Estudio del impacto de las concentraciones altas y bajas de CO_2 sobre el cultivo de trigo

Salvador Aljazairi López

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Memòria presentada per Salvador Aljazairi López per optar al títol de Doctor per la Universitat de Barcelona. Aquest treball s'emmarca dins el programa de doctorat "Biologia Vegetal", corresponent al bienni 2010/2014 del Departament de Biologia Vegetal de la Facultat de Biologia de la Universitat de Barcelona.

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Doctorand	Director de Tesi
Salvador Aljazairi López	Dr. Salvador Nogués Mestres

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“La utopía está en el horizonte.
Camino dos pasos, ella se aleja dos pasos
y el horizonte se corre diez pasos más allá.
¿Entonces para qué sirve la utopía?
Para eso, sirve para caminar”

Eduardo Galeano

“El viaje es lo que nos hace felices, no el destino”.
El guerrero pacífico

A mi familia y amigos

Índice:

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Abbreviations and symbols

a, discrimination coefficient during diffusion in air

A, net CO_2 assimilation rate

A_0, the photosynthesis determined at C_i of 360 µmol mol^{-1}

A_1, the photosynthesis determined at C_a of 360 µmol mol^{-1}

AB, aboveground biomass

A_m, atomic mass of an element

ANOVA, analysis of variance

AS, apical shoot

A_{sat}, light saturated net CO_2 assimilation rate

c_a, ambient CO_2 concentration (µmol mol^{-1})

c_i, intercellular CO_2 concentration (µmol mol^{-1})

DH, dehydrogenase

E, transpiration rate

EA, elemental analyser

EA-IRMS, elemental analyser – isotope ratio mass espectrometry

F_0, initial fluorescence

F_m, maximum fluorescence

F_v/F_m, maximum quantum yield of PSII

F_v'/F_m', efficiency of excitation energy capture by open PSII reaction centre

GC-C-IRMS, gas chromatography combustion isotope ratio mass spectrometry

GC-MS, gas chromatography mass spectrometer

GCM, general circulation models

g_s, stomatal conductance

HPLC, high performance liquid chromatography

IAEA, international atomic energy agency

IPCC, intergovernmental panel on climate change

IRGA, infrared gas analyzer

IRMS, Isotope Ratio Mass Spectrometer

Je(PSII), electron transport through photosystem II

J_{max}, maximum rate of photosynthetic electron transport (based on NADPH requirement)

l, stomatal limitation

LHCII, light-harvesting complex II

LWC, leaf water content

MS, mass spectrometry

PAR, photosynthetically active radiation

PPFD, photosynthetic photon flux density

PR, photorespiration

PSII, photosystem II

q_P, photochemical quenching

R_D, dark respiration

R_{leaf}, leaf respiration

Rubisco, Ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBP, ribulose-1,5-bisphosphate

ROS, reactive oxygen species

RWC (CHR), relative water content

SCT, serveis científico-tècnics

T0, determinations conducted before the labeling

T1, determinations conducted immediately after the labeling

T2 determinations conducted few days after the labeling

TCA, tricarboxylic acid

T_{leaf}, leaf temperature

TOM, total organic matter

TSP, total soluble protein

$V_{c,max}$, maximum carboxylation rate allowed by Rubisco

VPD, vapour pressure deficit

VPDB, Vienna Pee Dee Belemnite calcium carbonate

VSMOW, Vienna Standard Mean Oceanic Water

VSP, vegetative storage proteins

WS, mild water stressed

WW, well watered

$\delta^{13}C$, carbon isotope composition

$\delta^{15}N$, nitrogen isotope composition

$\Delta^{13}C$, carbon isotope discrimination

$\Delta^{15}N$, nitrogen isotope discrimination

Φ_{PSII}, relative quantum yield of PSII

Ψ, leaf water potential

Ψ_s, osmotic potential

δ_o, isotope composition of the outlet air

δ_s ,isotope composition of the N in the Hoagland solution

$\delta Y'$, carbon or nitrogen isotope compositions from labelled samples

δY, carbon or nitrogen isotope compositions from control samples

Introduction

1. Climate Change

As a consequence of Human activities (primarily due to consumption of fossil fuel and rapid deforestation) global atmospheric [CO_2] continue to accumulate (Pearson and Palmer, 2000; Aranjuelo et al., 2009; Pardo et al., 2009). This rising in atmospheric [CO_2] has a significant effect on global climate by increasing temperature and drought (Smith et al., 2012). Climate change poses risks for human and natural systems and have impacts on lives, livelihoods, health, ecosystems, economies, societies, cultures, services, and infrastructure due to the interaction of climate changes or hazardous climate events occurring within a specific time period, the vulnerability of an exposed society or system, floods, droughts and sea-level rise (IPCC, 2014).

1.1.Preindustrial CO_2

During the Last Glacial Maximum (i.e. 18000 – 20000 years ago) and previous glacial periods, atmospheric [CO_2] dropped to 180-190 ppm, which is among the lowest concentrations that occurred during the evolution of land plants (Fig. 1). Modern atmospheric [CO_2] are more than twice than glacier period and 45% higher than preindustrial concentrations (Gerhart and Ward, 2010). The significance of a CO_2 depleted atmosphere is substantial. First, the effects of low [CO_2] on primary productivity might have influenced all the terrestrial biota. Second, if plants were adapted to the low [CO_2] of recent geological time, these adaptations could constrain responses to future CO_2 enrichment. This would leave an evolutionary legacy that could affect plant performance into the future (Sage and Coleman, 2001).

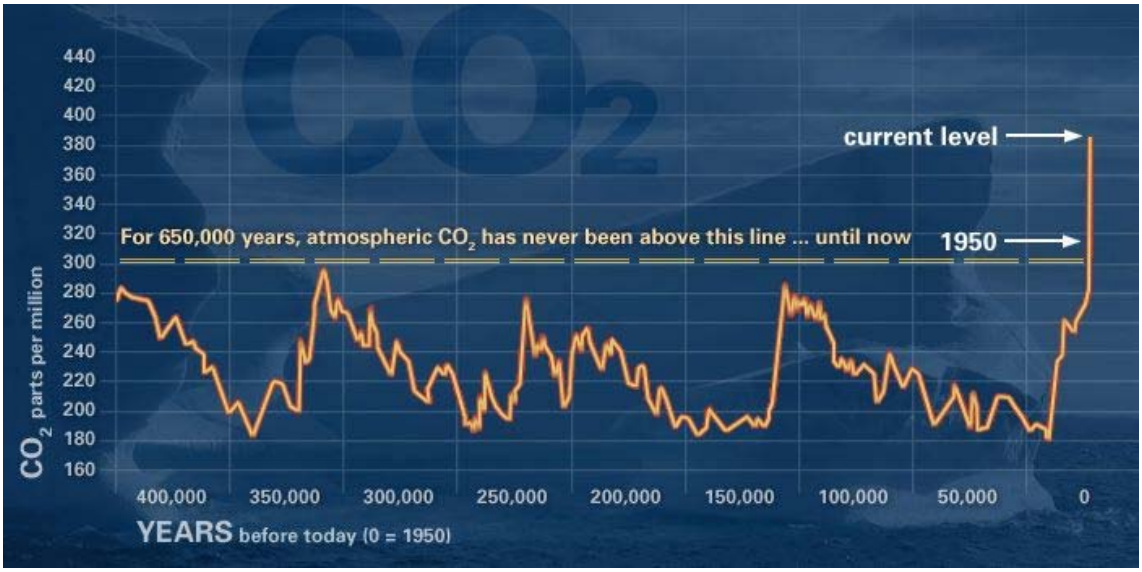

Figure 1: Atmospheric samples contained in ice cores and more recent direct measurements provide evidence that atmospheric CO_2 has increased since the Industrial Revolution (http://www.ncdc.noaa.gov/paleo/icecore/).

1.1.1. Effect of low [CO_2] on photosynthesis and productivity in C_3 plants

In C_3 plants, a reduction in atmospheric [CO_2] has direct effects on photosynthetic capacity of plants because CO_2 becomes more limiting as it is a substrate for the carboxylation reaction of Rubisco. Photorespiration is increased as the [CO_2] declines because CO_2 and O_2 compete for the same active site of Rubisco. A reduction in [CO_2]/[O_2] enhances oxygenation, resulting in carbon loss to the plant (Long and Drake, 1992). It is worth noting that unlike [CO_2], [O_2] has remained unchanged in the atmosphere for at least the last several million years (Berner et al., 2007). At optimal temperatures for C_3 photosynthesis (20–30ºC), higher temperatures enhance photorespiration and the degree to which a given low CO_2 level is limiting for Ribulose 1,5-bisphosphate (RuBP) carboxylation.

Over the past two decades, several studies (Sage, 1995; Sage and Coleman, 2001; Pagani et al., 1999; Ayub et al., 2014) have examined the effect of low CO_2 concentrations on plant productivity, generally under ideal growing conditions. Most studied species showed a proportional reduction in photosynthesis with declining CO_2. Notably, yield reductions parallel photosynthetic reductions, so that reducing CO_2 from 360 ppm to 180 ppm causes biomass to decline by 50%. This decline in potential productivity could have had significant consequences for much of the earth's biota, including human beings. The origin of agriculture, for example, might have been impeded by reduced ecosystem productivity during low CO_2 episodes of the late Pleistocene (Sage, 1995).

The predominance of low [CO_2] in the atmosphere in recent geological time and the yield reductions observed experimentally indicate that there might have been pronounced evolutionary selection pressure in response to low [CO_2]. If so, then plants of the late Pleistocene might have been adapted to lower [CO_2] than currently exist (Sage and Cowling, 1999). Given the short period of evolutionary time since low [CO_2], many plants might still be adapted to low [CO_2].

At the whole-plant level, traits conferring adaptation to low [CO_2] might include high allocation to leaf biomass at the expense of roots and a propensity to store carbohydrate during periods of carbon deficiency (Sage and Cowling, 1999). Within the photosynthetic apparatus, four clear adaptations to low [CO_2] are apparent. One is expression of high levels of Rubisco, to offset the limiting effects of low [CO_2] on carboxylation capacity. A second is the high carbonic anhydrase activity, which helps the diffusion of CO_2 from intercellular air spaces to the chloroplast. The third is the high expression of Rubisco activase, a regulatory protein that activates Rubisco by facilitating the binding of CO_2 to the active site (Coleman, 2000). A fourth adaptation to low [CO_2] might be low enzyme investment

in sucrose synthesis relative to what is needed to exploit the enhanced rates of carbon flux that can occur at high [CO_2] (Sharkey et al., 2000).

1.1.2. Photosynthetic acclimation to low [CO_2]

Photosynthetic acclimation is the physiological adjustment of plants to a given [CO_2] and can experience increases in photosynthesis at pre-industrial [CO_2] (i.e. up-regulation) and decreases in photosynthesis at future [CO_2] (i.e. down-regulation) through adjustments to the photosynthetic machinery (Nogués and Azcón-Bieto, 2013).

Many studies have shown up-regulation of photosynthesis in plants subjected to pre-industrial [CO_2] (Sage and Reid, 1992; Sage, 1994; Cowling and Sage, 1998; Anderson et al., 2001). A better understanding of plant responses to low [CO_2] will help us to understand how plants acclimated and adapted to changing carbon resources over geologic time scales. Studies of the effects of pre-industrial [CO_2] on plants are also fundamental for understanding plant evolution in response to changes in CO_2 availability over time (Ward and Strain, 1997; Ward et al., 2000; Gerhart and Ward 2010) and suggest that the influence of pre-industrial [CO_2] affected many levels, ranging from physiological effects (i.e. photosynthesis acclimation) on plants to changes in the functioning of ecosystems (i.e. C_3 species evolution; Dippery et al., 1995; Ward et al., 2000), and even played a major role in the emergence of agriculture (Gerhart and Ward, 2010).

One of the important issues in CO_2 research is the degree to which plants acclimate (exhibit phenotypic plasticity) to [CO_2] in a manner that improves performance and fitness. If acclimation completely compensates for the effects of [CO_2] on individual plant processes, evolutionary selection pressure could be minimized (Lehmeier et al., 2005). In this case, the acclimation response could be a robust predictor of effects of changing [CO_2] on the terrestrial vegetation.

In the absence of acclimation, selection pressure might be strong because the resource imbalances and inefficiencies associated with changing [CO_2] would remain in place. Identifying the nature of CO_2 acclimation is therefore crucial for understanding how the earth's vegetation responded to past changes in [CO_2] (Sage and Coleman, 2001). At the leaf level, theoretical models effectively predict differential effects of [CO_2] on the major components controlling C_3 photosynthesis (Long and Drake, 2001).

To be completely effective, an acclimation response should redress imbalances within a system caused by an environmental change and thus improve the efficiency with which resources are used. Upon transfer to low [CO_2] from the current ambient concentration, the C available to Rubisco and

the capacity of Rubisco becomes the predominant limitation (Sage and Reid, 1992). To reduce those limitations, Rubisco activity should be enhanced relative to a non-limiting process such as electron transport capacity. Because of these clear predictions, relative Rubisco content is a robust index for the potential of photosynthetic acclimation to redress imbalances caused by CO_2 variation. Many works indicates that Rubisco content can increase with prolonged exposure to low [CO_2] (Dippery et al., 1995; Pinto et al., 2014).

1.1.3. What controls plant acclimation to CO_2 variation?

[CO_2] is not the main signal for CO_2 acclimation of photosynthetic biochemistry, although it is directly important for stomatal regulation and might directly modulate carbonic anhydrase expression (Coleman, 2000). Instead, the acclimation mechanisms involve sensing of carbohydrate status by hexose flux, hexokinase activity or carbohydrate cycling. Carbohydrate signalling is a mechanism for CO_2 acclimation in part because it explains similar acclimation responses to a range of treatments perturbs source/sink ratios (Aranjuelo et al., 2009). Nutrients can also control the acclimation process and attenuate CO_2 effects, for example, N availability (Bloom et al, 2014).

1.1.4. Interaction of low levels of [CO_2] with other factors

CO_2 variation modulates the effects of environmental stress. If high CO_2 concentrations attenuate environmental stress then CO_2 depletion should increase the stress associated with a set of unfavourable conditions. The few studies that have tested this hypothesis indicate two contrasting outcomes: either CO_2 reduction enhances stress intensity or the stress attenuates the response of plants to CO_2 variation.

1.1.4.1. Temperature

Biomass reductions at high temperature and low [CO_2] in several C_3 species (i.e. wheat, tobacco, bean) was studied by Sage and Cowling (1999). They found that reductions were higher at low temperature and low [CO_2]. This response was attributed to a lower leaf expansion. It was also observed in other similar studies (Polley et al., 1993; Sage and Coleman, 2001; Vogan and Sage 2012) suggesting that altered leaf development has been implicated in the primary response to high temperature and low [CO_2]. The marked impact on leaf area production instead of photosynthesis per unit leaf area highlights the importance of understanding developmental effects of CO_2 variation.

1.1.4.2. Nutrients

Very little work has focused on the interactive effects of low [CO_2] with nutrients (Gerhart and Ward, 2010). On nutrient-deficient soils, plants often have a small growth response to CO_2 variation. This has been widely documented in high CO_2 experiments (Gutierrez et al., 2013) and has also been shown to occur at low [CO_2] as well (Lewis et al., 2010).

Effects of low nutrient supply on CO_2 sensitivity are interpreted to result from reduced sink strength and a shift of control over growth from carbon to mineral nutrient availability (Rogers et al., 2000). Whereas decreased nutrient availability can attenuate CO_2 effects, eventually at low [CO_2], a lack of carbon reduces the capacity of plants to assimilate nutrients, which contributes to growth failure (Farage et al., 1998).

1.1.4.3. Drought

The interactive effects of low CO_2 and low water availability during glacial periods (Petit et al., 1999; Ward, 1999) may have further altered the relative performance of plants. Based on physiological responses, it has been predicted that C_3 species may have been more negatively affected by the combination of low CO_2 and drought (Ward et al., 1999; 2006). C_3 species need to maintenance high stomatal conductance for increasing C uptake, but this response would simultaneously facilitate consumption and loss of water (Polley et al., 1995; Royer, 2001). In response of severe drought, C_3 plants could drop a large amount of leaf area and maintained higher leaf water potential in remaining leaves (Ward et al., 1999). Furthermore, C_3 plants grown at 180ppm CO_2 delayed reduction of stomatal conductance after the initiation of drought and retained greater leaf area (relatively to total biomass) compared to C_3 plants grown at the modern CO_2 value (Royer, 2001; Waring and Running, 2010).

1.2. Future [CO_2]

Since Industrial Revolution, CO_2 has been accumulating in the global atmosphere from 270 until 398 ppm in 2014 (NOAA-ESRL, 2014) and atmospheric concentrations continue to rise year after year (Fig. 2). The IPCC 2014 gives predictions with multi-model average indicating that by the end of the century, atmospheric CO_2 will have increases of 985 ± 95 ppm, with consequent increases in temperature (4 and 5 degrees) or drought periods.

Fossil fuels combustion is the main factor to increases [CO_2] and other greenhouse gas (GHG) accumulation in the atmosphere. Agriculture, forestry and other land uses also contribute to climate change. New FAO estimates of greenhouse gas data show that emissions from agriculture and

forestry have nearly doubled over the past fifty years and could increase an additional 30 percent by 2050, without greater efforts to reduce them (www.fao.org).

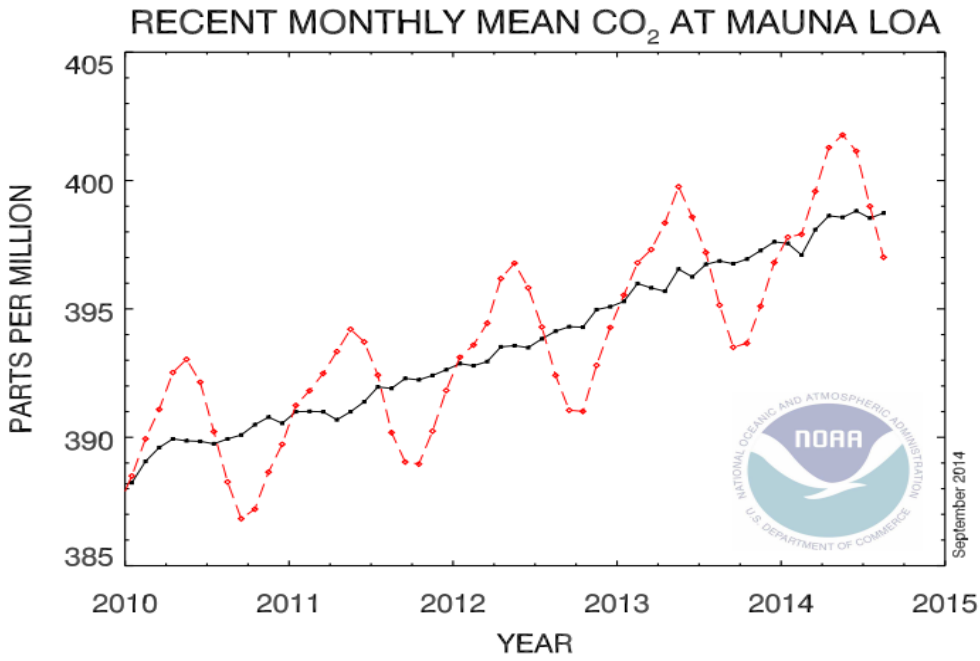

Figure 2. The last four complete years of the Mauna Loa CO_2 record plus the current year are shown. Data are reported as a dry air mole fraction defined as the number of molecules of carbon dioxide divided by the number of all molecules in air, including CO_2 itself, after water vapour has been removed. The mole fraction is expressed as parts per million (ppm). The dashed red line with diamond symbols represents the monthly mean values, centred on the middle of each month. The black line with the square symbols represents the same, after correction for the average seasonal cycle. (http://www.ncdc.noaa.gov/).

1.2.1. Effect of future [CO_2] on photosynthesis and productivity in C_3 plants

During short term (minutes to days) exposure to elevated [CO_2], the rate of light saturated assimilation (A) in many C_3 plants is generally stimulating (Sage et al., 1988).

Across a range of experiments, with a variety of plant species, they showed than plant growth at elevated [CO_2] increases leaf photosynthetic rates by an average of 40% (Ainsworth and Rogers, 2007). Carbon dioxide concentrations are also important in regulating the openness of stomata with the external environment. Open stomata allow CO_2 to diffuse into leaves for photosynthesis, but also provide a pathway for water to diffuse out of leaves. Plants therefore regulate the degree of stomatal opening (called stomatal conductance) as a compromise between maintaining high rates of photosynthesis and low rates of water loss. When [CO_2] increases, plants can maintain high photosynthetic rates with relatively low stomatal conductance (Ainsworth and Rogers, 2007). This would be expected to decrease overall plant water use, although the magnitude of the overall effect

of CO_2 will depend on how it affects other determinants of plant water use, such as plant size, morphology, and leaf temperature (Leakey et al., 2009). Plant growth also increases with future [CO_2]. This increase in growth is also reflected in the harvestable yield of crops, with wheat, rice and soybean all showing increases in yield of 12–14% under elevated [CO_2] (Ainsworth, 2008; Long et al., 2006; Taub, 2010).

1.2.2. Photosynthetic acclimation to elevated [CO_2]

Photosynthetic capacity acclimates to elevated [CO_2] in C_3 plants and the scale of down-regulation varies with genetic and environmental factors. However, despite acclimation of photosynthetic capacity, carbon gain is markedly greater (19–46%) in plants grown at the [CO_2] anticipated for the middle of this century (Leakey et al., 2009).

Elevated [CO_2] leads to Rubisco excess and plants are predicted to respond with reductions in Rubisco relative to RuBP regeneration capacity. Clearly, relative Rubisco content is a robust index for the potential of photosynthetic acclimation to redress imbalances caused by CO_2 variation (Sage and Coleman, 2001; Aranjuelo et al., 2009, 2014).

However, growth conditions play a major role, with nutrients, root volume, source/sink ratios, plant age and photoperiod acting as key modulators of the degree to which elevated [CO_2] acclimation occurs (Thomas and Strain, 1991). Conditions that enhance sink capacity relative to source strength generally attenuate acclimation responses to elevated [CO_2]. Young plants with high levels of nutrients and large rooting volumes show little acclimation and can even respond to elevated [CO_2] with increased Rubisco content (Sims et al., 1998).

Clearly, when Rubisco levels decline in response to elevated [CO_2], they are generally accompanied by reductions in other photosynthetic components such as chlorophyll binding proteins, photosystem II proteins, ATP synthases and carbonic anhydrase (Moore et al., 1999). This indicates that plants primarily reduce overall photosynthetic investment instead of simply reducing excess Rubisco (Sims et al., 1998).

1.2.3. What controls plant acclimation to [CO_2]?

In recent years, much work has focused on the mechanisms controlling photosynthetic acclimation to CO_2 variation. [CO_2] is not the main signal for CO_2 acclimation of photosynthetic biochemistry,

although it is directly important for stomatal regulation and might directly modulate carbonic anhydrase expression (Coleman, 2000). Instead, the acclimation mechanisms involve sensing of carbohydrate status by hexose flux, hexokinase activity or carbohydrate cycling (Moore et al., 1999). Carbohydrate signalling is a mechanism for photosynthesis acclimation in part because it explains similar acclimation responses to a range of source/sink ratios. Elevated [CO_2], long photoperiods and small sink capacity all lead to excessive source capacity (Aranjuelo et al., 2011). The common response to source excess is a general reduction in photosynthetic enzyme levels and an enhancement of root growth and the capacity for nutrient acquisition. Importantly, the reduction in Rubisco content that is predicted to occur under elevated [CO_2] typically occurs during acclimation caused by most treatments promote source excess (Drake et al., 1997). These adjustments can reduce the degree to which Rubisco and photosynthetic capacity are excessive under elevated [CO_2] (Sanz-Sáez et al., 2010).

