ at future [CO₂], indicating a clear down-regulation. Plants showed acclimation at future and pre-industrial [CO₂], with down- and up-regulation of photosynthesis, respectively. However, at future [CO₂], this photosynthetic acclimation was disrupted when a new C sink appeared during grain filling. The pre-industrial CO₂ treatment decreased growth and biomass production in both genotypes; however, these effects decreased over time, demonstrating a clear up-regulation of photosynthesis. Also, Blanqueta and Sula modulated the assimilation of ¹³C in accordance with CO₂ level, *i.e.* plants were less enriched in ¹³C at future [CO₂] and more enriched in ¹³C at pre-industrial [CO₂].

In our study, we observed the importance of the sink in terms of the response of plants to different CO₂ scenarios. Plants invested more C in shoots than roots at pre-industrial [CO₂], and specifically, in the case of the traditional genotype, vegetative parts were seen to be the main C sink. At current and future [CO₂], the source of C is higher and plants can redirect more from assimilation compartments (shoots) towards

non-assimilation compartments (roots). However, at future [CO₂], it is the modern genotype that has a larger capacity to increase the size of reproductive organs and harvest index. In future studies, it will be necessary to examine the interactions between varying CO₂ concentrations and other environmental factors (e.g. drought), as this will allow us to better understand and predict plant processes and the allocation of C and N in response to increases in anthropogenic [CO₂].

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SUPPORTING INFORMATION

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