1.2.4. Interaction of elevated [CO_2] with other factors.

As mentioned before, CO_2 variation modulates the effects of environmental stress. Elevated [CO_2] reduce the impact of moderate drought, salinity and temperature stress, and can indirectly reduce low nutrient stress by promoting root growth, nitrogen fixation and mycorrhizal infection (Lüscher al., 2000; Daepp et al., 2000; Hu et al., 2006).

1.2.4.1. Temperature

Temperature and atmospheric carbon dioxide (CO_2) are key environmental parameters affecting plant growth, development and function, and both have changed with the climate change. Since the effects of elevated [CO_2] and temperature on photosynthesis and respiration may be counteracting, the combined effects may differ from those of either factor alone, especially for C_3 plants (Eller et al., 2013). CO_2 and temperature interact in their effects on plants. Elevated [CO_2] can counteract the negative effects of high temperature (Lee, 2011) but high temperatures may also offset the stimulating effect of elevated [CO_2] (Clausen et al., 2011).

1.2.4.2. Nutrients

When nutrients are limiting, there is often a reduction in Rubisco concentration and in the amount of other key photosynthetic enzymes after growth at elevated [CO_2] (Sims et al., 1998).

In atmospheres of two to three times the current [CO_2], the amount of Rubisco required for a constant rate of CO_2 assimilation is predicted to be between half to a third of that needed in current atmospheres. At a constant nutrient supply, relative Rubisco concentrations generally vary by less

than 30% across a range of [CO_2] during growth. When large relative Rubisco reductions do occur, they are usually observed in nutrient deprivation.

Elevated [CO_2] also leads to changes in the chemical composition of plant tissues. Due to increased photosynthetic activity, leaf non-structural carbohydrates (sugar and starch) per unit leaf area increase on average by 30–40% under elevated [CO_2] (Ainsworth, 2008; Ainsworth and Long, 2005).

Leaf nitrogen concentrations in plant tissues typically decrease under elevated [CO_2], with nitrogen per unit leaf mass decreasing on average by 13% (Ainsworth and Long, 2005). This decrease in tissue nitrogen content is likely due to several factors: dilution of nitrogen from increased carbohydrate concentrations; decreased uptake of minerals from the soil, as stomatal conductance decreases and plants take up less water (Taub and Wang, 2008); and decreases in the rate of assimilation of nitrate into organic compounds (Bloom et al., 2010).

1.3.Water stress

Cereals are cultivated in Mediterranean areas as extensive crops where drought is the main yield limiting factor of these crops (Araus et al., 2002). On the other hand, the increase in atmospheric [CO_2] from approx. 260 before the beginning of the Industrial Revolution 250 years ago to the current [CO_2] of 398 ppm appears to have affected long term net assimilation and water use efficiency (Impa et al., 2005; Ferrio et al., 2009). Future scenarios are expected to involve a further steady in atmospheric [CO_2]. General Circulation Models (GCM) predicts that in Mediterranean area, an increase in evapotranspiration rates of plants. This increase will limit the water availability and water problems will be more serious in a region where annual evapotranspiration potential often doubles the rate of precipitation (Sabaté et al. 2002). The vegetative growth of cereals in Mediterranean areas takes place at low vapour pressure deficit (VPD) and (eventually) with well soil moisture conditions. Grain filling by contrast, may be affected by terminal water stress episodes, in particular associated with high irradiances and high temperatures (Tambussi et al 2007).

Water stress can affect almost every plant process, from membrane conformation, chloroplast organisation and enzyme activity, at a cellular level, to growth and yield reduction in the whole plant and increased susceptibility to other stresses. Reduction in photosynthetic activity and increases in leaf senescence are symptomatic of water stress and adversely affect crop growth.

Other effects of water stress include a reduction in nutrient uptake, reduced cell growth and enlargement, leaf expansion, assimilation, translocation and transpiration (Akinci and Losel, 2012).

1.3.1. Water stress in plant

Plants exposed to stress due to decreasing supply of water or other resources, or because of climatic changes, show different responses according to species and the nature and severity of the stress (Chaves et al. 2003). Water shortage significantly affects extension growth and the root-shoot ratio at the whole plant level (Passioura et al, 1993). Although plant growth rates are generally reduced when soil water supply is limited, shoot growth is often more inhibited than root growth and, in some cases; the absolute root biomass of plants in drying soil may increase water use efficiency relative to that of well-watered plants (Najarajan et al, 2010). Almost every plant process is affected directly or indirectly by water supply. Some of the most important processes for this work are listen in the following sections.

1.3.2. Osmotic adjustment mechanisms under water stress

Water is essential in the maintenance of the turgor. Turgor is also important in the opening of stomata. This is believed to be due to osmotic adjustment, the process in which solutes accumulate in growing cells as their water potential falls (Ψ_s) of osmotic potential arising from the net accumulation of solutes in response to by maintaining turgor in tissues (Blum, 2006).

Osmotic adjustment usually depends mainly on photosynthesis to supply compatible solute. As dehydration becomes more severe, photosynthesis is inhibited, resulting in a smaller solute supply for osmotic adjustment. With continued water limitation, osmotic adjustment delays, but cannot completely prevent, dehydration (Kramer and Boyer, 1995).

Wheat (and other cereals) shows other additional strategies: turgor loss and stomatal closure may occur at different relative water contents, while osmotic adjustment leads to rapid responses decreasing the effect of water stress (Richter et al, 1982). Water stress increases the osmotic pressure of the cell sap, increasing the percentage of sugar in sugar-cane and often in sugar beet, although the yield per acre may be reduced. Osmotic adjustment is usually not permanent and plants often respond rapidly to increased availability of water. Loss of osmotic adjustment can occur in less than 2 days in durum wheat, and both osmotic potentials and concentrations of some individual solutes have been shown to return to pre-stress levels within 10 days after watering (Kamely and Losel 1995).

1.3.3. Growth inhibition by drought

One of the first responses to the water stress in plants is the inhibition of cell elongation, and for that in plant growth. The growth of plants is controlled by rates of cell division and enlargement, as well as by the supply of organic and inorganic compounds required for the synthesis of new protoplasm and cell walls. Variation on leaf area is an important and fast response of plants to water stress (Passioura 1996). Secondary the root growth is also inhibited, but less than shoot area (Bradford y Hsiao, 1982) with a consequent increase of root/shoot (Muller y Whitsitt 1996).

1.3.4. Photosynthesis and respiration responses to drought

The inhibitory effect of drought over plants has been widely study (Chaves et al. 2002; 2003). The limitation of plant growth imposed by low water availability is mainly due to reductions in plant carbon balance, which is dependent on the balance between photosynthesis and respiration. Of the total carbon assimilated in photosynthesis, usually more than half is lost in respiratory processes necessary for growth and maintenance, but this balance may change under water stress (Flexas and Medrano, 2002).

Photosynthesis use to decrease under water stress. The response of photosynthesis to soil water shortage can be divided into two distinct phases: during the first stage, photosynthesis is mostly limited by restricted CO_2 diffusion (decreased stomatal conductance plus decreased mesophyll conductance); during the second stage, a general metabolic impairment eventually occurs (depending on species and conditions) (Flexas et al, 2006).

Studies examining the effects of water stress on respiration are scarcer than those analysing photosynthetic responses. Generally, the respiration rate decreases during water stress, due to reduced photosynthate assimilation and growth needs. The response of plant respiration remains within narrower ranges during stress, but the electron partitioning between the cytochrome and alternative pathways also changes in coincidence with the stomatal conductance threshold for photosynthetic impairment.

1.3.5. Water and CO_2

It has been predicted that low [CO_2] would have increased the water consumption of C_3 plants. Higher water loss is often a result of greater leaf biomass and/or area relative to total plant mass (proposed by Sage & Coleman, 2001), higher g, and greater stomatal density that enhance CO_2 uptake on a whole-plant basis, but produce greater water loss.

As with heat stress, CO_2 reduction is predicted to have a greater relative effect during drought stress because stomatal closure caused by water deficiency exacerbates limitations associated with low CO_2 (Sage and Cowling, 1999). Plants control water losses by stomata. The major function of stomata is to maximize the rate at which CO_2 can diffuse into the leaf for photosynthesis while minimizing the simultaneous loss of water vapour; so, plants regulate the amount of water that is transpired by stomata. Elevated [CO_2] lead to a reduction of stomatal conductance of C_3 plants (Leakey et al, 2009). Increases in WUE are the most common positive effect. The increase in WUE is the result of the stimulation of photosynthesis and the reduction of stomatal conductance (Urban 2003).

1.4. Nitrogen

Nitrogen (N) cycling is a fundamental ecological process, gaining in prominence because of concerns about the impact of excess N on ecosystems and the contribution to global warming (Robinson et al 2001). However, N is the main environmental factor that limits productivity in Mediterranean environment (Passioura 2002). The nitrogen cycle is being studied increasingly by measuring natural abundances of the stable isotope ^{15}N relative to that of the more abundant ^{14}N.

2. Stables isotopes in plant science

Since in the 50th, stable isotopes have started using as tracers and indicators in biology studies, this utilization have been strengthened especially in recent years as a tool for research work. Some authors (Farquhar and Sharkey. 1982; Farquhar and Richards 1984) established a detail usage of ^{13}C in leaf model. Since then, this technique has been developed and extended for research in plant physiology as a useful tool for studying flows of C, N, S, H and O through the plant in the different organs and metabolic pathways.

2.1. Theory and nomenclature

Isotopes are atoms of the same element with the same number of protons but different number of neutrons. Therefore, isotopes differ in atomic mass (Am, given by number of neutrons) but not in atomic number (Z, given by number of protons). Isotopes can be divided in stable and unstable (i.e. radioactive). The radioactive isotopes decay over time to other stable products, whereas the stable isotopes have not been observed to decay, though a few of them may be theoretically unstable with exceedingly long half-lives. Most of elements of biological interest such as hydrogen (H), carbon (C), nitrogen (N), oxygen (O) and sulphur (S) have two or more stable isotopes, the heavier one (higher Am) being in lower abundance (usually <1%, see Table 5.1.1).

Table 2.1.1. Stable isotopes and their relative abundance of the elements most commonly used in ecological and environmental research (H, C, N, O and S) (Mateo *et al*. 2004). IUPAC: International Union of Pure and Applied Chemistry

Element	Stable isotope	Relative abundance (%)
Hydrogen	^{1}H	99.9885
	^{2}H (D)	0.0115
Carbon	^{12}C	98.93
	^{13}C	1.07
Nitrogen	^{14}N	99.632
	^{15}N	0.368
Oxygen	^{16}O	99.757
	^{17}O	0.038
	^{18}O	0.205
Sulphur	^{32}S	94.93
	^{33}S	0.76
	^{34}S	4.29
	^{35}S	0.02

The isotope stable composition of a given sample is measured by Isotope-Ratio Mass Spectrometers, and it is usually expressed as the molar ratio of the two most abundant isotopes in the sample compared to the same ratio in an international standard (see Table 2.1.2), using the 'delta' (δ) notation (Coplen, 2011):

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}} \right) - 1 \qquad \text{(Eq. 1)}$$

where R_{sample} and $R_{standard}$ are the molar ratios of the heavy to light isotope (e.g. $^{13}C/^{12}C$) in the sample and in the international standard, respectively. Because the differences in ratios between the sample and standard are very small, δ values are expressed as parts per thousand or 'per mil' (‰).

Whereas the chemical properties of an atom or molecule are mostly determined by the number of electrons and its configuration, the physical properties are mainly determined by the atomic or molecular mass. Thus, isotopes are chemically identical but physically different. Hence, heavier isotopes tend to react more slowly than lighter isotopes of the same element during the physical, chemical and biological reactions.

Table 2.1.2. International standards, notation, abundance, typical range in plants and mean analytical error of the stable isotopes most commonly used in plant physiology, ecological and environmental research. Adapted from Mateo *et al.* (2004). Original data from Barbour *et al.* (2001), Ehleringer and Rundel (1988), Farquhar *et al*. (1989) and (Handley and Raven 1992).

	Isotope pair			
	$^{13}C/^{12}C$	**$^{15}N/^{14}N$**	**$^{18}O/^{16}O$**	**$^{2}H(D)/^{1}H$**
Standard	PDB[b]	Air N_2	SMOW[c]	SMOW[c]
Differential notation	$\delta^{13}C$	$\delta^{15}N$	$\delta^{18}O$	δD
Mean abundance (%)[a]	1,1	0,37	0,20	0,015
Observed range δ (‰)	-35 to -5	-10 to +10	+15 to +35	-300 to +20
Analytical error[d] (‰)	0.1	0.2	0.05-0.2	4-7

[a] Abundances of the heavier isotope against the total pool of the element
[b] PDB, Pee-Dee Belemnite (limestone): already used up, replaced by secondary standards
[c] SMOW, Standard Mean Ocean Water
[d] Overall analytical precision (standard error: sample preparation + internal error of mass spectrometer)

Isotopic composition of a given element varies considerably between the different pools of the biosphere. This phenomenon is called isotopic fractionation, and is determined by isotope effects occurring during the cleavage or formation of atomic bonds, as well as during other processes affected by atomic mass (e.g. diffusion). Thus, some substances are enriched in the heavier isotope, while others become depleted (i.e. lighter). We can distinguish two kinds of isotopic effects: kinetic and thermodynamic. The former are due to differences between isotopes in the rate of a given reaction and the latter reflect divergences in the equilibrium constants of the reaction. Kinetic isotope effects of a chain of reactions are generally non-additive, whereas thermodynamic effects are additive. Since isotope effects have values usually very close to unity, they are often expressed in terms of isotopic discrimination (Δ), defined as its deviation from unity:

$$\Delta(‰) = a - 1 = \frac{\delta_r - \delta_p}{1 + \delta_p / 1000} \qquad \text{Eq. 2}$$

where "a" is the isotope effect associated with the reaction, and δ_r and δ_p stand for the isotopic composition of reactives and products, respectively. Moreover of being a more intuitive expression of the consequences of a given process, it allows an easier comparison of the results obtained by different researchers.

Stable isotopes are powerful non-invasive probes in a number of scientific disciplines:

• Ecology (food webs)

• Biology (integrators of biological processes)

• Biochemistry (metabolic pathways)

• Palaeoclimatology (reconstruction of past climates)

• Palaeobotany (origin of cultivated plants and reconstruction of palaeovegetation)

• Food industry (detection of alteration on food and beverage products)

• Forensic medicine

In what follows the potential applications of stable carbon and nitrogen isotopes in plant sciences are presented.

2.2. Carbon isotopes in plant science

Since carbon is the most abundant element in the biosphere, and potentially participates in many biological reactions, including photosynthetic metabolism, the measurement of carbon isotope composition ($\delta^{13}C$) of organic and inorganic compounds is useful for studying e.g. processes that control C cycling within and between plants, animals, and ecosystems, and exchanges between these and other reservoirs, the atmosphere and hydrosphere (Mateo *et al*. 2004; Bowling *et al*. 2008) (Fig. 2.2.1). The utility of using C isotopes as an ecological index of plant function stems from the ability to integrate the close interplay between environmental conditions and the biochemical discriminations during C assimilation (Dawson *et al*. 2002). Therefore the $\delta^{13}C$ of source air, the environmental conditions in which plants are grown, and the fractionation processes during the C assimilation and the consequent post-photosynthetic reactions during the biosynthesis of organic compounds are reflected in the $\delta^{13}C$ of plant matter.

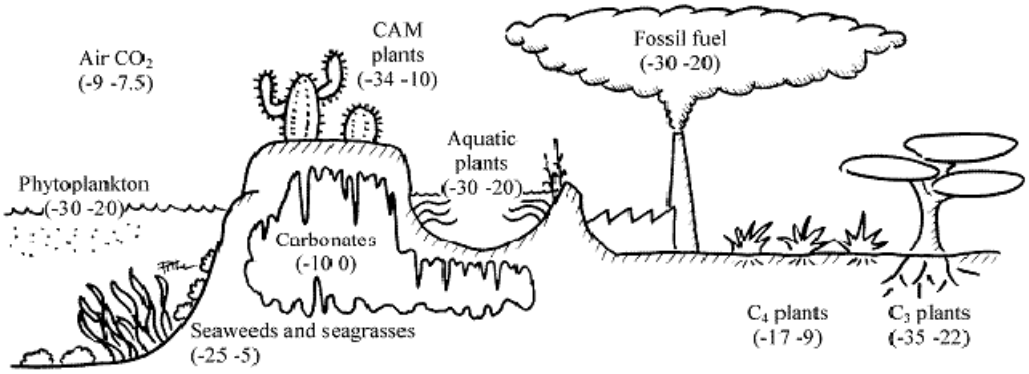

Figure. 2.2.1. Range of carbon isotopic composition ($\delta^{13}C$) within the main compartments of environment and biosphere. Redraw from Mateo et al. 2004. Original data from Ehleringer and Rudell (1988) and Vogel 1993.

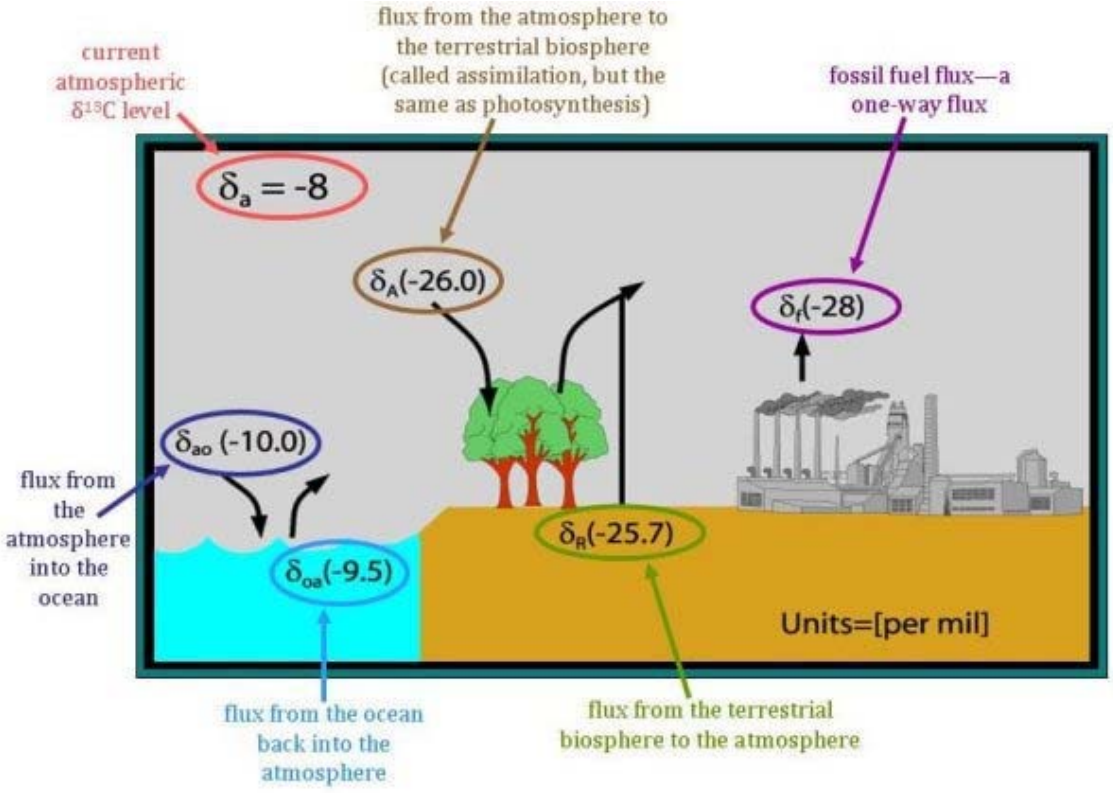

Figure 2.2.2. The $\delta^{13}C$ values of the different fluxes both into and out of the terrestrial biosphere and ocean. These differences are disequilibrium fluxes caused by differences in time in the atmospheric carbon dioxide composition.

2.2.1. Isotopic composition of source air

The $\delta^{13}C$ of atmospheric CO_2 ($\delta^{13}Ca$) averages a value of -8‰, although an overall decrease in this value has been observed since the beginning of the Industrial Revolution during the last part of the XVIII century, when values of about 6.6‰ were present (Keeling *et al*. 1979; Xu and Chen *et al* 2006). The slow, but progressive, decrease in $\delta^{13}Ca$ is caused by the synergic effect of the anthropogenic deforestation and the burning of fossil fuels of organic origin ($\delta^{13}C \approx$ -26‰, see Fig. 1.2.2) (Keeling *et al.* 1979; Farquhar *et al*. 1989).

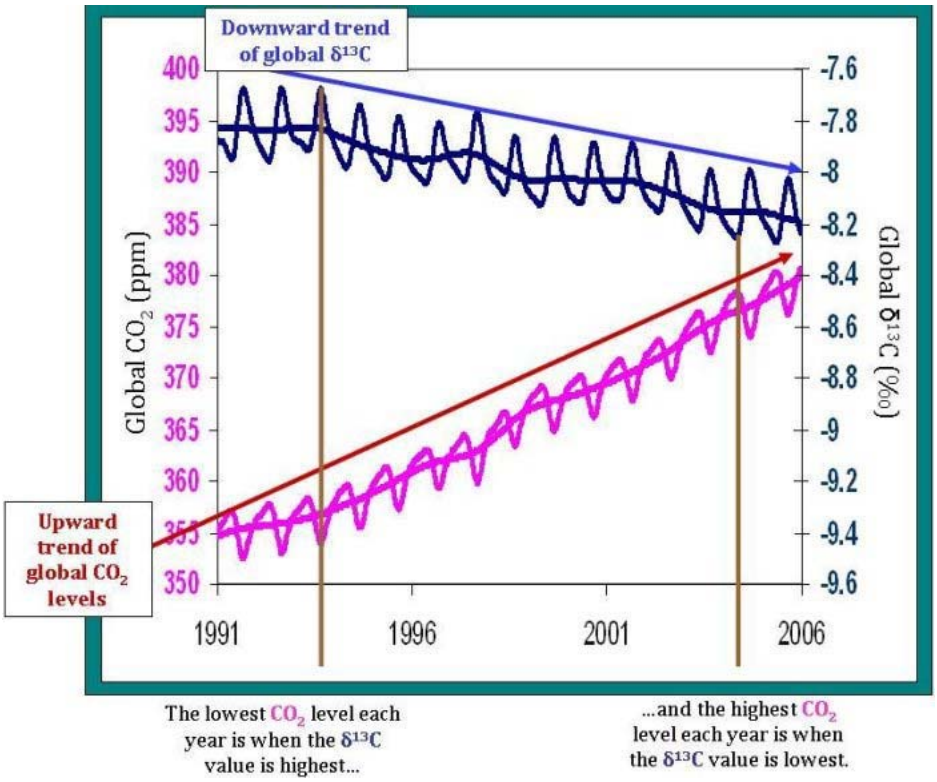

Figure 2.2.3 The evolution and comparison of global total atmospheric carbon dioxide trends and global carbon isotope composition ($\delta^{13}C$) of air at the South-West Pacific station of Mauna Loa Station, Hawaii (19.54ºN, 155.58ºW, 3397) during the period covering 1990 to 2006. Plotted with data from NOAA/GMD (http://www.cmdl.noaa.gov/index.html).

2.2.2. Discrimination against $^{13}CO_2$

Plants are normally depleted in the heavier isotope (^{13}C) compared with the atmospheric CO_2 (C source), because of the physical and chemical processes involved in CO_2 uptake. In C_3 plants, $\delta^{13}C$ values in their organic matter (OM) typically vary between -22 to -35‰. Then carbon isotope discrimination ($\Delta^{13}C$) of plant parts is calculated as Eq. 3 (Farquhar *et al.* 1989).

$$\Delta^{13}C\,(‰) = a - 1 = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p / 1000} \qquad \text{Eq. 3}$$

where $\delta^{13}C_a$ and $\delta^{13}C_p$ refer to air and the plant carbon isotope compositions, respectively.

Carbon isotope discrimination in C_3 plants

As mentioned before, there are two stable isotopes of carbon in the nature, ^{12}C and ^{13}C. ^{12}C is the most abundant isotope with the 98.9% of the atoms (Table 5.1.1.). C_3 plants are likely to dominate world's flora representing approximately the 95% of species.

There are two main steps in CO_2 uptake in C_3 plants: (i) diffusion of CO_2 from the air to the internal gas space through the boundary layer and the stomata, and the consequently dissolution in the cell sap and diffusion to chloroplast; and (ii) carboxylation reaction by Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) (Farquhar *et al.* 1982). The model of Farquhar *et al*. (1982) has been one of the most extensively used for describing carbon isotope discrimination ($\Delta^{13}C$) in leaves of C_3 plants, and is given in its simplest form as:

$$\Delta^{13}C = a\frac{c_a - c_i}{c_a} + b\frac{c_i}{c_a} = a + (b-a)\frac{c_i}{c_a} \qquad \text{Eq. 4}$$

Where "*a*" is the discrimination coefficient during diffusion in air (4.4‰), c_i and c_a are the CO_2 partial pressures in the intercellular air spaces and in the atmosphere, respectively. The term "*b*" refers to the net fractionation caused by carboxylation, and is given by:

$$b = (1-\beta)b3 + \beta b4 = b3 - \beta(b3 - b4) \qquad \text{(Eq. 5)}$$

where *b*3 is the discrimination coefficient during carboxylation by Rubisco (the main

carboxylase in C_3 plants, ≈29‰), β is the proportion of CO_2 fixation via phospho*enol*pyruvate carboxylase (PEPC) and *b*4 is the discrimination coefficient by PEPC (-5.7‰). A value of ≈27‰ is given to *b* according to empirical approximations.

2.2.3. Plant variation in $\Delta^{13}C$

Variation in $\Delta^{13}C$ is caused by genetic and environmental factors that combine to influence gas exchange through morphological and functional plant responses. However, the causes of such variation are complex and not straightforward (Dawson *et al*. 2002). Therefore, $\Delta^{13}C$ has been observed to vary in response to soil moisture (Ehleringer and Cooper 1988; Ehleringer and Vogel 1993; Stewart *et al*. 1995; Araus *et al* 1997, 2003; Korol *et al*. 1999), air humidity (Panek and Waring 1997), light (Ehleringer *et al*. 1986; Zimmerman and Ehleringer 1990), temperature (Panek and Waring 1997), nitrogen availability and source (Lopes and Araus 2006; Serret *et al*. 2008), salinity (Yousfi *et al*. 2009), atmospheric CO_2 concentration (Aranjuelo *et al*. 2008) and environmental pollutants (Bassin *et al*. 2009).

Moreover post-photosynthetic fractionation processes related with differences between autotrophic and heterotrophic tissues, respiration and transport also occur altering the original isotopic signature

imprinted in the autotrophic tissues (Ghashghaie *et al.* 2003, Nogués *et al.* 2004, 2006, 2014; Badeck *et al*. 2005; Bathellier *et al*. 2008; Cernusak *et al*. 2009).

Among the mentioned factors that may affect $\Delta^{13}C$, water availability is one of the most important (and most studied). It is well known that plants close stomata as a response to drought before any change in leaf water potential and/or leaf water content is observed (Flexas and Medrano 2002; Medrano *et al*. 2002). Thus, a decrease in $\Delta^{13}C$ with drought may be evidence of long-term stomatal limitation of photosynthesis, indicating a decrease in C_i with stress (Farquhar *et al*. 1989).

When stomata are open (Fig. 2.2.4.a), CO_2 diffuses easily into the intercellular space, and C_i is closer to C_a; thus, $\Delta^{13}C$ approaches the value of b (about 30‰). In other words, Rubisco is not limited by CO_2 and thus discrimination takes place mostly during the carboxylation step. In contrast, when stomatal conductance is reduced (Fig. 2.2.4.b), CO_2 flux is limited and C_i is significantly lower than C_a. Therefore, photosynthesis is strongly limited by stomatal conductance, and $\Delta^{13}C$ becomes closer to a, the value of the discrimination during CO_2 diffusion in air (about 4.4‰).

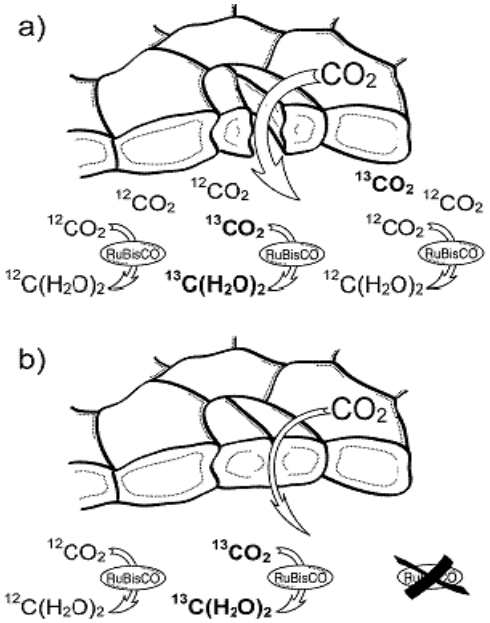

Figure 2.2.4. Simplified scheme of the relationship between carbon isotopic discrimination ($\Delta^{13}C$) in C_3 plants and stomatal conductance; a) high stomatal conductance, high discrimination: CO_2 diffuse easily into the intercellular space, the activity of the carboxylation enzyme Rubisco, is not limited by [CO_2] and thus it has more changes to discriminate against ^{13}C; b) low stomatal conductance and low discrimination: the flux of CO_2 is reduced, and the limiting factor of photosynthesis is stomatal conductance. In this case, Rubisco is forced to fix higher proportion of ^{13}C. Redraw from Ferrio *et al,* (2003).

There are several environmental factors that can modify the isotopic composition of plant tissues through their influence on either leaf conductance or photosynthetic rate, or both parameters simultaneously. Changes in irradiance levels, CO_2 concentration, and plant water status (often derived from human activities) are clearly reflected in $\delta^{13}C$ variations. The considerable variation in C_i due to environmental factors is reflected in $\delta^{13}C$ of plants.

Exist a positive linear relationship between $\Delta^{13}C$ and the ratio C_i/c_a in C_3 plants (Fig. 2.2.4), and therefore $\Delta^{13}C$ has been proposed as an integrated record of the ratio c_i/c_a over time. The c_i/c_a ratio is determined by the balance between stomatal conductance and photosynthetic rate (A/g_s ratio). Because c_i/c_a may increase by lowering *A*, increasing *g*s or by a combination of both processes (Farquhar *et al*. 1989; Condon *et al*. 2004), the ratio c_i/c_a and the ratio A/*g*s are negatively related (see Condon *et al* 2004). Therefore $\Delta^{13}C$ and A/*g*s should also be negatively related.

2.3. Nitrogen isotopes in plant science

Stable isotope of nitrogen (^{15}N) is now being used widely in research on N cycling in organisms and ecosystems. ^{15}N natural abundances are used in fundamentally different ways from traditional ^{15}N tracers by integrating N cycle processes via N isotope fractionations and the mixing of various N-containing pools. Using ^{15}N natural abundances still requires certain technical and conceptual advances before it can be used routinely in ecological research (Robinson, 2001). Natural abundance of ^{15}N has been also used to compare plant species patterns of N uptake (Dawson et al., 2002).

The composition on nitrogen stable isotope studies ($\delta^{15}N$) with natural abundance but also with enriched abundance are giving relevant information about his utility to study the N metabolism in plants (Yakir 2003) and metabolic fluxes(Yoneyama et al. 2003).

2.3.1. Source of nitrogen

The majority of nitrogen on earth is in the form of gas N_2 that makes 78% of the atmosphere. However, a vast majority of plants obtain N directly from the environment via root absorption of soil NO^{3-} or NH^{4+} (Wiliams and Miller, 2001). Plant performance its fitness, yield, nutrient efficiency or susceptibility to biological or environmental stress, generally depend on the plant ability to obtain this mineral nitrogen (Epstein and Bloom, 2005).

Isotope studies are also changing the classical view that plants use only inorganic materials for their nutrition (Schimel and Bennett, 2004). Many studies had revealed that plant use of organic N forms,

either by direct uptake from the soil solution or indirectly through mycorrhizal symbiosis, is a common feature in ecosystems (e.g., Chapin et al., 1993).

2.3.2. Discrimination against ^{15}N

Anthropogenic activity has altered the amount and relative abundance of the forms of nitrogen (NH_4^+, NO_3^-) that are available for plant absorption (Evans and Belnap, 1999). The forms of nitrogen absorbed by plants can have different isotope compositions, and many studies now routinely measure foliar $\delta^{15}N$ in an attempt to understand differences in patterns of nitrogen use among co-occurring species (Robinson, 2001).

Many studies assume that ^{15}N at natural abundance levels acts as a tracer (i.e. the isotope ratio of source nitrogen is preserved during nitrogen absorption, assimilation and translocation, and that the $\delta^{15}N$ of leaf tissues reflects that of the nitrogen source in the soil). This assumption is important because although the reported variation in plant $\delta^{15}N$ can be between −10‰ and +10‰, the difference among co-occurring species is often less (0–10‰), and biologically significant differences can be ~1‰ (Handley and Raven, 1992). However, it is clear that this assumption could be invalid because physiological factors, such as different nitrogen uptake mechanisms, different pathways of assimilation, and recycling of nitrogen in the plant, can discriminate against ^{15}N.

There are different steps (Bloom, 1988; Evans, 2001; Bloom et al., 2010) into the mechanism of NH_4^+ and NO_3^- uptake, assimilation and distribution (Fig. 2.3):

- Uptake of nitrogen from the soil solution.
- Translocation to the site of assimilation.
- Assimilation of inorganic nitrogen into organic nitrogen
- Distribution among organs

As mentioned before, physiological transformations of nitrogen can influence whole-plant and leaf ^{15}N. Besides, significant discrimination has been observed for plants grown on NO_3^- using open nutrient systems. A general pattern is that the discrimination increases with external NO_3^- concentration and decreases with plant age. Similar patterns have been observed for plants grown with NH_4^+ as their sole nitrogen source (Evans 2001; Tcherkez and Hodges 2008).

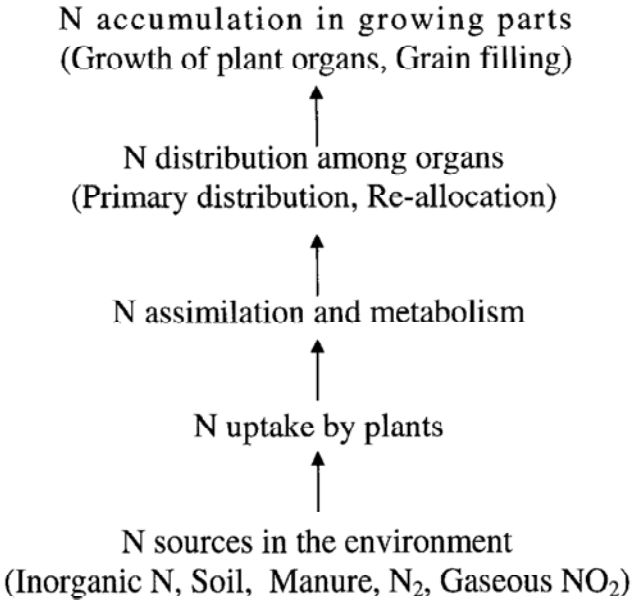

Figure 2.3. Framework on plant nitrogen, which was traced in enriched and natural ^{15}N by Yoneyama et al,(2003).

2.3.3. Plant variation of nitrogen isotope composition

Whole-plant and leaf nitrogen isotope composition are determined by the isotope ratio of the external nitrogen source and physiological mechanisms within the plant. Whole-plant isotope composition can reflect that of the nitrogen source when plant demand exceeds nitrogen supply. Uptake by mycorrhizae can cause the isotope ratio of the plant to deviate from the source. Intra-plant variation in isotope composition can be caused by multiple assimilation events, organ specific loss of nitrogen, and resorption and reallocation of nitrogen. It is important to know the role of plant to address acquisition of organic nitrogen from the soil solution, the role of mycorrhizae, and internal transformations within the plant (Evans 2001).

3. Importance of cereals (wheat)

Agriculture in the world started about 10000 years ago, coinciding with the beginning of the Holocene. From this time up to the present, C_3 cereals such as bread (*Triticum aestivum* L.) and durum (*Triticum turgidum* L. var. *durum*) wheat as well as barley (*Hordeum vulgare* L.) have remained the outstanding crops in terms of area cultivated and food source (Araus et al, 2002; Evans et al, 1999).

Food and Agriculture Organization (FAO) of the United Nation's latest estimate for world cereal production in 2013 stands at a record 2515 million tonnes (including rice in milled terms) (Fig. 3.1). At the current level, wheat production is estimated to account for 716 million tonnes (www.fao.org), 8.5 percent up from 2012, while output of coarse grains is put at 1305 million tonnes, a year-on-

year increase of almost 13 percent (Fig 3.2). Global rice production is seen to rise moderately in 2013, by less than 1 percent to 494 million tonnes.

World cereal market					
	2010/11	2011/12	2012/13	2013/14 estimate	2014/15 forecast (08 May 2014)
	(million tonnes)				
Production[1]	2 257.4	2 354.3	2 307.3	2 518.8	**2 458.2**
Supply[2]	2 779.9	2 854.2	2 826.4	3 021.5	**3 032.1**
Utilization	2 272.0	2 327.0	2 327.3	2 420.6	**2 465.7**
Trade[3]	288.9	319.4	310.2	335.7	**330.8**
Ending Stocks[4]	499.9	519.1	502.7	573.9	**565.8**
	(percent)				
World stock-to-use ratio	21.5	22.3	20.8	23.3	**22.7**
Major exporters' stock-to-disappearance ratio[5]	17.3	18.0	16.9	18.4	**17.7**

Figure 3.1: Prospect for world cereal production in 2014/2015 (source: www.fao.org)

World wheat market					
	2010/11	2011/12	2012/13	2013/14 estimate	2014/15 forecast (08 May 2014)
	(million tonnes)				
Production[1]	653.8	702.4	659.7	715.1	**701.7**
Supply[2]	844.1	887.0	839.9	872.0	**879.3**
Utilization	658.3	698.7	685.6	687.9	**699.2**
Trade[3]	128.0	147.2	140.8	150.5	**149.5**
Ending Stocks[4]	184.6	180.2	156.9	177.5	**179.5**
	(percent)				
World stock-to-use ratio	26.4	26.3	22.8	25.4	**25.2**
Major exporters' stock-to-disappearance ratio[5]	20.7	17.9	14.1	15.3	**14.2**

Figure 3.2: Prospect for world wheat production in 2014/2015 (source: www.fao.org)

Wheat is widely grown around the world under diverse climatic conditions and has been the staple food of the major civilizations in Europe, Asia and North Africa for 8,000 years. In 2007 it was the third most produced cereal after maize and rice.

Wheat (Triticum) is clearly an important crop. Recent data summarized by FAO indicate that wheat is harvested from more than 15% of global cropland and contributes directly about 20% of calories

and 22% of protein to the human food supply. Moreover, one-third of wheat harvested is fed to animals (IFPRI. 2013), so it also contributes to calories and protein in meat and milk products.

Because cereal and wheat´s importance, effects of ongoing regional and global environmental changes on wheat yield need to be better understood. For example, due to an increase in CO_2 concentrations of Earth´s atmosphere stimulates or inhibits photosynthesis in C_3 plants in wheat.

As an important staple food used in a wide variety of products, post-production operations play an important role in creating a stable food supply. Wheat is also used to produce animal feedstuffs, starch and ethanol.

Durum wheat is one of the most important crops cultivated in the south and east Mediterranean basin (www.//fao.org/). In this area, water and nitrogen limitations are the major constraints on wheat production (Araus et al, 2002).

Objectives:

El objetivo principal de esta Tesis ha sido estudiar el efecto de las diferentes [CO_2] atmosférico (preindustriales, actuales y futuras) de sobre el trigo duro. Para ello han sido necesarias diferentes aproximaciones multidisciplinares como son las mediciones del crecimiento, rendimiento, fisiología, fluorescencia de la clorofila, análisis moleculares y/o el uso de los isótopos estables.

Así, esta Tesis se enfocó desde dos puntos de vista uno teórico y otro funcional:

-Teórico: Entender los mecanismos fisiológicos que se producen debido a la variación en las [CO_2] de la atmósfera.
-Funcional: Estudiar la capacidad de adaptación de las plantas a diferentes [CO_2] y su habilidad para adaptarse a los estreses.
Por ello, plantas de trigo duro fueron colocadas en cámaras de crecimiento controladas donde se sometieron a diferentes [CO_2] y se describieron y compararon las medidas fisiológicas, de crecimiento, e isótopos estables. Los siguientes aspectos fueron estudiados:

1. Estudiar el efecto de la baja [CO_2] atmosférica en la fisiología y el crecimiento de las plantas de trigo duro para entender las adaptaciones que han sufrido las plantas desde niveles preindustriales [CO_2] hasta la actualidad.
2. Estudiar el efecto de la alta [CO_2] atmosférica en la fisiología y el crecimiento de las plantas de trigo duro y comprender las adaptaciones de las plantas que se podrán dar en el futuro a altas [CO_2].
3. Estudiar la importancia de las diferencias genéticas de una variedad de trigo antigua y otra moderna, y la selección de variedades para la mayor producción en la adaptación a diferentes [CO_2] en el ambiente.
4. Estudiar el efecto de la variación en las [CO_2] en la eficiencia en el uso del agua (WUE) y el eficiencia en el uso del N (NUE) en las plantas.
5. Estudiar las variaciones de los isótopos estables ^{13}C y ^{15}N a diferentes [CO_2] en la atmósfera.
6. Estudiar la variación genotípica en el $\delta^{13}C$ y $\delta^{15}N$ entre una variedad moderna y una variedad antigua de trigo duro modulado por las diferentes [CO_2] en la atmósfera.
7. Estudiar el efecto del estrés hídrico en la fisiología y el crecimiento de plantas de trigo duro modulado por las diferentes [CO_2] en la atmósfera.
8.

Informe del director de la Tesis:

El Dr. Salvador Nogués Mestres com a director de la Tesi que porta per títol: "**Estudio del impacto de las concentraciones altas y bajas de CO_2 sobre el cultivo de trigo"** que ha dut a terme el doctorand Salvador Aljazairi López, informa sobre l'índex d'impacte i la participació del doctorand en cadascun dels articles inclosos en la memòria de la Tesi. En tots els articles, el doctorand és el primer autor dels treballs.

Capítol 1.

Article 1: "EFFECTS OF PRE-INDUSTRIAL, CURRENT AND FUTURE CO_2 LEVELS IN TRADITIONAL AND MODERN WHEAT GENOTYPES" publicat a la revista *Journal of Plant Physiology*, amb un índex d´impacte de 2.770. En aquet estudi es va avaluar l'efecte de diferents [CO_2], en dues varietats de blat dur (una tradicional anomenada "Blanqueta" i una altre moderna anomenada "Sula"). Aquest va ser el primer experiment realitzat pel doctorand, incloent la part experimental i l'anàlisi dels resultats així com la redacció de l´article científic prèviament esmentat, essent per tant el primer autor del treball. Aquest treball suposa també un pas mes en la formació i en l'assimilació els fonaments científics i metodològics que el doctorand ha desenvolupat al llarg de la seva Tesi.

Capítol 2:

Artícle 2: "C AND N ALLOCATION AND PARTITIONING IN TRADITIONAL AND MODERN WHEAT GENOTYPES UNDER PRE-INDUSTRIAL AND FUTURE CO_2 CONDITIONS" publicat a la revista *Plant Biology*, amb un índex d'impacte de 2.405. Després del resultants obtinguts en el Capítol 2 sobre la fisiologia i creixement en condicions controlades, en aquest Capítol es va a profunditzar en l'efecte combinat de las diferents [CO_2] en dues varietats de blat dur sobre la composició isotòpica de ^{13}C i ^{15}N, i la utilització d'aquests paràmetres com a caràcters integradors relacionats amb el rendiment i l'ambient. El doctorand va participar activament durant l'experiment realitzat en lescambres de creixement i en el laboratori i l'anàlisi dels resultats així com en la redacció de l'article científic, essent primer autor del treball.

Capítol 3.

Artícle 3: "EFFECTS OF WATER STRESS IN WHEAT PLANTS GROWN UNDER DEPLETED, CURRENT AND ELEVATED CO_2 LEVELS, ha estat enviat a la revista *Environmental and Experimental Botany*, amb un índex d´impacte de 3.003 i en aquest moment està en procés de revisió.

En aquet estudi es va avaluar l'efecte combinat de la limitació hídrica i de diferents [CO_2] i la relació d'aquests factors amb el creixement i el metabolisme fotosintètic. El doctorand va participar activament duent a terme la part experimental i el l'anàlisi posterior del resultats així com la redacció de l'article científic, essent el primer autor del treball.

Results:

Chapter 1:

EFFECTS OF PRE-INDUSTRIAL, CURRENT AND FUTURE [CO_2] IN TRADITIONAL AND MODERN WHEAT GENOTYPES

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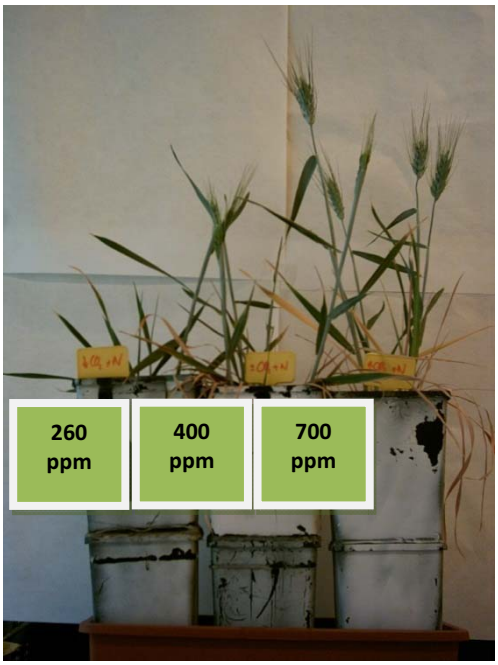

EFFECTS OF PRE-INDUSTRIAL, CURRENT AND FUTURE [CO_2] IN TRADITIONAL AND MODERN WHEAT GENOTYPES

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Running title: Photosynthetic acclimation to different [CO_2].

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 [CO_2]. 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 [CO_2] (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 [CO_2]. Moreover, we have demonstrated that at future [CO_2] the growth response will depend on the ability of plants to develop new sinks or expand existing ones.

Keywords: Chlorophyll fluorescence; Climate change; leaf nitrogen; photosynthetic acclimation; pre-industrial, current and future [CO_2]; Rubisco; wheat acclimation.

Abbreviations: $A_{max,}$ light and CO_2-saturated net assimilation rate; $A_{sat,}$ light-saturated net assimilation rate; cm, centimeter; cm^2, square centimeter; F, flag leaf; $F_v/F_{m,}$ maximum quantum yield of PSII; $F'_v/F'_{m,}$ efficiency of the capture of excitation energy by open PSII reaction centers; FxS, flag per spike; g, gram; g_s, stomatal conductance; HI, Harvest Index; ITE, Instantaneous transpiration of efficiency; J_{max}, rate of photosynthetic electron transport; NsS, number of spikelets per spike; PPFD, photosynthetic active photon flux density; PSII, Photosystem II; Φ_{PSII}, relative quantum yield of PSII ; q_p, photochemical quenching; q_N non-photochemical quenching coefficient; *NPQ,* non-photoquemical 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; $V_{c,max}$, maximum carboxylation velocity of Rubisco.

INTRODUCTION

Global atmospheric [CO_2] 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 CO_2. These models have shown that [CO_2] were 30-50% lower than currently (between 180 to 260μmol mol^{-1}) and that atmospheric [CO_2] had remained stable in the period from 150 to 1,200 years ago standing at around 260 μmol mol^{-1} (Jouzel et al., 1993 and Cowling and Sage, 1998). Since the Industrial Revolution, increases in atmospheric [CO_2] have been produced at an alarming rate and currently, [CO_2] stand at around 398 μmol mol^{-1} (NOAA-ESRL, 2014). Increases in atmospheric [CO_2] 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 [CO_2] will have reached 985±95ppm (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 CO_2 levels. Knowledge of these adaptations may help us to better understand how plants will respond to future increases in CO_2 levels (Prentice et al., 2001; Sage and Coleman, 2001; Nogués and Azcón-Bieto, 2013).

Specifically, photosynthesis in C_3 plants is usually affected by changes in [CO_2]. Moreover, there is a wide variation of responses to these changes in different species such as the acclimation of photosynthesis to different atmospheric [CO_2] after a long period of exposure (Aranjuelo et al., 2009, 2011; Pardo et al., 2009). Acclimation is the physiological adjustment carried out by plants in response to a given level of CO_2, where photosynthesis can either decrease (down-regulation) in response to high [CO_2] or increase (up-regulation) in response to low [CO_2] through adjustments made to the photosynthetic machinery (Sage, 1994; Anderson et al., 2001; Nogués and Azcón-Bieto, 2013).

Many studies suggest that the influence of low CO_2 during pre-industrial periods may have affected plants at many different levels ranging from the physiological effects on plants to changes in 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 [CO_2] (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 [CO_2] in short-term experiments. However, in long-term experiments at future [CO_2], 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., 2009; Sanz-Sáez et al., 2010). When plants which are exposed to future [CO_2] 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 [CO_2], 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 [CO_2] allow for the characterization of the effects of limited CO_2 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 [CO_2] (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 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 CO_2 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, 2007; 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 CO_2. 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 [CO_2] has been demonstrated in many experiments conducted in controlled environmental chambers and under field conditions in free air CO_2 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 [CO_2] (700 μmol mol^{-1}). This may help us to understand how plant species adapted in the past to pre-industrial [CO_2] and may be important in determining the potential of plants to evolve in response to rising [CO_2]. 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.

MATERIAL 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-litre 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 (Conviron E15, Controlled Environments Ltd, Winnipeg, Canada) at a temperature of 22/18°C (day/night) and 60% relative humidity. Plants were supplied with a photosynthetic photon flux density (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 distillated 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 [CO_2] in the air within the chambers were monitored continuously by a sensor (CMP3243 Controlled Environments Ltd, Winnipeg, Canada) over the period of the experiment at intervals of every 5 minutes 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 [CO_2] (i.e. 700, 400 and 260 μmol mol^{-1}) for the entire life cycle (from September to January) at the Experimental Field Service of Barcelona University, Barcelona, Spain. Forty-eight plants were placed in the first plant-growth chamber, which was maintained at future [CO_2] (ca. 700 ± 18 μmol mol^{-1}) by injecting CO_2

into the chamber from an external bottle (Carburos Metálicos SA, Barcelona, Spain). Another forty-eight plants were placed in the second plant-growth chamber, which was maintained at current [CO_2] (ca. 400 ± 20 μmol mol^{-1}). Finally, the same number of plants was located in the third plant-growth chamber, which was maintained at pre-industrial [CO_2] (ca. 260 ± 28 μmol mol^{-1}). Air in this chamber was maintained at pre-industrial [CO_2] by using a pump to send the air inside the chamber through a 1-litre column filled with soda lime (Soda lime with indicator QP Panreac Quimica 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 (LI6400-40) was used to perform simultaneous measurements of gas exchange and chlorophyll fluorescence. A-Ci curves with chlorophyll fluorescence determinations were conducted in fully expanded flag leaves from each treatment of CO_2 and for each genotype, Sula and Blanqueta. The A-Ci curves were repeated in four different plants per treatment and genotype, and were measured from 0 to 2000 μmol mol^{-1} of CO_2. The curves were made at 1200 μmol m^{-2} s^{-1} of PPFD and at a temperature of 25 ºC. 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 Farquhar (1981). Estimations of the maximum carboxylation velocity of Rubisco ($V_{c,max}$), the rate of photosynthetic electron transport based on NADPH requirement (J_{max}) and the rate of day respiration (Rd) were made by fitting a maximum likelihood regression below and above the inflexion of the A-Ci response using the method 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-photoquemical quenching coefficient (q_N) and non-photoquemical quenching (NPQ) (Nogués and Baker, 2000).

Instantaneous transpiration of efficiency (ITE) was calculated for samples as Assimilation/stomatal conductance (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 g-number for 5 minutes. An aliquot of each extract was used to measure soluble protein by spectrometry, with reference to a standard line which was calculated with BSA (Bobine 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 ºC 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 ± 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 Harvest Index (HI) (0.26; 0.24; 0.31) than Sula (0.56; 0.54; 0.55) in future, current and pre-industrial [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 [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 [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 [CO_2] than did Sula and at current [CO_2], the effects on biomass were not significant. A similar effect was found in Sula plants grown at pre-industrial [CO_2] (Fig. 1; Table S1).

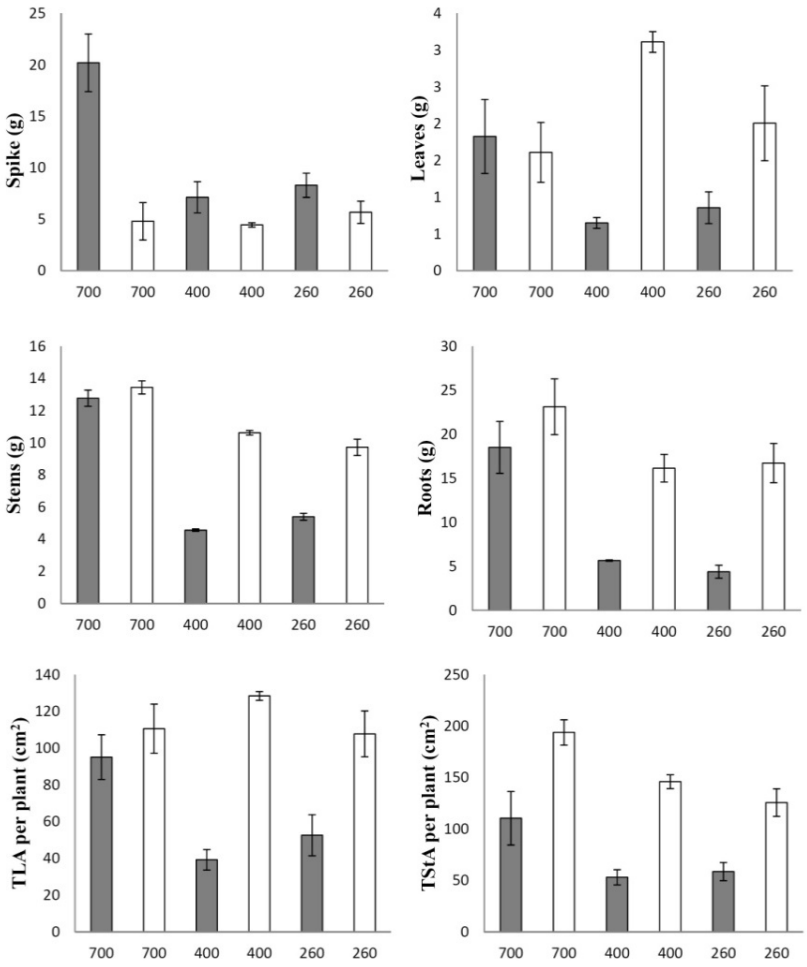

Figure 1: Total spike biomass per plant (g), total leaf biomass per plant (g), total stem biomass per plant (g), total root biomass per plant (g), total leaf area per plant TLA (cm^2) and total stem area per plant TStA (cm^2) in durum wheat genotypes Sula (grey bars) and Blanqueta (white bars) under three CO_2 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 [CO_2], 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 [CO_2] than at current [CO_2]. 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 than in Blanqueta (Table 1). At current [CO_2], 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 [CO_2], thereby showing up-regulation of photosynthesis. A decrease in the respiration rate was also observed when [CO_2] 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 [CO_2], these were studied by means of chlorophyll fluorescence measurements. Future [CO_2] 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 [CO_2]. On the other hand, at pre-industrial [CO_2], with respect to current [CO_2] 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 CO_2 treatments. Only in the case of pre-industrial [CO_2] did the modern genotype have lower photochemical efficiency and higher NPQ than the traditional genotype (Fig. 2).

Table 1: Physiological parameters in durum wheat genotypes (Sula and Blanqueta) at three CO_2 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). 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, A_{360}: Assimilation rate at Ci of 360, A_{max}: maximum Assimilation rate at light and CO_2 saturation; Ci/Ca: internalCO_2/ambientCO_2 ratio, *R*d: dark respiration.

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

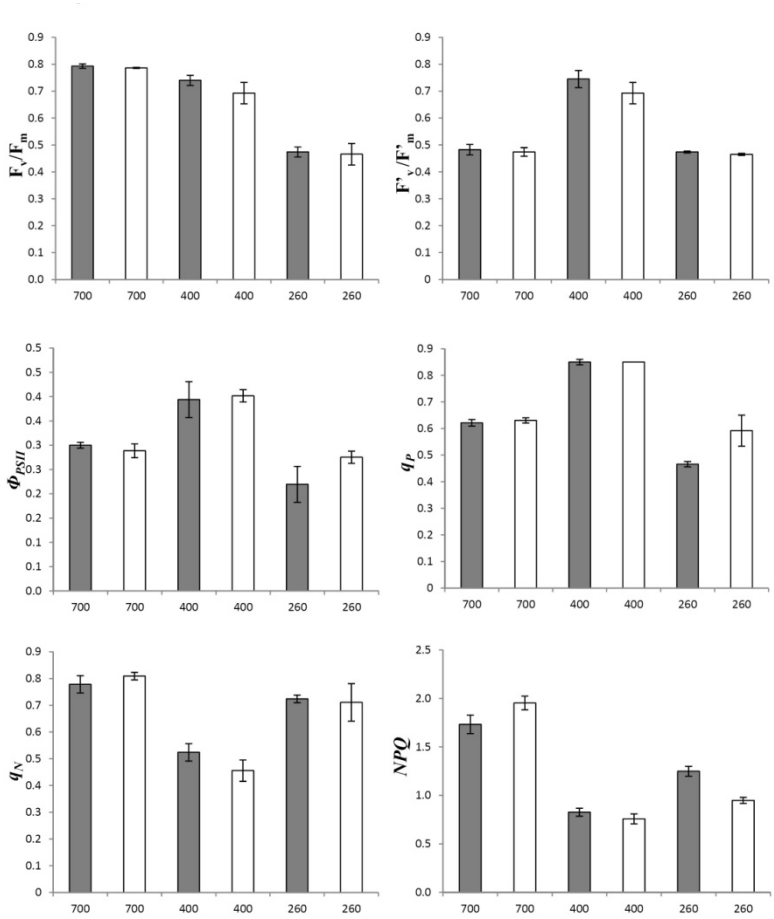

Figure 2: Fluorescence parameters (F_v/F_m: maximal photochemical efficiency in the dark-adapted stage; Φ_{PSII}: quantum yield of Photosystem II electron transport; F'_v/F'_m: maximal photochemical efficiency in light; q_P: photochemical quenching; *NPQ*: non-photochemical quenching, and q_N: non-photochemical quenching coefficient) in durum wheat genotypes Sula (grey bars) and Blanqueta (white bars) under three CO_2 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 S4. Data are means ± SE, n = 4.

Instantaneous transpiration efficiency (ITE)

Although no significant differences in ITE between CO_2 treatments were observed, there were significant differences between genotypes (P<0.05). Lower ITE in Sula was directly proportional to lower levels of CO_2 suggesting that the ability to scale this response may be relatively straightforward. On the other hand, stomatal conductance (g_s) decreased when [CO_2] 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 [CO_2] (Fig. 3).

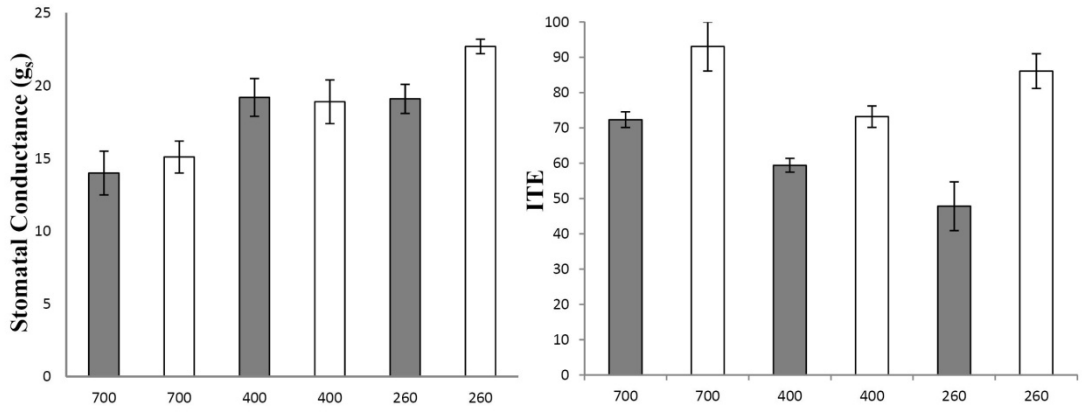

Figure 3: CO_2 effects (700, 400 and 260 µmol mol^{-1}) on stomatal conductance (gs) and Instantaneous transpiration of efficiency efficiency (ITE) in durum wheat genotypes Sula (grey bars) and Blanqueta (white bars). Statistical analyses are presented in table S4. Data are means ± SE, n = 4.

Leaf nitrogen content and nitrogen-use efficiency

At future [CO_2], plants have less N concentration in leaves (32 and 37% for Blanqueta and Sula respectively) than at current [CO_2], whereas at pre-industrial [CO_2] 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 [CO_2]. Furthermore, these differences were much greater at pre-industrial and current [CO_2] than at future [CO_2]. Specifically, NUE in Sula at 260 and 400 µmol mol^{-1} was 76% lower than Blanqueta whereas at future [CO_2] it was only 7% lower (Fig. 4).

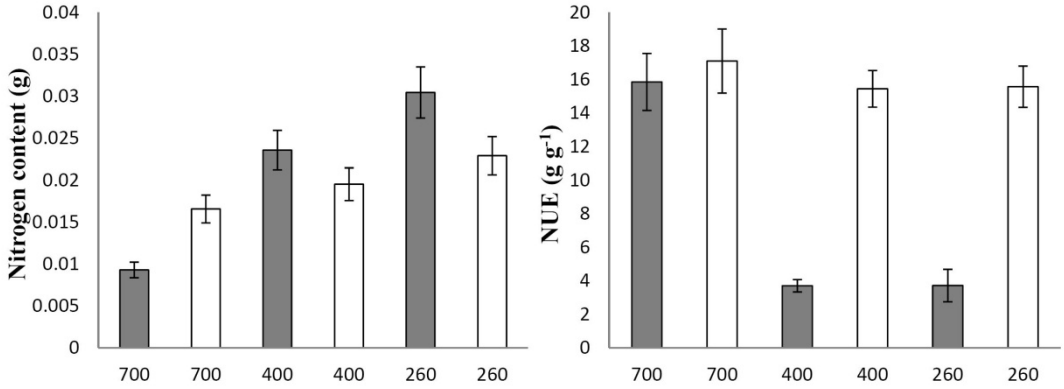

Figure 4: CO_2 effects (700, 400 and 260 µmol mol^{-1}) on N content (g) and nitrogen use efficiency (NUE) in durum wheat plants genotypes Sula (grey bars) and Blanqueta (open bars) during grain filling. Statistical analyses are presented in table S4. Data are means ± SE, n = 4.

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 [CO_2] than at current [CO_2] (22% for Sula and 31% for Blanqueta) whereas levels were greater at pre-industrial [CO_2] than at current [CO_2] (20% for Sula and 29% for Blanqueta). On the other hand, levels of protein concentrations were lower in Sula than Blanqueta across all CO_2 treatments (Table S2A).

Both genotypes showed greater concentrations of Rubisco at pre-industrial [CO_2], however, only in the case of Sula were concentrations of Rubisco lower at future [CO_2]. The biggest differences between CO_2 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 [CO_2].

At the beginning of the experiment, it was seen that at future [CO_2] there was an increase in C assimilation and growth parameters of plants, however, at pre-industrial [CO_2] a decrease in all these parameters was observed. Nevertheless, after a few weeks, we found that plants underwent an acclimation process at different [CO_2]. Acclimation is the physiological adjustment carried out by plants where photosynthesis can decrease with elevated [CO_2] (down-regulation) or increase with low [CO_2] (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 [CO_2] (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, Reynols et al., 1999) and (ii) the availability of C and N to plants (Fuertes-Mendizabal et al., 2010). 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-Felix, 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 CO_2 is one of the most important issues in CO_2 studies (Sage and Coleman, 2001) and this acclimation at pre-industrial [CO_2] (increases in photosynthesis i.e. up-regulation) and future [CO_2] (decreases in photosynthesis i.e. down-regulation) during long-term exposure can compensate for the effects of CO_2 variation on plant processes. Many studies have shown that photosynthesis decreases at future [CO_2] 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 [CO_2], 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 [CO_2] 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. 2009). 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 [CO_2], 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 [CO_2] 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 [CO_2] since Sula has been shown to have the capacity to expand its sinks as previously mentioned (Table 1; Aranjuelo et al. 2009; Sanz-Sáez, et al., 2010). This is because, firstly, there were no effects on biomass and assimilation

in traditional plants and, secondly, the aversion of the down-regulation of photosynthesis in modern genotypes during grain filling at future [CO_2] 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 [CO_2] plants had less Rubisco and photosynthesis was down-regulated (Sicher et al., 1997; Moore et al., 1998; Moore et al., 1999; Urban, 2003; Pandurangam et al., 2006; Aranjuelo et al., 2011; Aranjuelo et al., 2013). In our case, at future [CO_2] Sula plants had lower levels of Rubisco than at current [CO_2], 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 [CO_2] Blanqueta showed no decrease in Rubisco and maintained low assimilation rates during grain filling. Many studies have shown that the pre-industrial CO_2 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 C_3 plants, at optimal temperatures and nutritional conditions, pre-industrial [CO_2] can lead to a reduction in photosynthetic capacity (Sage, 1995). However, the effect of pre-industrial [CO_2] 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 [CO_2] for C_3 plants and observed an up-regulation of photosynthesis in response to pre-industrial [CO_2] 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 [CO_2] 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 [CO_2] than those grown at current [CO_2] (Maherali et al. 2002). Gesch et al. (2000) observed increased Rubisco small subunit gene expression in *Oryza sativa* L. exposed to pre-industrial [CO_2]. Other studies have also found evidence for photosynthetic acclimation at pre-industrial [CO_2] (Sage and Reid, 1992; Cowling and Sage, 1998). In Sula at future [CO_2], 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 [CO_2], 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 [CO_2] (Table 1) .

We could also observe the effect of [CO_2] treatments in chlorophyll fluorescence measurements, where it was shown that PSII activity is less affected at future than at pre-industrial [CO_2]. 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 [CO_2] (F_v/F_m is higher), therefore, at future [CO_2] 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 [CO_2], 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 [CO_2] 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 [CO_2] indicating that limitations in carbon assimilation caused a decrease photochemical quenching. The highest rates of *NPQ* and q_N were observed at future [CO_2] 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 [CO_2] had higher q_N than plants at pre-industrial [CO_2]. Thus, there was greater protection of PSII at future [CO_2]. At pre-industrial [CO_2], 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 CO_2, the dissipation of energy as heat was not very efficient (with lower values of *NPQ* and q_N than at future [CO_2]) 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 [CO_2].

In terms of the potential of plants and leaves to avoid stress, this can be indicated by Instantaneous transpiration of efficiency (ITE) (Farquar and Sharkey, 1982). The exchange of

water vapor and CO_2 is controlled mainly by the stomatal aperture. This, as well as conductance of the leaf depend on irradiance, temperature, air humidity and internal [CO_2] (Kutsch et al., 2001). Plant ITE and stomatal aperture is strongly dependent on atmospheric CO_2, this being lower at pre-industrial [CO_2] (Cowling and Sykes, 1999). Furthermore, Polley et al., (1993; 1995) observed that at pre-industrial [CO_2] *Triticum aestivum* had reduced ITE values. In contrast however, for the same plant species ITE was higher at future [CO_2] (Aranjuelo et al., 2011). Experimental data indicate that ITE increases from pre-industrial to future [CO_2] (Polley et al., 1995; Gerhart et al., 2010). Moreover, plants can regulate stomatal conductance (g_s) in accordance with the [CO_2] in the environment. Plants subjected to pre-industrial [CO_2] tended to open more stomata than plants at future [CO_2] (increased stomatal closure and so conductance was lower) (Fig. 3). These results indicate that plants growing at pre-industrial [CO_2] need to keep the stomata open in order to assimilate more [CO_2]. Thus, plants have a lower ITE at pre-industrial than at future [CO_2]. On the other hand, Blanqueta increased its ITE at pre-industrial [CO_2] since plants were able to increase their assimilation rates.

The leaf is a major storage organ for nitrogen. At future [CO_2] 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 [CO_2] 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 [CO_2], 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 [CO_2] after long-term exposure on two durum wheat genotypes (traditional and modern). Plants underwent acclimation processes under long-term exposure at future [CO_2] 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 μmol mol^{-1} and decreasing at 700 μmol 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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SUPLEMENTARY MATERIAL

Table S1. Biomass parameters in durum wheat genotypes (Sula and Blanqueta) under three CO_2 growth conditions (future 700 µmol mol^{-1}, current 400 µmol mol^{-1} and pre-industrial 260 µmol mol^{-1}) and three measuring periods (Pre-anthesis, T0; grain filling, T1; and end of grain filling, T2). Anova Duncan (ns non-significant, *<0.05; **<0.01; ***<0.001) Data are means ± SE, n = 4. T: measuring periods, SN: spike number, SL: Spike Length, NsS: Number of spikelets per spike, StN: Stem Number, StL (cm): Stem length, Zadok: zadok scale, F(g): total flag biomass per plant, S(g): total spike biomass per plant, FxS(g): flag biomass per spike, L(g): Leaf biomass, St(g): Stem biomass, R(g): Root biomass, TFA(cm^2): total flag area, TSA(cm^2): total spike area, TLA(cm^2): Total leaf area, TStA(cm^2): total stem area.

CO_2	Genotype	T	SN	SL(cm)	NsS	StN	StL(cm)	Zadok	F (g)	S(g)	FxS(g)	L(g)	St (g)	R (g)	TFA(cm^2)	TSA(cm^2)	TLA(cm^2)	TStA(cm^2)
700	Blanqueta	T0	3.3±0.3	5.3±0.5	16.0±0.6	4.3±0.3	62.7±9.5	59.7±3.5	0.4±0.1	3.0±0.3	0.13±0.01	2.1±0.5	12.2±2.2	21.1±6.1	22.7±8.5	49.2±4.9	94.7±17.7	147.1±29.9
	Sula		3.0±0.6	6.7±0.1	15.3±0.3	3.0±0.5	55.6±0.3	77.3±0.8	0.5±0.1	12.4±2.3	0.19±0.02	1.9±0.4	10.5±1.9	22.9±0.9	28.9±3.8	69.1±12.2	95.0±24.8	113.3±31.3
400	Blanqueta		6.0±1.2	6.4±0.2	18.0±0.0	6.7±0.8	64.0±0.9	57.7±0.6	1.2±0.3	5.4±1.1	0.19±0.03	2.9±0.5	20.2±3.4	33.7±2.3	74.7±21.3	101.8±23.3	136.2±21.8	202.5±36.9
	Sula		2.6±0.3	5.7±0.1	13.3±0.7	2.7±0.7	45.0±0.3	85.0±0.0	0.4±0.0	6.6±0.7	0.17±0.01	0.9±0.0	4.5±0.5	6.8±0.5	24.1±3.5	55.4±8.4	38.6±10.1	44.8±6.0
260	Blanqueta		3.3±0.3	6.2±0.2	17.0±0.6	4.3±0.3	64.5±2.0	59.7±0.3	0.4±0.1	3.1±0.3	0.13±0.01	3.6±0.3	9.9±0.8	17.5±1.0	25.9±2.6	55.5±2.3	186.2±10.3	103.3±10.2
	Sula		2.6±0.6	6.5±0.2	14.6±0.3	2.7±0.6	42.7±2.6	80.0±0.0	0.5±0.1	8.1±0.5	0.20±0.05	1.3±0.4	4.7±0.6	5.6±1.1	24.6±1.0	64.1±9.5	74.4±20.4	52.1±9.2
700	Blanqueta	T1	2.3±0.8	5.9±0.2	16.3±1.4	4.3±0.3	72.4±4.6	64.7±2.0	0.4±0.1	2.9±0.8	0.20±0.06	1.5±0.8	10.8±2.4	17.7±4.6	17.3±5.3	41.3±9.9	130.2±46.3	112.5±28.7
	Sula		3.0±0.0	6.3±0.2	14.3±1.2	3.0±0.7	54.1±2.3	81.7±1.7	0.6±0.1	11.8±0.7	0.19±0.03	1.3±0.4	9.1±0.7	13.7±1.9	28.9±3.8	74.7±7.2	57.1±21.6	87.4±12.1
400	Blanqueta		4.0±0.6	6.6±0.1	19.6±0.3	4.3±0.7	70.0±3.3	67.7±0.3	1.2±0.3	5.8±1.2	0.29±0.04	3.2±0.6	16.6±3.2	26.3±3.7	62.3±17.1	112.9±44.3	173.7±32.1	207.4±29.2
	Sula		3.6±0.6	6.5±0.1	15.3±0.3	3.6±0.7	51.4±2.7	85.7±0.7	0.6±0.0	13.1±1.6	0.18±0.04	0.8±0.1	7.5±1.1	6.6±1.2	32.8±1.7	86.5±11.4	49.6±4.9	74.7±9.9
260	Blanqueta		4.6±0.3	6.7±0.5	16.6±0.8	6.3±0.7	64.8±2.6	71.3±1.8	0.6±0.0	4.8±0.2	0.13±0.01	3.9±1.0	14.4±1.6	23.6±2.5	37.1±2.9	68.8±4.9	230.1±42.3	186.7±31.0
	Sula		3.3±0.3	6.3±0.1	14.0±0.6	3.3±0.3	40.5±0.3	83.7±3.3	0.4±0.0	6.4±0.9	0.13±0.00	0.6±0.1	4.4±0.7	4.6±1.2	26.1±2.7	49.6±11.2	38.7±8.4	44.7±9.3
700	Blanqueta	T2	3.3±0.8	5.6±0.3	17.3±1.2	4.3±1.0	81.5±4.8	72.7±4.3	0.4±0.3	4.8±1.8	0.12±0.02	1.6±0.8	13.4±5.9	23.1±8.1	38.9±5.9	88.6±39.8	110.6±33.4	193.9±12.3
	Sula		4.6±0.6	7.0±0.7	16.6±1.8	4.7±0.7	53.9±1.4	87.0±1.1	1.0±0.3	20.2±4.8	0.22±0.02	1.8±0.5	12.8±2.6	18.5±2.9	52.3±7.6	106.3±21.0	95.2±24.2	110.5±26.0
400	Blanqueta		3.3±0.3	6.5±0.3	19.3±0.9	4.3±0.8	79.9±2.4	72.0±1.0	0.5±0.0	4.4±0.2	0.17±0.02	3.1±0.1	10.6±0.7	16.1±1.5	31.8±2.8	65.9±5.1	128.4±2.3	146.0±6.7
	Sula		3.3±0.6	5.4±0.2	13.0±0.6	3.3±0.0	49.8±4.2	87.0±0.0	0.5±0.0	7.1±1.5	0.16±0.03	0.6±0.1	4.6±0.4	5.6±0.0	30.6±2.9	56.5±4.9	39.3±5.6	53.1±7.4
260	Blanqueta		4.0±0.0	6.8±0.8	18.6±2.2	5.3±0.3	62.6±2.6	74.3±0.7	0.8±0.1	5.7±1.1	0.21±0.02	2.0±0.6	9.7±1.0	16.7±3.2	46.4±6.1	91.8±30.6	107.8±32.4	125.8±13.4
	Sula		4.0±0.5	5.5±0.1	12.6±0.3	4.0±0.6	41.9±0.5	85.0±0.0	0.7±0.1	8.3±1.1	0.17±0.04	0.8±0.2	5.4±1.0	4.4±0.7	39.6±7.8	67.8±7.3	52.6±11.2	58.7±8.8
CO_2 treatment			n.s.	n.s.	n.s.	n.s.	***	n.s.	n.s.	*	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.	***	***
CO_2treatment* genotype			*	***	***	n.s.	***	n.s.	*	***	**	n.s.	**	**	**	*	**	*

Table S2: Total soluble protein concentrations TSPC, (table A) and the % of Large (rbcL) and Small (rbcS) subunit of Rubisco (B) on leaves of two varieties of wheat in response to three CO_2 treatments. Data is the mean of (at least) three replicates ± SE. rbcL and rbcS data is normalised at 400 μmol mol^{-1}. Data are means ± SE, n = 4.

A

TSPC (μg protein g^{-1} leaf)	700 μmol mol^{-1}	400 μmol mol^{-1}	260 μmol mol^{-1}
Sula	0.313±0.002	0.397±0.001	0.479±0.007
Blanqueta	0.408±0.002	0.590±0.002	0.759±0.004

B

Genotype	Rubisco	700 μmol mol^{-1}	400 μmol mol^{-1}	260 μmol mol^{-1}
Sula	rbcL	70.76	100	109.75
Sula	rbcS	46.09	100	152.21
Blanqueta	rbcL	122.66	100	117.08
Blanqueta	rbcS	114.94	100	176.04

Table S3: Statistical analysis of CO_2 effects (700, 400 and 260 ppm) on $\delta^{13}C$ (‰),atom % ^{15}N, F_v/F_m, F'_v/F'_m, Φ_{PSII}, q_P, g_s, *ITE, N content (g), NUE and protein content.* Anova Tukey-b (ns: non-significant; *<0.05; **<0.01; ***<0.001) was made. Where differences were found, the Duncan multiple tests were carried out and values with different letters indicate significant differences at $P < 0.05$.

	Organ	CO_2 Treatment	Genotype	CO_2Treatment * Genotype	CO_2 Treatment		
					700	400	260
$\delta^{13}C$ (‰)	**Leaves**	***	0.472	0.634	a	b	c
	Spikes	***	0.592	0.619	a	b	b
	Stems	***	0.496	0.740	a	a	b
	Roots	***	0.467	0.986	a	b	b
atom % ^{15}N	**Leaves**	**	0.636	0.885	a	ab	b
	Spikes	**	0.568	0.770	a	b	b
	Stems	**	0.365	0.560	a	b	b
	Roots	**	0.859	0.737	a	b	b
F_v/F_m	**Flag Leaf**	0.06	*	*	a	b	c
F'_v/F'_m	**Flag Leaf**	0.45	*	*	b	a	b
Φ_{PSII}	**Flag Leaf**	0.77	*	*	b	a	b
q_P	**Flag Leaf**	0.136	0.119	*	b	a	cb
NPQ	**Flag Leaf**	0.126	0.490	0.356	a	b	a
q_N	**Flag Leaf**	0.909	0.165	0.77	a	c	bc
g_s	**Flag Leaf**	*	0.268	*	c	b	ab
ITE	**Flag Leaf**	0.089	*	*	ab	bc	ac
N content (g)	**Leaves**	*	0.876	0.258	cb	b	ab
NUE	**leaves**	*	*	*	a	ab	ab
Protein content	**leaves**	***	***	***			

Chapter 2:

C AND N ALLOCATION AND PARTITIONING IN TRADITIONAL AND MODERN WHEAT GENOTYPES UNDER PRE-INDUSTRIAL AND FUTURE CO_2 CONDITIONS.

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C AND N ALLOCATION AND PARTITIONING IN TRADITIONAL AND MODERN WHEAT GENOTYPES UNDER PRE-INDUSTRIAL AND FUTURE CO_2 CONDITIONS.

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Running title: C and N allocation under different CO_2 concentrations

ABSTRACT

The results of a simultaneous ^{13}C and ^{15}N labeling experiment with two different durum wheat cultivars, Blanqueta (a traditional wheat) and Sula (modern) are presented in this manuscript. Plants were grown from the seedling stage in three fully controllable plant-growth chambers for one growing season and at three different CO_2 levels (i.e. 260, 400 and 700 ppm). Short-term isotopic labeling (*ca*. 3-days) was performed at the anthesis stage using $^{13}CO_2$ which was supplied with the chamber air and $^{15}NH_4$-$^{15}NO_3$ applied with the nutrient solution, thereby making it possible to track the allocation and partitioning of ^{13}C and ^{15}N in the different plant organs. We found that photosynthesis was up-regulated at pre-industrial CO_2 levels, whereas down-regulation occurred under future CO_2 conditions. ^{13}C labeling revealed that at pre-industrial CO_2 carbon investment by plants was higher in shoots, whereas at future CO_2 more carbon was invested in roots. Furthermore, the modern genotype invested more carbon in spikes than did the traditional genotype, which in turn invested more in non-reproductive shoot tissue. ^{15}N labeling revealed that the modern genotype was better adapted to assimilating N at higher CO_2 levels, whereas the traditional genotype was able to assimilate N more efficiently at lower CO_2 levels.

Key words: CO_2, climate change, carbon and nitrogen partitioning, stable isotopes.

Abbreviations: $\delta^{13}C$, carbon isotope composition; $\Delta^{13}C$, carbon isotope discrimination; A_n, net photosynthetic rate; C_{new}, new labeled C in respired CO_2 ; IRMS, isotope ratio mass spectrometry; PPFD, photosynthetic photon flux density; R_d, dark respiration; TOM, total organic matter.

INTRODUCTION

The rapid increase in the concentration of atmospheric CO_2 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 greater negative impact on wheat than other crops as for example potato, maize and rice (IFPRI 2007; 2013).

The concentration of atmospheric CO_2 remained stable at 260 ppm for thousands of years prior to the Industrial Revolution, however, since then, CO_2 has steadily been accumulating in the atmosphere (Pagani *et al.* 1999; Pearson & Palmer 2000). Currently, CO_2 concentrations 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 the century atmospheric CO_2 will have reached 985 ± 95 ppm (IPCC, 2013). This is expected to result in increases in temperatures (up to 5 ^{o}C) and more frequent and prolonged periods of drought. Therefore, in order to provide answers as to how plants will adapt to future CO_2 levels, it is essential to understand how plants have adapted from past to current CO_2 levels (Prentice *et al.* 2001; Sage & Coleman 2001).

In C_3 plants, photosynthesis is usually affected by changes in CO_2 levels. Plants undergo a wide variety of changes in response to increases in CO_2 levels and can acclimatize to different atmospheric CO_2 after prolonged periods of exposure (Pardo *et al*. 2009; Aranjuelo *et al*. 2009b, 2011a). Photosynthetic acclimation can be defined as the physiological adjustment of plants to a given CO_2 concentration where plants undergo increases in photosynthesis at pre-industrial CO_2 (up-regulation) and decreases in photosynthesis at future CO_2 levels (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 which 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 CO_2 (Sage & Reid 1992; Sage 1994; Cowling & Sage 1998; Anderson *et al*. 2001). Studies of the effects of pre-industrial CO_2 on plants are also fundamental in order to understand plant evolution in response to changes in CO_2 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 CO_2 during pre-industrial eras affected plants at many levels ranging from physiological effects

(i.e. photosynthesis acclimation) to changes in the functioning of ecosystems (i.e. the evolution of C_3 species, Dippery *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 the photosynthetic rate in response to future CO_2. However, in experiments with prolonged exposure to future CO_2 (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 the modification of the source-sink ratio (Urban 2003). For example, if increases in carbohydrate production (source) associated with elevated CO_2 concentration exceed the capacity of the plant to produce new sinks, net photosynthetic 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 2011a). Moreover, the utilization of photoassimilates is influenced by the genotype and growth conditions such as stresses and/or increases in [CO_2] (Tambusi *et al*. 2007, Aranjuelo *et al.* 2009).

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 metabolisms 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 maximizing the response of cereal to growth under different C and N availability conditions (Aranjuelo *et al.* 2012). 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 CO_2 levels have stimulated the allocation of C towards leaves and shoots because of increasing C demand (Gerhart & Ward 2010). However, at future CO_2 levels 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 (Fuertes-Mendizábal et al. 2012). N is remobilized from different parts of the plant (stored before grain filling) or new N is taken up that same period (Dupont & Altenbach 2003). N content in plants

is modulated by CO_2 and it is lower at future than at current or pre-industrial CO_2 (Mitsutoshi 2005). Explanations for this decline in N content under elevated CO_2 include different processes: i) dilution as a result of greater plant growth, ii) down-regulation and lower protein content in the form of Rubisco induced by increases in carbohydrate, iii) limitations in the nitrogen available to plants due to carbon enrichment of the rhizosphere and iv) inhibition of nitrate assimilation from the soil due to elevated CO_2 (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 climatic scenarios (Farrar & Jones, 2000; Fisher *et al.* 2010).

The $^{13}CO_2$ isotope labeling technique was used in this paper to study recently fixed carbon in wheat organs and the respiratory metabolism. This allowed for the calculation of the contribution of stored carbon vs. current photoassimilates to the production of CO_2 through respiration (Schnyder *et al.* 2003; Nogués *et al*. 2004; Nogués *et al*. 2014). Labeling with $^{13}CO_2$ permitted us to calculate the proportion of "new" (i.e. recently fixed) C in Total Organic Matter (TOM) and respired CO_2 (Nogués *et al*. 2004).

Similarly, the $^{15}NH_4$-$^{15}NO_3$ isotope labeling technique is used to understand the N cycle in plants under ambient conditions (Robinson 2001). ^{15}N can act as a powerful tool to assess whether processes in the N cycle are influenced by the increasing concentration of atmospheric CO_2. The IPCC (2013) predict that with climate change there will be a reduction in N availability (N-limitation) with increasing CO_2. A large part of the uncertainly in models predicting climate-change feedbacks lies in the role of the N-cycle in modulating the exchange of CO_2 between plants, the ecosystem and the atmosphere (Hungate *et al.* 2003). More importantly, the pattern of changes in plant $\delta^{15}N$ in response to future CO_2 could guide future studies in the 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 characterize the carbon and nitrogen allocation and its implications in terms of biomass, photosynthesis and reserves in traditional and modern wheat genotypes grown in pre-industrial, current and future CO_2 environments. To date, the mechanism conditioning C and N allocation responses to pre-industrial CO_2 in wheat has not been sufficiently documented and studying this may help us to understand the behavior 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 CO_2, double labeling with $^{13}CO_2$ and $^{15}NH_4$-$^{15}NO_3$ was conducted.

MATERIAL AND METHODS

Plant material

Wheat seeds were germinated in Petri dishes. After 4 days, seedlings were transferred to 4-litre 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, 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 approximately 400 μmol m^{-2} s^{-1} for a 16h light period (day) and the remaining 8h in darkness (night). Plants were watered with Hoagland complete nutrient solution. Each plant-growth chamber was maintained at a different CO_2 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 sensorial properties of this variety. It is characterized by its tall stature, high tillering capacity, medium to 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. It is characterized 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 CO_2 levels in the chamber air were continuously monitored at 5 minute intervals with a combined sensor (CMP3243 Controlled Environments Ltd, Winnipeg, Canada) and compared every two weeks with separate sensors (HMP75: humidity and temperature, and GMP222: for 0-2000ppm CO_2; Vaisala MI70; Vaisala, Helsinki, Finland) in order to maintain a good characterization of environmental parameters.

Plants were grown during the whole life cycle (from September to January) under three different levels of CO_2 (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) CO_2 concentration level (*ca.*700 ± 18 ppm) during the whole life cycle. In order to raise the CO_2 level in the chamber, commercial CO_2 (99.5 % pure CO_2, without

H_2O, O_2, N_2, CO or hydrocarbons) was used (Carburos Metálicos S.A. Barcelona, Spain). An IRGA analyzer connected to the chamber continuously monitored CO_2 levels. When the level of CO_2 dropped below 700 ppm, commercial CO_2 was injected into the chamber, thus maintaining CO_2 concentration at 700 ppm. The air in the three plant-growth chambers (e.g. future, current and pre-industrial CO_2) was collected using 10-mL vacutainers and analyzed using gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS). Mixing of the commercial CO_2 ($\delta^{13}C$ *ca.* -38.2‰) with the ambient air ($\delta^{13}C$ *ca.* -12.5‰) resulted in a $\delta^{13}C$ of CO_2 of *ca.* -22.6 ± 0.9‰ inside the plant-growth chamber. Another forty-eight plants were placed in the second plant-growth chamber and were maintained at current CO_2 concentration levels (*ca.* 400 ± 20 ppm) during whole life cycle, with a $\delta^{13}C$ of CO_2 of *ca.* -11.2 ± 0.6‰. Finally, the same number of plants was placed in the third plant-growth chamber and were maintained at pre-industrial CO_2 concentration levels (*ca.* 260 ± 28 ppm) during whole life cycle, with a $\delta^{13}C$ of CO_2 of *ca.* -10.8 ± 0.5‰. In this chamber, CO_2 was removed using a pump which sent the air inside the chamber through a 1-litre column filled with soda lime (Soda lime with indicator QP, Panreac Quimica SA., Barcelona, Spain). The soda lime was changed every two weeks. The CO_2 levels of these two chambers (400 and 260 ppm) were also continuously monitored by an IRGA analyzer. Plants were rotated in the plant-growth chamber each week to avoid chamber influences in the treatments.

Isotope labeling procedures with ^{13}C and ^{15}N

Simultaneous C and N labeling was conducted in the plants and at different CO_2 levels. Double labeling with ^{13}C and ^{15}N was carried out over three days during the anthesis period in order to ensure that both genotypes were labelled with the same amount of ^{13}C and ^{15}N in the different plant-growth chambers. All plants assimilated the same amount of labeled CO_2 (*ca.* 3000 mmol C m^{-2}) during approximately three days but the assimilation time varied between the different CO_2 treatments and was calculated according to their net assimilation rates (Table 3; Nogués et al 2014).

The ^{13}C composition of air inside the three plant-growth chambers was modified during the labeling period. In each of the chambers, mixing of commercial CO_2 (^{13}C *ca.* 99.9% Euriso-top, Saint-Aubin, France) with the ambient air ($\delta^{13}C$ *ca.* -22.6, -11.2 and -10,8‰ in future, current and pre-industrial plant-growth chambers, respectively) resulted in a $\delta^{13}C$ of CO_2 of *ca.* 165‰. Air samples from the chambers and air respired by plants in darkness were taken before and after labeling in order to analyze the $^{13}CO_2$ isotopic composition using GC/C/IRMS according to Nogués *et al.* (2004).

^{15}N labeling was also applied during the same period by replacing the $^{14}NH_4$- $^{14}NO_3$ in the Hoagland solution with double ^{15}N labeled ammonium nitrate ($^{15}NH_4$- $^{15}NO_3$) that had a ^{15}N excess atom fraction of 5%. After labeling, ^{15}N was removed by washing the quartz sand with distilled water. Plants were then irrigated with normal Hoagland solution.

C and N isotope compositions of Total Organic Matter (TOM)

Samples from different parts of the plant (i.e. leaves, stems, roots and spikes) were collected before, 1 day and 10 days after labelling, dried in an oven at 60º C for 48 hours and ground to a fine powder. Then, 1 mg was weighed in tin capsules and carbon and nitrogen isotope composition was determined using an elemental analyzer (Flash EA 112 Carlo Erba, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C with Conflo III, Termo Finnigan, Bremen, Germany).

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

$$\delta^{13}C(‰) = \left(\frac{R_{sample} - R_{standard}}{R_{standard}} \right) x1000 \qquad \text{(Eq.1)}$$

Where R is the $^{13}C/^{12}C$ ratio.

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

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

Where a and p refer to $\delta^{13}C$ of air-CO_2 and plant material, respectively.

Nitrogen results were also expressed in $\delta^{15}N$ notation (‰), using the international secondary standards with known $^{15}N/^{14}N$ ratio (IAEA N_1 and IAEA N_2 ammonium sulphate and IAEA NO_3 potassium nitrate) with reference to the international primary standard air-N_2, which has a $\delta^{15}N$ value of 0‰ (Werner and Brand 2001):

$$\delta^{15}N(‰) = \left(\frac{R_{sample} - R_{standard}}{R_{standard}} \right) x1000 \qquad \text{(Eq.3)}$$

Where R is the $^{15}N/^{14}N$ ratio.

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

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

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

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

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

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

Open system for isotopic dark respiration determinations

The $\delta^{13}C$ of the CO_2 respired after 20 minutes in darkness of the different plant organs (i.e. flag leaf, the remaining leaves, spikes, stems, and root) was studied in a respiration chamber which has been previously described (Nogués *et al.* 2004). The chamber was connected in parallel to the sample air hose of a portable gas-exchange analyzer (LI-COR 6400, LI-COR Inc., Lincoln, NE, USA). The PPFD inside the chamber was maintained at 0 μmol photon m^{-2} s^{-1} by covering the chamber with a black piece of cotton which kept the chamber in darkness. The organ was first placed in the chamber with ambient air ($\delta^{13}C$ *ca*. -10.3 ± 0.5‰). The chamber was then flushed with CO_2-free air and the CO_2 respired by the organ was allowed to accumulate over a period of 10 minutes. 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 CO_2) was also sampled using 10-mL vacutainers in order to know isotopic composition of the air (the source of C). The CO_2 inside the vacutainers was analyzed 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.

Proportion (*p*) of new C and N calculation

We assumed that 100% of carbon and nitrogen supplied during short-term labeling 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 labeling) in CO_2 respired in darkness after illumination and the proportion of 'new' carbon and nitrogen in TOM was calculated as described in Nogués *et al.* 2004:

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

where $\delta_{control}$, δ_{fixed} and δ_{after} are the isotope compositions of the fraction of interest (CO_2 and TOM) of the control (not labeled), of C and N atoms fixed during labeling and of the sample after labeling respectively. The isotope composition of fixed C and N was calculated as:

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

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

Leaf carbon and nitrogen 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. One mg of dry powder samples were analyzed for the C (mg C mg^{-1}) and N content (mg N mg^{-1}) using an elemental analyzer 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-Ci curves with chlorophyll fluorescence and dark respiration rates were determined. A-Ci curves determinations were conducted on totally expanded flag leaves for each CO_2 treatment and in each genotype. The A-Ci curves were repeated in four different plants for each treatment and genotype and were measured from 0 to 2000 µmol mol^{-1} of CO_2. The curves were generated at 1200 µmol photon $m^{-2}s^{-1}$ PPFD and 25 ºC. Measurements were carried out before anthesis (T0). CO_2 assimilation rates (A),

CO_2 assimilation rate at light saturation (A_{sat}), the maximum CO_2 assimilation rates at CO_2 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 ($V_{c,max}$), 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-Ci response using the method by Nogués *et al*. (2000).

Biomass parameters

Biomass parameters in durum wheat genotypes grown at three different CO_2 levels (700, 400 and 260 ppm) were analyzed. Leaves were scanned with a commercial scanner (HP ScanJet 3400C Scanner, Hewlett-Packard, Palo Alto, California, USA) and images were analyzed with a leaf area meter software (Comprises WinDIAS, Delta-T Devices Ltd. Cambridge, United Kingdom) to obtain the total leaf area (TLA) (cm^2). 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 CO_2 in both genotypes 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 ±standard errors (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 labeling (T0), TOM was more ^{13}C enriched at pre-industrial than at current CO_2 (with increases of 5.4 and 7.3‰ in spikes, 7.7 and 6.3‰ in leaves, 7.1 and 8.6‰ in stems, and 8.7 and 7.4‰ in roots for Blanqueta and Sula, respectively) and more ^{13}C depleted at future CO_2 than at current CO_2 (with decreases of 9.7 and 8.2‰ in spikes, 6.4 and 9.5‰ in leaves, 8.6 and 8.6‰ in stems, and 8.3 and 9.7‰ in roots for Blanqueta and Sula respectively) (Figures 1 and S1). In general, the Blanqueta genotype was between 2.7 to 5.9‰ more ^{13}C enriched than Sula in all the organs and CO_2 treatments with some exceptions as for example root and stem at elevated CO_2. It is worth noting that plants at future CO_2 levels were grown with a $\delta^{13}C$ of ca. -22.6 ± 0.9‰ inside the plant-growth chamber while the $\delta^{13}C$ of the other two plant-growth chambers were *ca.* -10.8 ± 0.5 ‰ and -11.2 ± 0.6 ‰ for 260 and 400 respectively (see Material and Methods).

During labeling, the $\delta^{13}C$ of the air in the three plant-growth chambers was *ca.* 165 ‰. After labeling (T1), the $\delta^{13}C$ of TOM in labeled plants was more ^{13}C enriched than the corresponding non-labeled plants in both wheat genotypes (Figure 1) where spikes were the main C sink. In general, Blanqueta was more ^{13}C enriched than Sula in all organs and CO_2 treatments. However, plants were more ^{13}C depleted at future than at current CO_2 conditions and more ^{13}C enriched at pre-industrial than current CO_2 (F=23.05; P<0.001). We also observed that on the last sampling day (T2), plants were less ^{13}C enriched at higher CO_2.

The $\delta^{13}C$ of respired CO_2 ($\delta^{13}CO_2$_Respired) in the dark by the different plant organs was analyzed with a GC-C-IRMS. It was observed that, ^{13}C losses through dark respiration (Figure 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 labeling (T0), $\delta^{13}CO_2$_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 CO_2 treatments, respired CO_2 was more ^{13}C enriched. After labeling (T1), the $\delta^{13}CO_2$_Respired was positive in all organs indicating that plants had assimilated labeled C, however the largest quantities were found in spikes and stems. Moreover, the $\delta^{13}CO_2$_Respired values increased with decreasing CO_2 concentration (Figure 2). We also observed that on the last sampling day (T2), the $\delta^{13}CO_2$_Respired was negative once again.

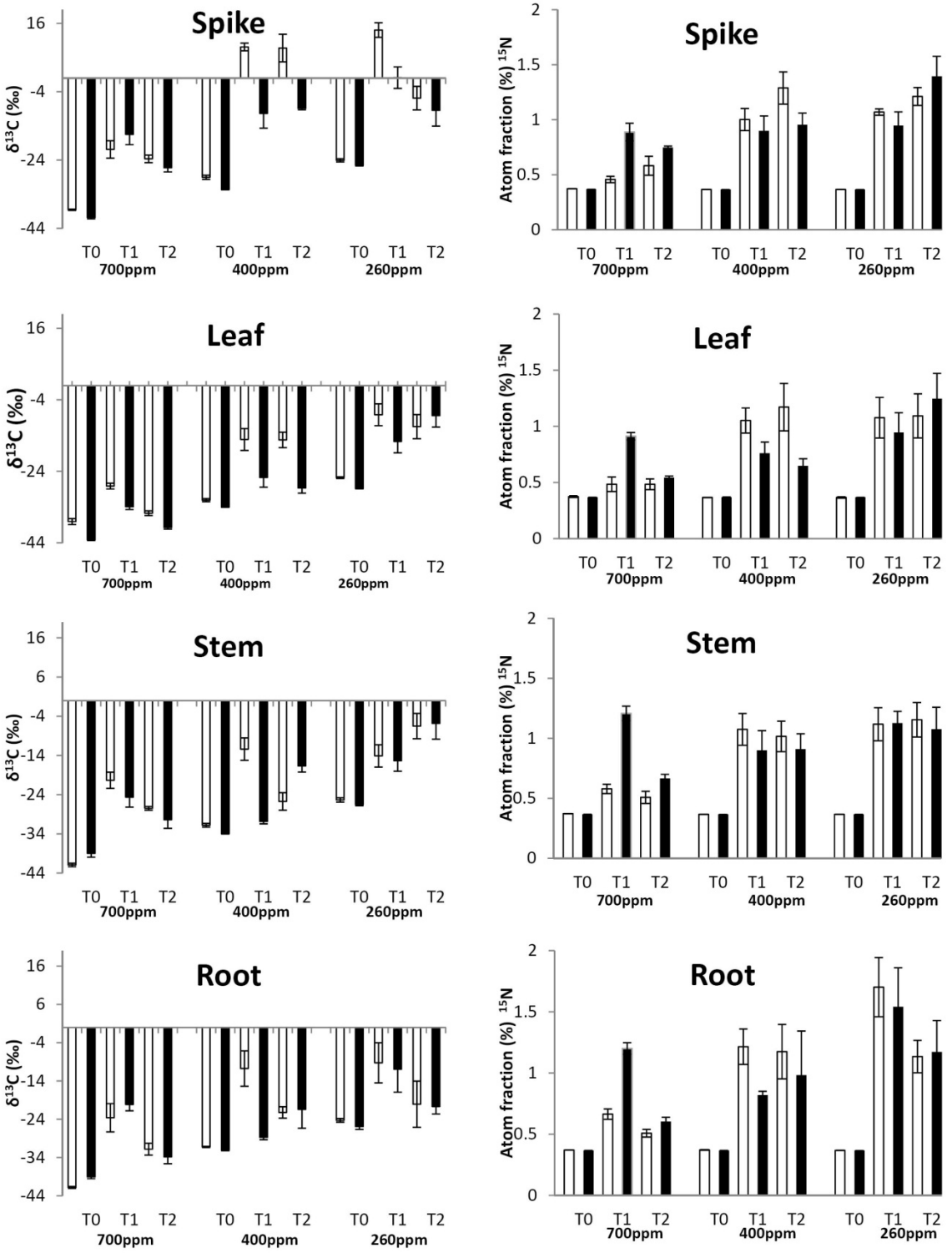

Figure 1. CO_2 effects (700, 400 and 260 ppm) on $\delta^{13}C$ (‰) and ^{15}N atomic fraction (%) values of Total Organic Matter (TOM) in spikes, leaves, stems and roots in two different genotypes of durum wheat: Blanqueta (open bars) and Sula (close bars) before labeling (before anthesis, T0), 1 day after labeling (beginning of grain filling, T1) and 10 days after labeling (end of grain filling, T2). Labeling was made during anthesis. Statistical analysis is presented in supplementary data table S1. Data are means ± SE, n = 4.

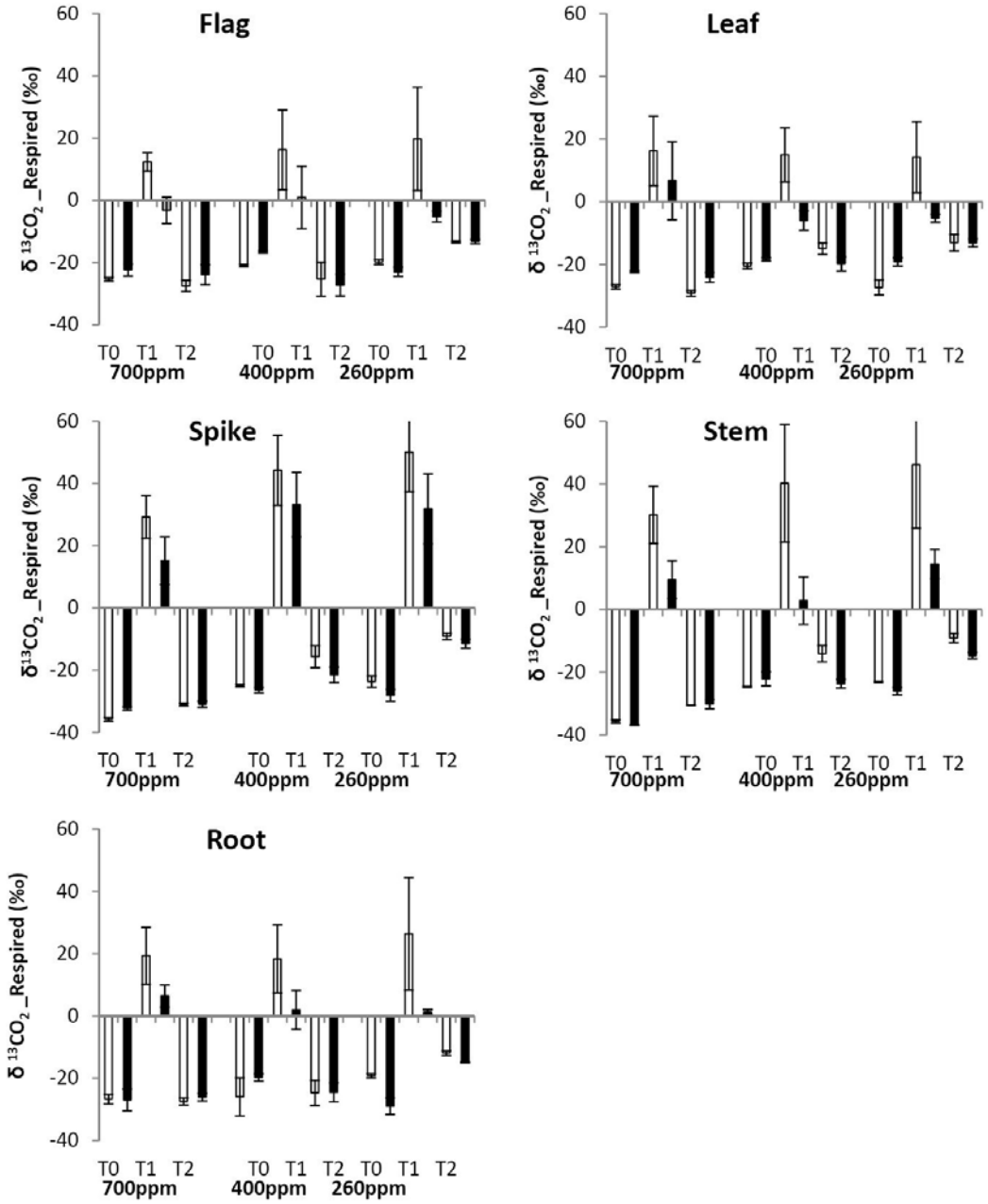

Figure 2: CO_2 effects (700, 400 and 260 ppm) on δ^{13} values (‰) of respired CO_2 ($\delta^{13}CO_2$_Respired) in spikes, leaves, stems and roots in two different genotypes of durum wheat: Blanqueta (open bars) and Sula (close bars) before labeling (before anthesis, T0), 1 day after labeling (beginning of grain filling, T1) and 10 days after labeling (end of grain filling, T2). Labeling was made during anthesis. Statistical analysis is presented in supplementary data table S1. Data are means ± SE, n = 4.

The figure 3 shows a simplified diagram of recently fixed C and respired CO_2 for wheat at 700 (Fig. 3A), 400 (Fig. 3B) and 260 ppm (Fig. 3C). We assumed that 100 % of carbon supplied during short-term labeling 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 CO_2 respired

after the labeling 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 greater percentage of new carbon in pre-industrial CO_2, up to seven times more in current CO_2 and almost the same value at future CO_2 than Sula. However, 10 days after labeling (T2), the percentages of new carbon in both varieties decreased. Plants at future CO_2 had less new labeled C than treatments at current CO_2 and plants at pre-industrial CO_2 had a higher percentage of new carbon overall. After labeling, plants exposed to future and pre-industrial CO_2 had higher percentages of new carbon in respired CO_2 than at current CO_2 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 CO_2), 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 labeling through respiration were between 20-35% at 700 ppm and 10-30% at 260 ppm.

Before ^{15}N labeling (Figure S1), significant differences in $\delta^{15}N$ were found between CO_2 treatments (F=9.61; P<0.05), however not between genotypes (F=0.527; P=0.471). At pre-industrial CO_2 organs were more ^{15}N depleted than at current CO_2 (0.7 and 0.5‰ in spikes, 0.4 and 1‰ in leaves, 0.6 and 1‰ in stems and, 5 and 4‰ in roots) and more ^{15}N enriched at future CO_2 levels than at current CO_2 (18 and 2.7‰ in the spike, 21 and 2.2‰ in leaves, 14 and 4‰ in the stem and, 3 and 2‰ in roots, for Blanqueta and Sula respectively) (Figure S1). After labeling with $^{15}NH_4$-$^{15}NO_3$, at future CO_2 Sula had a higher $\delta^{15}N$ than Blanqueta. In the other CO_2 treatments, Blanqueta was more ^{15}N enriched. Furthermore, at 260 ppm, between labeling and the end of the grain filling, spikes and leaves were more ^{15}N enriched, while roots and stems showed decreases in $\delta^{15}N$. In this same period, plants at pre-industrial CO_2 had more ^{15}N than plants in current conditions, which in turn were more ^{15}N enriched than plants at future CO_2 (Figure 1).

The percentage of N absorbed during the labeling ($^{15}NH_4$-$^{15}NO_3$) was calculated as a percentage of new N in T1 and T2. Overall, the percentage of new N found in the different plants organs and genotypes ranged from 0.6% (i.e. the spike in Blanqueta at future CO_2) to 8.8% (i.e. roots in Blanqueta at pre-industrial CO_2) (Table 2). Sula presented a higher percentage of new N in the spike at future CO_2 (i.e. 3.6 %) than Blanqueta (i.e. 0.6 %), whereas Blanqueta presented higher percentages of new N than Sula in the other treatments (i.e. 2.8 and 4.7 % higher at current CO_2 and, 3.6 and 4.8% at pre-industrial CO_2 in Sula and Blanqueta respectively). This percentage of new N was higher in plants growing at lower CO_2 concentration 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 CO_2 since N was allocated to the leaf or the spike where it was used for storage or the production of new proteins and metabolites. This was not the case at pre-industrial CO_2, where the percentage of new N increased in the stem.

Table 1: CO_2 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). Data are means ± SE, n = 4.

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**					
Organ	Leaf						Leaf						Leaf					
N content ± SE	1.6 0.3	1.9 0.3	2.5 0.13	1.3 0. 2	2.4 0.2	3.5 0. 2	1.7 0. 1	2.5 0. 3	3.5 0. 2	2.0 0. 1	2.4 0. 5	3.6 0. 3	1.6 0. 4	3.2 0. 2	2.4 0. 8	1.4 0. 2	2.7 0. 2	2.0 0. 5
Organ	Spike						Spike						Spike					
N content ± SE	1.9 0.1	2.1 0.1	2.0 0.0	1.2 0.1	1.5 0.0	1.6 0.0	1.4 0.2	2.3 0.1	2.6 0. 1	0. 8 0.0	1.7 0.1	2.1 0.2	1.6 0.4	2.2 0.0	2.3 0.4	1.1 0.1	2.2 0.3	3.4 0.4
Organ	Stem						Stem						Stem					
N content ± SE	1.5 0.4	0.8 0.0	1.2 0.1	0.9 0.2	0.5 0.0	0.8 0.2	1.4 0. 1	1.4 0. 3	1.5 0. 3	1.2 0. 3	0.5 0. 0	1.2 0. 2	1.2 0.0	2.6 0.1	1.8 0.2	0.7 0.1	1.5 0.8	1.8 0.2
Organ	Root						Root						Root					
N content ± SE	2.0 0.2	1.1 0.0	1.9 0.2	0.9 0.3	1.6 0.3	2.1 0.3	2.1 0. 2	2.2 0. 3	3.8 0. 4	1.3 0. 2	1.5 0. 1	2.8 0. 3	2.1 0.3	1.7 0.3	2.0 0.3	1.4 0.2	1.9 0.3	2.5 0.4

All genotypes showed higher leaf and spike N content (mg N g^{-1}) at lower CO_2 at T0 and T1. However, at T2, leaf N content was greater at current CO_2 than in any other CO_2 treatment (Table 1). On the other hand, data was more variable in stems with the lowest N content at current CO_2, at T0 and T1 but not at T2. In roots, the trend was more constant where N content decreased with higher levels of CO_2 for both Sula and Blanqueta genotypes except for Blanqueta at T2. The relationship between $\delta^{15}N$ and $\delta^{13}C$ in TOM in Sula and Blanqueta genotypes before and after labeling and at the different CO_2 levels (700, 400 and 260ppm) showed a positive correlation (r^2= 0,834; P<0.05) (Figure 4). Table 3 shows that plants underwent an acclimation process under the different CO_2 treatments which can also explain in part C allocation. At future CO_2, we observed that carboxylation activity was diminished by the reduction in A_{max} and A_{sat}. Furthermore, plants were observed to have 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 CO_2, although differences between CO_2 treatments and genotypes were not significant.

Table 2: CO_2 effects (700. 400, 260 ppm) on the % 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 labeling. Labeling was carried out during the anthesis period. Data are means ± SE, n = 4.

% New N		700		400		260	
Genotype	**Organ**	**T1**	**T2**	**T1**	**T2**	**T1**	**T2**
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

In terms of biomass parameters, significant differences were found between CO_2 treatments and genotypes. It was observed that CO_2 treatments had a greater effect on the biomass of Sula than Blanqueta (Table 4), although there were no significant differences in TLA between CO_2 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 CO_2 levels. Sula showed higher TLA, spike biomass, shoot and root DW at future CO_2 (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 CO_2 compared with current CO_2. 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 CO_2 treatments were characterized by means of ^{13}C and ^{15}N labeling.

Photosynthetic acclimation at different CO_2 concentrations

Plant photosynthesis showed acclimation to the different CO_2 levels. Currently, photosynthetic acclimation to different CO_2 levels is one of the key issues in CO_2 research and it has been demonstrated that the acclimation to pre-industrial and future CO_2 during long-term exposure can compensate for the effects of CO_2 variation in plant processes (Sage & Coleman 2001). Plants used in this study, which were maintained at optimal water and nutritional conditions showed an 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 CO_2 (Sage & Reid 1992; Cowling & Sage 1998; Anderson *et al*. 2001). On the other hand, many studies have shown that photosynthesis also acclimates at future CO_2 over long-term experiments through down-regulation (Urban 2003; Ainsworth *et al.* 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 3) were lower at future CO_2 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 CO_2 and decreases at future CO_2) 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.

Table 3: CO_2 effects (700. 400 and 260 ppm) on physiological parameters in the flag leaf of durum wheat Blanqueta and Sula genotypes. Anova Tukey-b (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; R_d: rate of day respiration; g_s: stomatal conductance; A_{sat}: Assimilation rate of saturation; A_{360}: assimilation rate at 360ppm C_i of CO_2; A_{max}: maximum assimilation rate; C_i/C_a: internal CO_2 concentration/ambient CO_2 ratio. Data are means ± SE, n = 4.

CO_2 Treatment	Genotype	$V_{c,max}$	J_{max}	Rd	g_s	A_{sat}	A_{360}	A_{max}	C_i/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		ns	ns	***	*	ns	ns	ns	ns
Genotype		ns	ns	**	ns	ns	ns	ns	ns
CO_2 Treatment * Genotype		ns	ns	*	*	ns	ns	ns	ns

Different plant responses are conditioned by the genetics of the different genotypes (Reynols *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 CO_2 treatments. Sula plants showed a large capacity to increase the biomass of the spike and root at future CO_2, but less capacity at current or pre-industrial CO_2 (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 an increased C allocation from assimilation areas to the spike, especially when C was limited, thereby resulting in an increase of the shoot/root ratio. Lehmeier *et al.* (2005) found changes in shoot/root ratios at future and pre-industrial CO_2. Optimal C allocation favors those parts of the plant which 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 CO_2, from future to pre-industrial CO_2 (Table 4). Plants at pre-industrial CO_2 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 CO_2, however, this effect was not seen at pre-industrial CO_2. 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 CO_2.

Table 4: CO_2 effects (700, 400 and 260 ppm) on biomass parameters in durum wheat Blanqueta and Sula genotypes at the end of grain filling (T2). Anova Tukey-b (ns: non-significant; *<0.05; **<0.01; ***<0.001). TLA: total leaf area (cm^2); 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.

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_2treatment		ns	*	*	***	*	ns
Genotype		***	***	ns	***	***	***
CO_2treatment*Genotype		**	***	***	***	*	ns

C and N dynamics

New ^{13}C and ^{15}N was allocated to all the organs of the plant in both genotypes in the different CO_2 treatments. The largest C sinks were the spike and respiration since a large percentage of ^{13}C was quickly respired in the first 24 hours after labeling (Tcherkez *et al.* 2003; Nogués *et al.* 2004). In our case, 10 days after labeling, plants had lost most ^{13}C through respiration and a large proportion of the remaining ^{13}C had been allocated to the spike. Plants at future CO_2 were less enriched in ^{13}C 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 CO_2 discriminated less against $\delta^{13}C$ because the availability of CO_2 was lower than in plants grown at higher levels of CO_2 (Farquhar *et al*. 1989). Thus, $\delta^{13}C$ in TOM of plant organs increased proportionally with lower CO_2 concentrations in the environment.

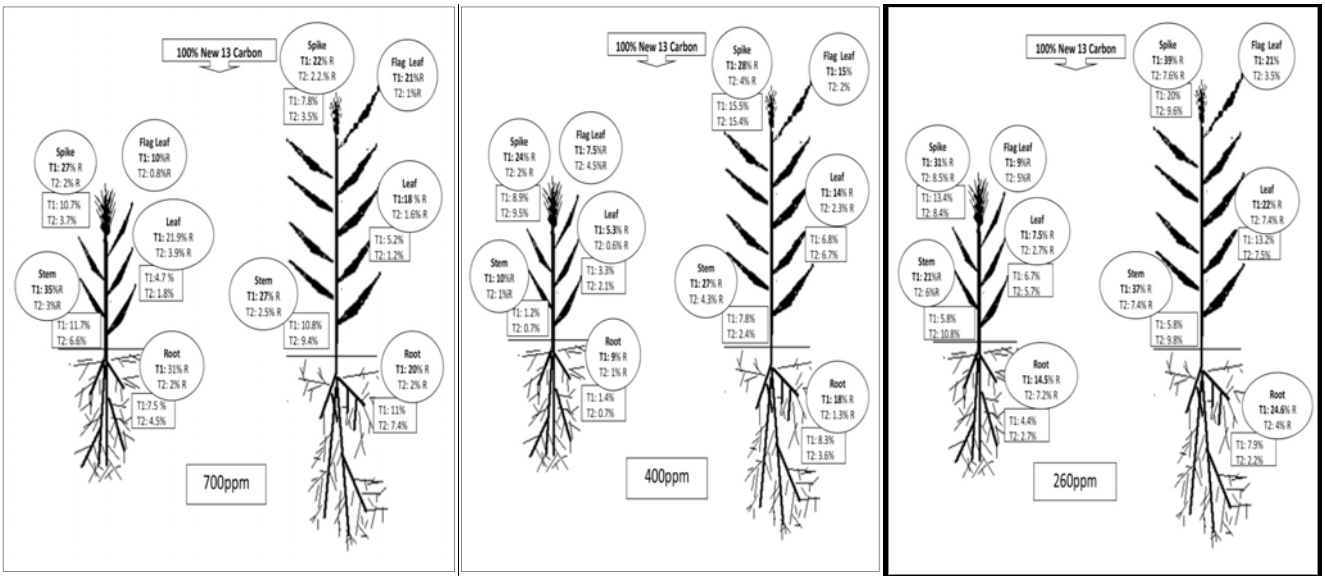

Figure 3: CO_2 effects at 700ppm, 400ppm and 260ppm on the plant carbon allocation in spikes, flag leaf, leaves, stems and roots in two genotypes, Sula (left) and Blanqueta (right) 1 day after labeling (beginning of grain filling, T1) and 10 days after labeling (end of grain filling, T2). Labeling was carried out during anthesis with $^{13}CO_2$. Rectangular shapes: % stored C after labeling. Cloud shapes: % respired C after labeling. 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.

The percentage of new carbon in TOM after the labeling confirmed that less than 20% of C in the different organs, genotypes and treatments was derived from recently assimilated C (Figure 3; Aranjuelo *et al*. 2009a; Nogués *et al*. 2004; 2014). 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 labeling (T2). This suggests that in modern plants the spike was the largest C sink, whereas this was respiration in the traditional genotype. $\delta^{13}C$ increased in leaves and stems at T2, but decreased in the spike due to respiration and in roots by means of translocation and respiration. This suggests that at pre-industrial CO_2 photoassimilates are stored in stems and/or leaves as plants cannot increase the capacity of the spike as a new sink.

CO_2 respired by Blanqueta and Sula (exposed to the three levels of CO_2) was enriched in ^{13}C at T1 (Figure 2), implying that some of the C assimilated during labeling was immediately respired (Nogués *et al*. 2014). However, the fact that respired CO_2 had low levels of ^{13}C enrichment implies that a large part of the respired C originated in C stored in the organs prior to labeling (Figure 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 photoassimilates to grain filling (Evans *et al*. 1975; Schnyder 1993). Ten days after labeling (T2), $\delta^{13}CO_2$_Respired values were similar to those of $\delta^{13}CO_2$_Respired before labeling suggesting that leaves and other organs had used almost all of the labeled C substrate. The $\delta^{13}C$ values of TOM at T1 suggest that plant organs used the labeled carbon, and the $\delta^{13}C$ of TOM at T2 shows that some ^{13}C 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 CO_2 modified the ^{13}C enrichment of respired CO_2, as the CO_2 used for establishing the 700 ppm atmosphere had an approx. 10‰ more negative $\delta^{13}C$ value, which is also reflected in the respired CO_2 of the plants grown at 700 ppm which was lower than at current and pre-industrial CO_2 prior to labeling (Figure 2). However after labeling, plants exposed to pre-industrial CO_2 had higher values of $\delta^{13}C$ (in TOM and respired CO_2) and needed more time to lose the enriched ^{13}C in TOM after labeling because in pre-industrial environments plants discriminate less and are more enriched in ^{13}C (Figure 1) than at current CO_2.

Labeled ^{15}N was applied with the $^{15}NH_4$-$^{15}NO_3$ of the solution. Spikes and roots were the main N sinks. Plants were less enriched in ^{15}N at future CO_2 than at pre-industrial CO_2 (Figure 1) as photosynthesis was down-regulated and N demand was lower. This suggests that acclimation and CO_2 treatments also affected the allocation and distribution of N. We found significantly higher levels of leaf N in both genotypes at pre-industrial CO_2. Anderson *et al.* (2001) showed that the up-regulation of assimilation may be related to increases in leaf N content as well as to the re-allocation of N within leaves. Besides, increases in leaf N and Rubisco would be needed for plants grown at 260 ppm CO_2 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 CO_2 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 CO_2, both genotypes had lower ^{15}N than at current or pre-industrial CO_2 (Figure 1). However, Blanqueta was more ^{15}N-depleted than Sula at future CO_2, whereas in the other CO_2 treatments the opposite was true (with Sula being more $\delta^{15}N$ depleted). Also, we found different $\delta^{15}N$ levels between organs. These data suggest that changes in organ $\delta^{15}N$ (i.e. leaves, stems, roots or spikes) can be attributed to internal processes related to the assimilation and loss of ^{15}N (as for example, 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 $\delta^{15}N$ signal between roots and shoots, and between different CO_2 levels: i) changes in the fractionating processes within the plant-mycorrhizal system and/or changes in the nitrate assimilation enzymes which discriminate heavily against ^{15}N (Bassirirad *et al.* 2003); ii) the influence of the C availability and soil moisture to the microbial activity thereby enriching plant-available N (Dijkstra & Cheng 2008); iii) the correlation between the influence of the plant transpiration in the N acquisition from the soil (Cernusak *et al.* 2009); and iv) isotopic fractionations along metabolic reactions (Gauthier *et al.* 2013; Tcherkez 2010). Furthermore, there is evidence that plant assimilation of nitrate can vary at different CO_2 levels (Bloom *et al*. 2014). In our experiment, we assume a reduction of nitrate assimilation in the shoot under future CO_2 in agreement with previous work (Robinson 2001; Kruse 2002). With regard to the percentage of new N, the same pattern was found in plants at future CO_2 (i.e. at future CO_2 Sula had a higher percentage of new N than Blanqueta). This suggests that the modern plant (with large C sinks) at future CO_2 conditions will be better adapted to assimilate more N than traditional plants. However, at current and pre-industrial CO_2 traditional plants had higher percentages of new N, suggesting that Blanqueta is better adapted to the assimilation of N at these levels of CO_2.

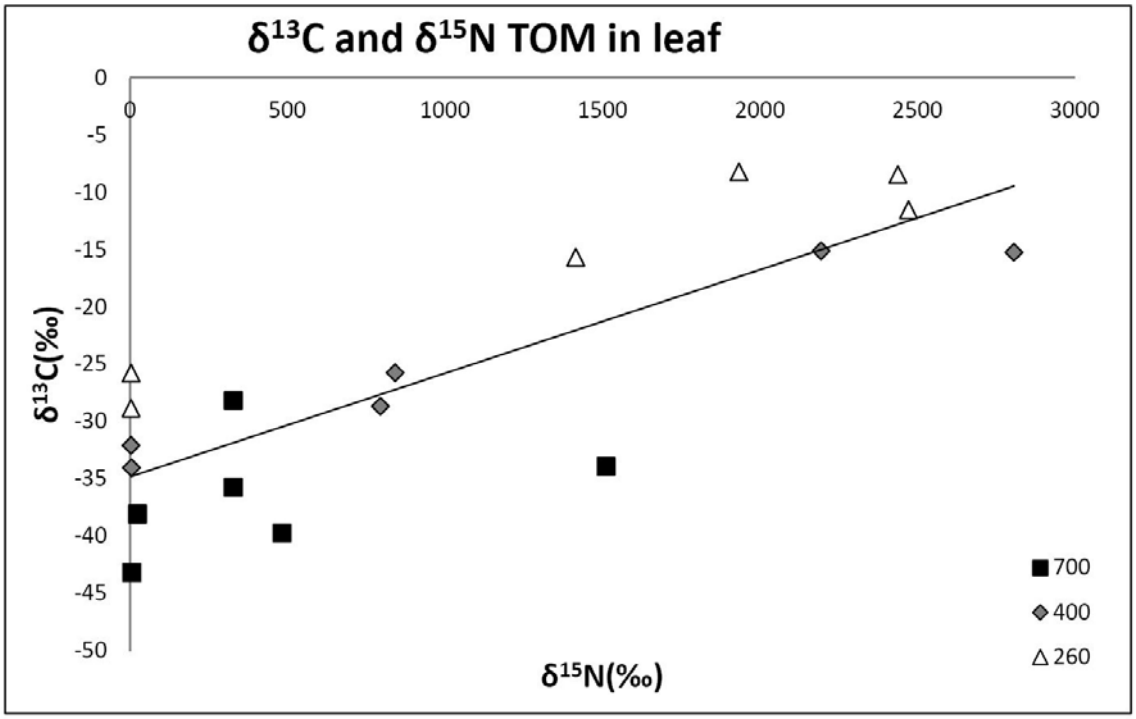

Figure 4. $\delta^{13}C$ and $\delta^{15}N$ correlation in TOM of the leaf in wheat plants and three CO_2 treatments (700, 400 and 260 ppm), before labeling (before anthesis, T0), 1 day after labeling (beginning of grain filling, T1) and 10 days after labeling (end of grain filling, T2). Labeling was carried out during three days in anthesis. Linear regression is significant ($r^2 = 0.834$; $P<0.05$)

Furthermore, the correlation between $\delta^{15}N$ and $\delta^{13}C$ ($r^2 = 0.834$; $P<0.05$) (Figure 4) confirms that the distribution of labeled 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 CO_2 growth conditions. This allocation is reflected in Figure S2 where the N/C ratio decreases as the overall CO_2 increases in leaves, suggesting that at future CO_2 leaves are more N and C limited. This is in accordance with Cernusak *et al.* (2011).

CONCLUSIONS:

The effects of future and pre-industrial CO_2 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 CO_2 concentrations indicating a clear down-regulation. Plants showed acclimation at future and pre-industrial CO_2 with down- and up-regulation of photosynthesis respectively. However, at future CO_2, this photosynthetic acclimation was disrupted when a new C sink appeared during grain filling. The pre-industrial CO_2 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 ^{13}C in accordance with CO_2 levels, i.e. plants were less enriched in ^{13}C at future CO_2 and more enriched in ^{13}C at pre-industrial CO_2 levels.

In our study, we observed the importance of the sink in terms of the response of plants to different CO_2 scenarios. Plants invested more C in shoots than roots at pre-industrial CO_2, and specifically, in the case of the traditional genotype, vegetative parts were seen to be the main C sink. At current and future CO_2, the source of C is higher and plants can redirect more from assimilation compartments (shoots) towards non-assimilation compartments (roots). However, at future CO_2, it is the modern genotype that has a greater capacity to increase the size of reproductive organs and harvest index. In future studies, it will be necessary to examine the interactions between varying CO_2 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 CO_2.

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SUPPLEMENTARY MATERIAL

Table S1: Statistical analysis of CO_2 effects (700, 400 and 260 ppm) on $\delta^{13}C$ (‰) and atom% ^{15}N values of Total Organic Matter (TOM), and $\delta^{13}CO_2$_Respired (‰) in flag, spikes, leaves, stems and roots in two different genotypes of durum wheat: Blanqueta and Sula, before labeling (before anthesis, T0), 1 day after labeling (beginning of grain filling, T1) and 10 days after labeling (end of grain filling, T2). Labeling was made during anthesis. Anova Tukey-b (ns: non-significant; *<0.05; **<0.01; ***<0.001) was made. Where differences were found, the Duncan multiple tests were carried out and values with different letters indicate significant differences at $P < 0.05$.

	Organ	CO_2 Treatment	Genotype	CO_2 Treatment * Genotype	CO_2 Treatment		
					700	400	260
$\delta^{13}C$ (‰)	**Leaves**	***	0.472	0.634	a	b	c
	Spikes	***	0.592	0.619	a	b	b
	Stems	***	0.496	0.740	a	a	b
	Roots	***	0.467	0.986	a	b	b
atom % ^{15}N	**Leaves**	**	0.636	0.885	a	ab	b
	Spikes	**	0.568	0.770	a	b	b
	Stems	**	0.365	0.560	a	b	b
	Roots	**	0.859	0.737	a	b	b
$\delta^{13}CO_2$_Respired(‰)	**Flag**	0.267	0.187	0.769	ns	ns	ns
	Leaves	0.767	0.747	0.267	ns	ns	ns
	Spikes	0.154	0.474	0.863	ns	ns	ns
	Stems	0.111	0.407	0.767	ns	ns	ns
	Roots	0.190	0.342	0.966	ns	ns	ns

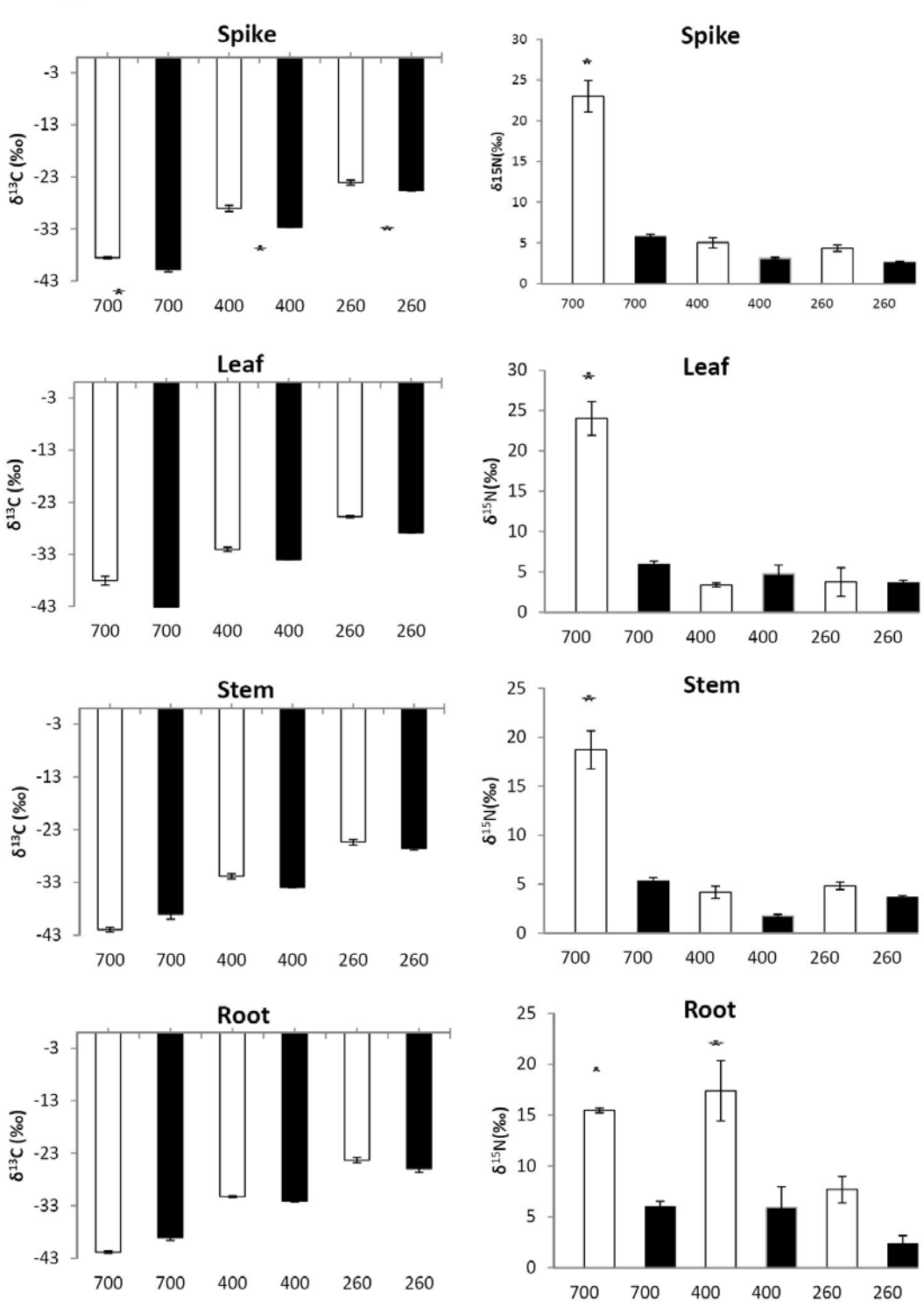

Figure S1 : Effects of three different CO_2 treatments (700ppm, $\delta^{13}CO_2$_air -22.6‰; 400 ppm, $\delta^{13}CO_2$_air -11.2‰ and 260 ppm $\delta^{13}CO_2$_air -10.8‰) on the natural abundance of $\delta^{13}C$ and $\delta^{15}N$ in Total Organic Matter (TOM) of flag leaf, other leaves, spikes, stems and roots in two different genotypes of durum wheat: blanqueta (open bars) and sula (close bars).

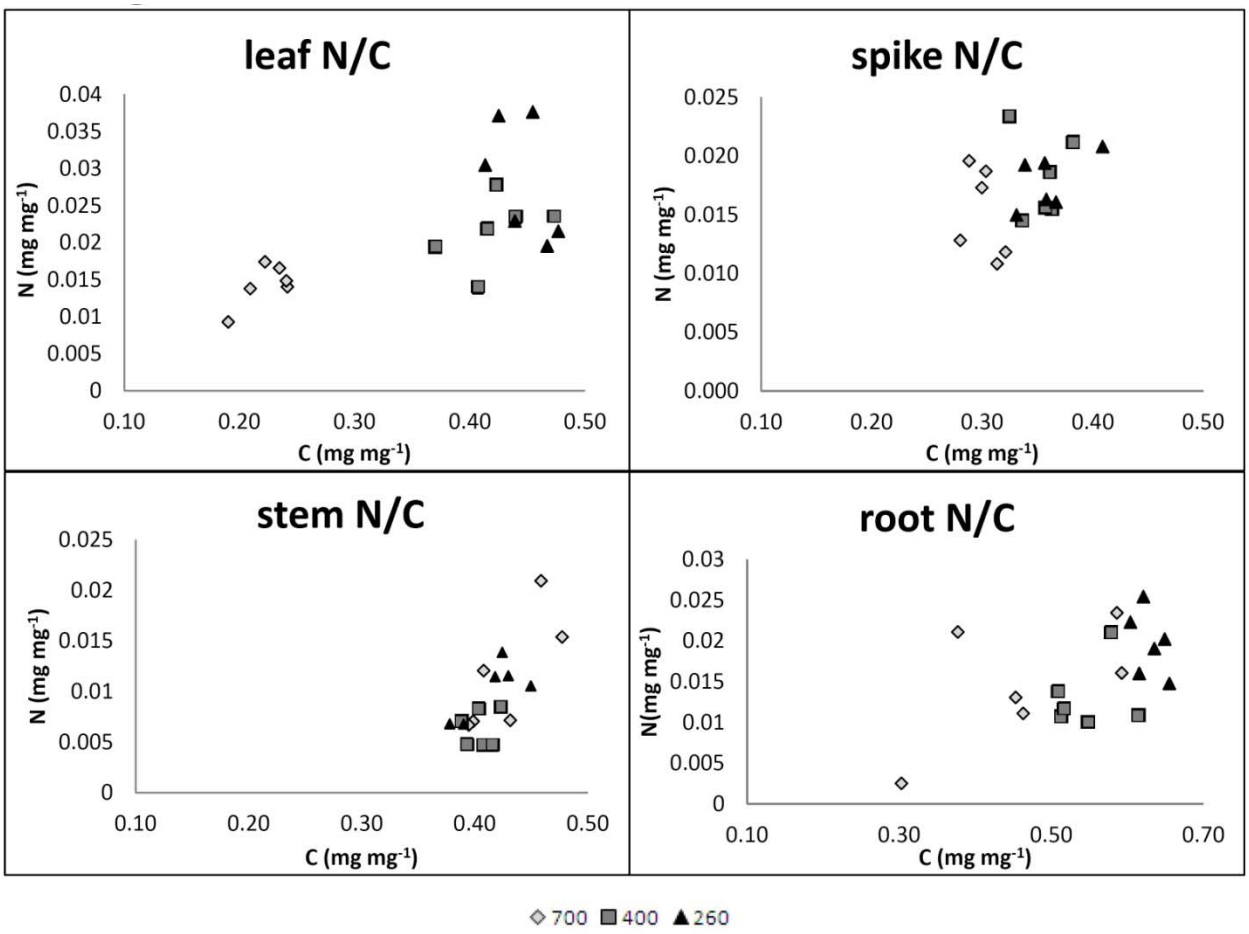

Figure S2: CO_2 effects (700, 400 and 260 ppm) on C and N content (mg mg^{-1}) in spikes, leaves, stems and roots of durum wheat.

Chapter 3:

THE EFFECTS OF DEPLETED, CURRENT AND ELEVATED GROWTH [CO_2] IN WHEAT ARE MODULATED BY WATER AVAILABILITY.

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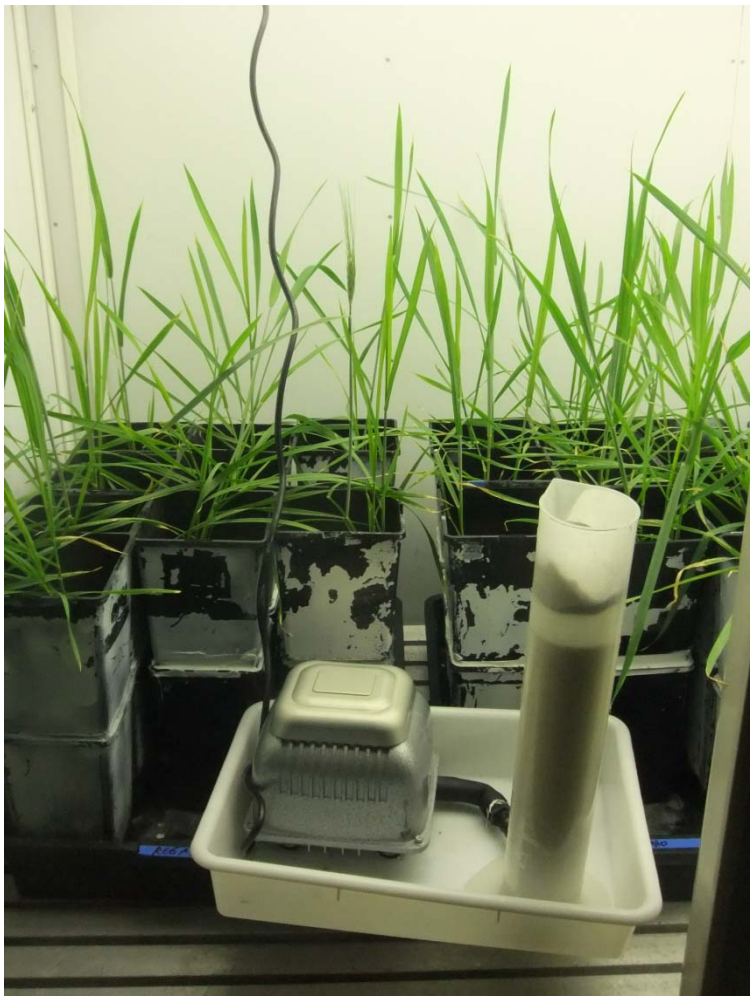

THE EFFECTS OF DEPLETED, CURRENT AND ELEVATED GROWTH [CO_2] IN WHEAT ARE MODULATED BY WATER AVAILABILITY

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Running title: Photosynthetic acclimation to different [CO_2] and water stress

ABSTRACT

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

Key words: Climate change; depleted, current and elevated [CO_2]; nitrogen content; photosynthetic acclimation; Rubisco; wheat acclimation; water stress.

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

INTRODUCTION

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

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

The increases in atmospheric CO_2 concentration from ca. 260 ppm before the beginning of the industrial revolution (i.e. 250 years ago) to the current level ca. 398 ppm (NOAA-ESRL, 2014) have affected long-term net assimilation rates (Araus and Buxó, 1993; Araus, 2002). Future scenarios are predicting a further steady increase in atmospheric CO_2 concentrations, due to the burning of fossil fuels and biomass (Pagani et al., 1999; Pearson and Palmer, 2000). By the end of this century, according to predictions using multi-model averages, atmospheric [CO_2] will have reached 985±95ppm (IPCC, 2013), with consequent increases in temperature (4 and 5 degrees) and drought periods. While photosynthesis in C_3 plants is usually affected by changes in [CO_2], there is a wide variation of responses in different species such as the acclimation of photosynthesis to different atmospheric CO_2 concentrations after a long period of exposure (Aranjuelo et al., 2009, 2011; Pardo *et al*., 2009). Many studies suggest that the influence of low [CO_2] during pre-industrial periods may have affected plants at many different levels, ranging from the physiological effects on plants to changes in ecosystem functioning (Gerhart and Ward, 2010). Some studies have even shown an increase in photosynthesis in plants subjected to pre-industrial [CO_2] (Sage and Reid, 1992; Sage, 1994; Cowling and Sage, 1998; Anderson et al., 2001). Furthermore, many other studies have shown how plants respond to future CO_2 in short-term experiments (days or weeks) by increasing photosynthetic rates. However, responses to long-term experiments (i.e. weeks, months or years) show a process of acclimation of photosynthesis at projected future CO_2 levels (Long et al., 2004; Leakey et al., 2004). Acclimation is a physiological adjustment carried out by plants in

response to a given [CO_2]. Photosynthesis acclimation can undergo up-regulation with depleted [CO_2] or down-regulation with elevated [CO_2] or through adjustments to the photosynthetic machinery (Sage, 1994; Anderson et al., 2001; Nogués and Azcón-Bieto, 2013).

Photosynthetic acclimation is explained by different processes. Mechanisms to explain within-species variation often focus on changes in the balance between carbohydrate production (source) and the capacity to use and store carbohydrates (sink). For example, if increased carbohydrate production associated with future [CO_2] exceeds the capacity to use or store it, net photosynthetic rates may decline in order to balance the source activity with the sink capacity (Thomas and Strain, 1991; Aranjuelo et al., 2013; Aljazairi et al., 2014b). Other basic mechanism leading to photosynthesis acclimation are changing the Rubisco content caused by decreases in leaf N content in the case of down-regulation (Ellsworth et al., 2004; Aranjuelo et al. 2005; Aranjuelo et al., 2007; Kant et al., 2011; Aljazairi et al., 2014a), increases in Rubisco and leaf N content in the case of up-regulation (Gesch et al., 2000; Anderson et al., 2001) or reallocation of N within the plant (Nakano et al., 1997). Extensive evidence that nitrogen limits the growth response of plants at future [CO_2] has been demonstrated in many experiments conducted in controlled environmental chambers and under field conditions in free air CO_2 enrichment (FACE) experiments (Ainsworth and Long, 2005; Rogers et al., 2006; Gutierrez et al., 2013; Bloom et al., 2010, 2014).

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

Atmospheric CO_2 reductions can enhance stress intensity, for example, in the case of drought stress, because depleted [CO_2] tends to open stomata and exacerbates limitations associated with drought (Sage and Cowling, 1999; Sage and Coleman 2001). However, elevated [CO_2] plants have lower stomatal conductance, which should ameliorate the water deficits and even in some cases increase

plant growth significantly in dry conditions (Morgan et al., 2004, 2011; Perry et al., 2013). Also, other studies have reported that elevated [CO_2] can compensate for water stress-induced reduction in growth (Aranjuelo et al., 2009). Elevated [CO_2] also increases WUE in many plants under dry conditions, thus reducing their overall demand for water (Ainsworth and Long, 2005). Most studies on the impact of climate change on plants have been conducted with high [CO_2] and water-stress (West et al., 2005; Erice et al., 2007; Aranjuelo et al., 2009); however, the interaction between depleted, current and elevated CO_2 and water-stress has received little attention and studies on photosynthesis and chlorophyll fluorescence are generally scarce and are lacking for cereals (Lopes et al., 2004).

The aim of this study was to explore the combined effects of [CO_2] and water availability on wheat physiology (i.e. growth, photosynthesis and chlorophyll fluorescence). A better understanding of up- and down-regulations of photosynthesis in these plants during grain filling was also studied.

MATERIAL AND METHODS

Plant material

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

Experimental design

Plants were grown in three fully controlled plant-growth chambers (Conviron E15, Controlled Environments Ltd, Winnipeg, Canada) at a temperature of 22/18°C (day/night) and 60% relative humidity. Plants were supplied with a photosynthetic photon flux density (PPFD) of about 400 ± 30 μmol m^{-2} s^{-1} during the 16h light period (day) and 8h dark period (night). Plants were watered with Hoagland complete nutrient solution (Arnon and Hoagland, 1939) alternating with distilled water in order to avoid salt accumulation over the whole life cycle. Humidity, temperature and [CO_2] in the air within the chambers were monitored continuously by a sensor (CMP3243 Controlled Environments Ltd, Winnipeg, Canada) over the period of the experiment at intervals of every 5 minutes by a sensor and compared every two weeks with a separate sensor (HMP75 humidity and temperature; and GMP222; 0-2000 μmol mol^{-1} carbon dioxide; Vaisala MI70 Helsinki, Finland) in order to capture a complete record of environmental parameters (Table 1).

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

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

The plants were grown in three plant-growth chambers under three different [CO_2] (i.e. 700, 400 and 260 μmol mol^{-1}) for the entire life cycle (from September to January) at the Experimental Field Service of Barcelona University, Barcelona, Spain. Forty-eight plants were placed in the first plant-growth chamber, which was maintained at future [CO_2] (ca. 731.7 ± 16.9 μmolmol^{-1}) by injecting CO_2 into the chamber from an external bottle (Carburos Metálicos SA. Barcelona, Spain). Another forty-eight plants were placed in the second plant-growth chamber, which was maintained at current [CO_2] (ca. 409.3 ± 2.5 μmolmol^{-1}). Finally, the same number of plants was located in the third

plant-growth chamber, which was maintained at pre-industrial [CO_2] (ca. 259.4 ± 13.6 μmolmol^{-1}). Air in this chamber was maintained at pre-industrial [CO_2] by using a pump to send the air inside of the chamber through a 1-litre column filled with soda lime (Soda lime with indicator QP Panreac Química SA, Barcelona, Spain). The soda lime was changed every two weeks. Plants were rotated inside the chamber each week and between chambers every three weeks in order to avoid chamber influences in the treatments.

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

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

Gas exchange and chlorophyll fluorescence measurements

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

Modulated chlorophyll fluorescence measurements allowed for the estimation of the relative quantum yield of photosystem II (PSII), the efficiency of the capture of excitation energy by open PSII reaction centres (F'_v/F'_m), the maximum quantum yield of PSII (F_v/F_m), photochemical

quenching (q_p), the non-photochemical quenching coefficient (q_N) and non-photochemical quenching (NPQ) determined in the flag leaf after 30 min of dark adaptation (Nogués and Baker, 2000).

SPAD and Chlorophyll content

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

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

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

Rubisco and Protein Determination

Total soluble protein content (TSPC) was determined using the Bradford method (Bradford, 1976). One hundred mg of frozen leaf tissue was ground with PBS solution and centrifuged at 13,000 rpm for 5 minutes. An aliquot of each extract was used to measure soluble protein by spectrometry, with reference to a standard line that was calculated with BSA (Bovine Serum Albumin). Another aliquot of the same extract was used for protein separation using SDS-PAGE. Gel images were scanned and analysed using the Motic Images Plus2.0 program. The concentration of Rubisco Large (L) and Small (S) subunits was measured against a Rubisco standard protein (Bio-Rad Laboratories, Inc. Berkeley, California).

Growth parameters

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

Data analysis

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

RESULTS

[CO_2] effects on plants.

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

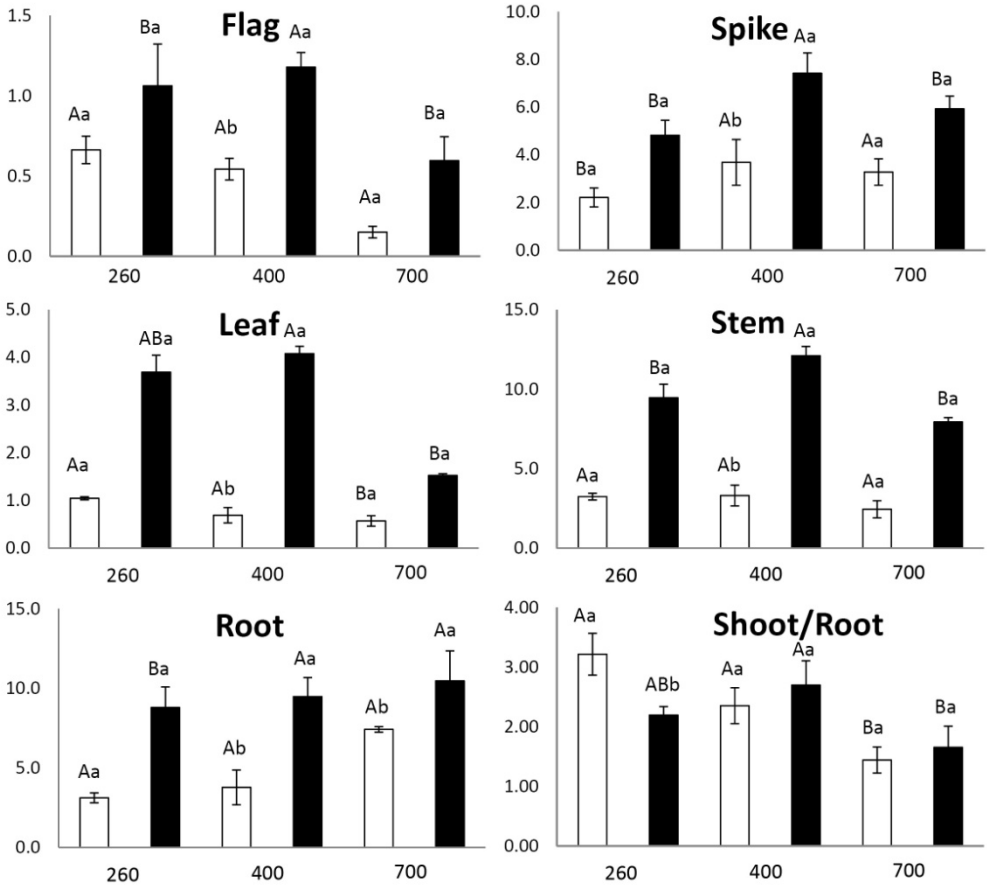

Figure 1: CO_2 effects (260, 400 and 700 ppm) on Biomass (flag leaves, leaves, spikes, stems and roots) and the Shoot/Root ratio under well-watered (WW, close bars) and mild water-stress (WS, open bars) conditions. Uppercase letters are differences between CO_2 treatments. Lowercase indicates differences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

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

Table 2: CO_2 effects (260, 400 and 700 ppm) on V_{cmax}, J_{max}, Stomatal limitation and A_{sat} under the two water treatments, well-watered (WW) and mild water-stress (WS), before anthesis (T0), at the beginning of grain filling (T1) and the end of grain filling (T2). Each value represents means of 4 plants. Statistical analysis is in Table S2.

CO_2	Timing	H_2O	$V_{c.max}$	J_{max}	Stom. Lim.	A_{sat}
700	T0	WW	68.9 ± 5.1	166.4 ± 4.5	22.7 ± 3.5	15.2 ± 0.8
		WS	93.0 ± 5.9	197.9 ± 2.9	30.5 ± 4.0	16.6 ±1.9
	T1	WW	152.5 ± 18.5	296.1 ± 56.1	15.1 ± 1.3	27.3 ± 2.1
		WS	113.6 ± 23.3	234.2 ± 71.5	21.3 ± 4.9	21.1 ± 5.7
	T2	WW	83.4 ± 3.8	232.1 ± 12.2	25.0 ± 1.3	16.0 ± 1.0
		WS	98.8 ± 14.0	220.1 ± 29.9	17.9 ± 1.3	21.2 ± 3.1
400	T0	WW	104.7 ± 19.0	201.9 ± 27.8	20.3 ± 3.3	18.0 ± 1.8
		WS	85.6 ± 18.9	213.9 ± 46.4	28.8 ± 5.6	15.7 ± 3.8
	T1	WW	157.8 ± 8.8	267.3 ± 7.0	14.3 ± 1.3	28.6 ± 0.6
		WS	137.6 ± 16.1	274.7 ± 32.8	14.0 ± 2.2	28.4 ± 2.7
	T2	WW	102.1 ± 4.6	225.8 ± 21.0	18.6 ± 1.4	22.5 ± 2.7
		WS	105.2 ± 3.2	243.2 ± 19.4	22.4 ± 3.0	21.8 ± 0.7
260	T0	WW	90.5 ± 3.8	182.3 ± 13.9	20.2 ± 1.4	18.3 ± 1.3
		WS	19.8 ± 4.8	34.8 ± 6.9	15.1 ± 5.0	4.7 ± 1.3
	T1	WW	123.4 ± 8.0	277.2 ± 28.1	20.2 ± 0.4	26.1 ± 1.9
		WS	109.8 ± 4.2	250.1 ± 10.9	20.3 ± 2.3	22.9 ± 0.7
	T2	WW	101.6 ± 6.3	229.4 ± 6.3	22.9 ± 5.1	20.9 ± 1.8
		WS	94.5 ± 12.4	208.8 ± 32.7	19.6 ± 2.0	20.0 ± 2.1

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

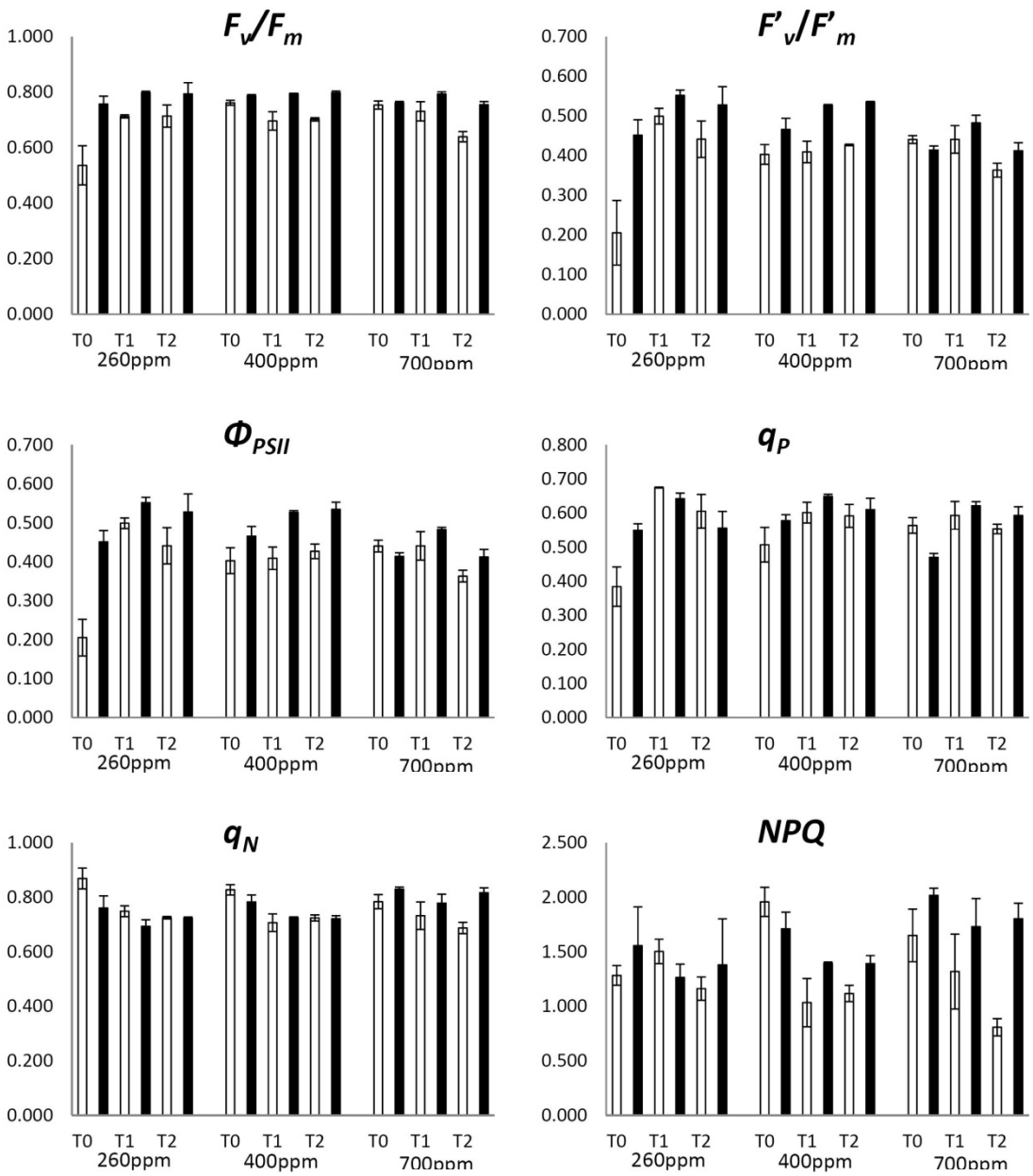

Figure 2: CO_2 effects (260, 400 and 700 ppm) on leaf fluorescence under well-watered (WW, close bars) and mild water-stress (WS, open bars) conditions before anthesis (T0), the beginning grain filling (T1) and the end of grain filling (T2). Each value represents means of 4 plants. Statistical analysis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

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

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

Large and Small subunit Rubisco concentrations (Rbc_L and Rbc_S, respectively) were measured during the experiment. Plants showed higher concentrations of Rubisco at depleted CO_2 in both subunits. Plants under elevated [CO_2] showed lower Rubisco content than the current [CO_2] (Table 2). On the other hand, leaf total soluble protein content (TSPC) decreased in plants when the [CO_2] increased, with this trend in TSPC change occurring across the three [CO_2]. Plants at depleted [CO_2] were seen to have greater concentrations than at current [CO_2]. At elevated [CO_2] WW plants had lower leaf TSPC levels than at current [CO_2] and WS plants had higher leaf TSPC levels than current and lower leaf TSPC levels than depleted [CO_2] (Fig. 4).

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

Water availability effects on plants.

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

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

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

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

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

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

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

[CO_2] effects on plants modulated by water availability.

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

Analyses of gas exchange parameters showed that the [CO_2] effects were modulated by water treatments. At current [CO_2], WW conditions resulted in higher $V_{c,max}$, A_{sat}, A_{max} and lower J_{max} than mild WS. At depleted [CO_2], lower values were found than under current [CO_2], however,

some of the parameters increased, such as $V_{c,max}$ or A_{max} during grain filling, thus indicating up-regulation of photosynthesis. However, we did not find any significant differences between CO_2xWater treatments.

Regarding CO_2xWater, we found significant differences in the interaction between in terms of chlorophyll fluorescence parameters (F_v/F_m, F'_v/F'_m, Φ_{PSII} and q_p). Besides, significant differences in pigment contents (chlorophyll a, chlorophyll b, total chlorophyll and carotenoids) were found between CO_2xWater treatments. The pigment contents showed effects of [CO_2] modulated by water availability, being higher in WW plants at elevated [CO_2] than at depleted [CO_2]. While the quantity of pigments was lower under the future [CO_2] than under depleted [CO_2] when combined with mild WS, the differences were not large (Fig. 3; table S2).

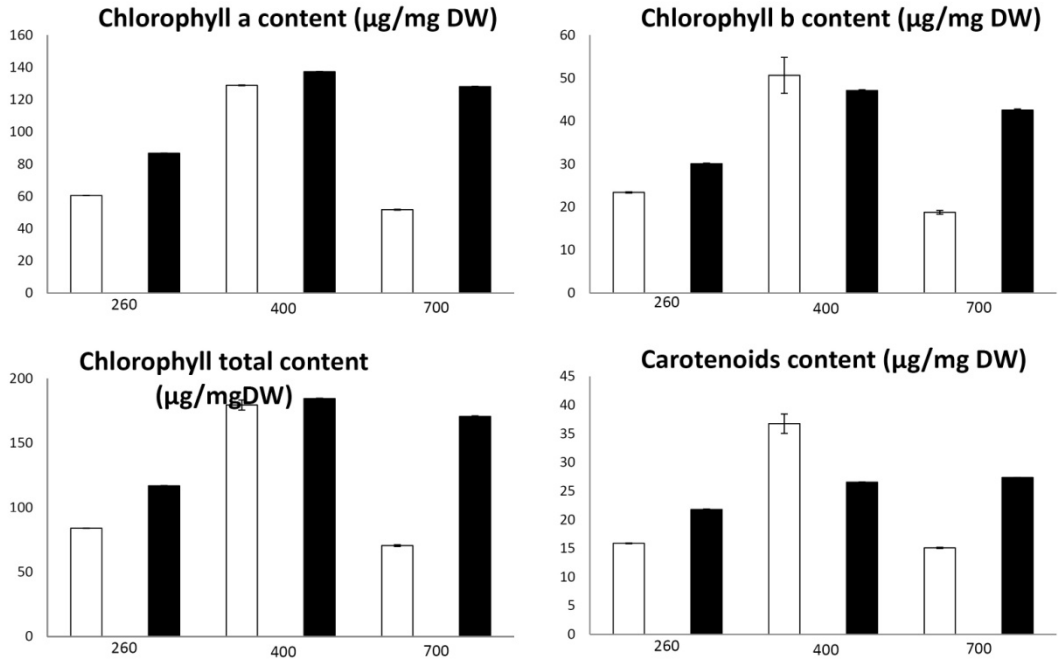

Figure 3: CO_2 effects (260, 400 and 700 ppm) on pigment contents (Total chlorophyll, Chlorophyll a, Chlorophyll b and carotenoids) under well-watered (WW, close bars) and mild water-stress (WS, open bars) conditions. Each value represents means of 4 plants. Statistical analysis is in Table S2.

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

differences were for the Rbc_S at elevated [CO_2] and WS, and the Rbc_L at depleted [CO_2] and WW (Table 3).

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

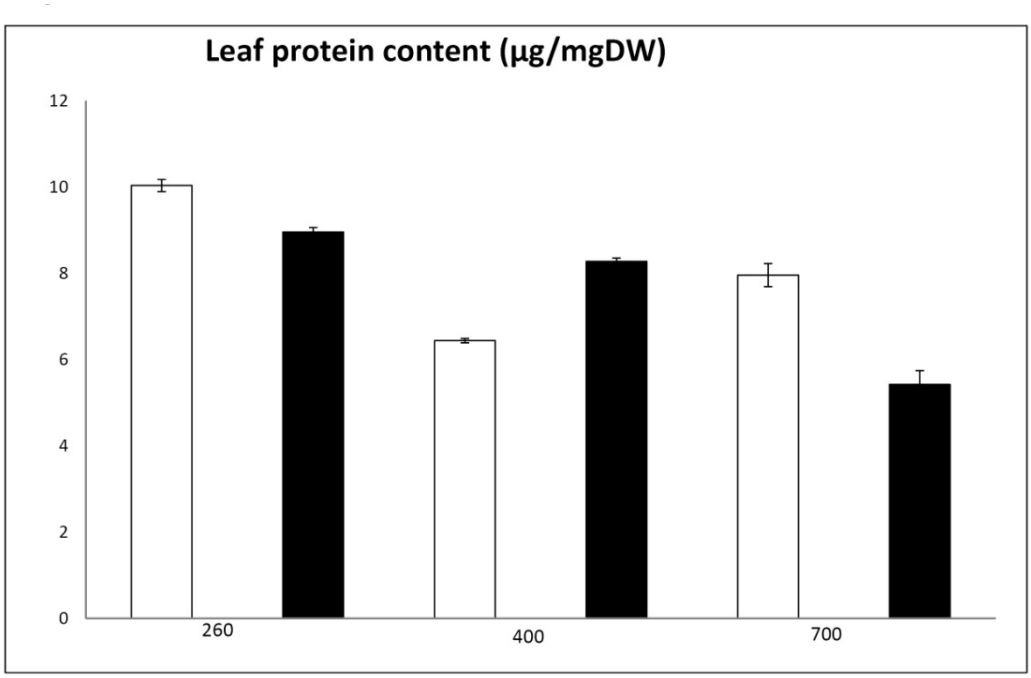

Figure 4: CO_2 effects (260, 400 and 700 ppm) on leaf protein content under well-watered (WW, close bars) and mild water-stress (WS, open bars) conditions. Each value represents means of 4 plants. Statistical analysis is in Table S2.

Leaf carbon and nitrogen content, and NUE and PNUE were affected with the [CO_2] and those effects were modulated by water availability. Plants showed higher leaf C content at elevated [CO_2] (i.e. 5% and 3 % under WW and WS respectively) and lower leaf C content at depleted [CO_2] (i.e. 3% and 5 % under WW and WS respectively) than current [CO_2], but the differences were not significant. At depleted and elevated [CO_2], plants had a lower leaf N content, with the larger difference at elevated [CO_2] (48% and 6% less N for WW and WS, respectively) than at current [CO_2], whereas at depleted [CO_2], plants were seen to have also a higher N content (16% and 4% for WW and WS, respectively). NUE increased with the [CO_2] in WW plants (155%, 323% and 451% for 260, 400 and 700ppm respectively). In addition, WS plants had lower NUE than WW plants, and these differences were much greater at elevated [CO_2] than at depleted [CO_2]. PNUE

also increase significantly at elevated [CO_2] in WW plants. However, no differences were observed in WS plants between [CO_2] (Fig. 5).

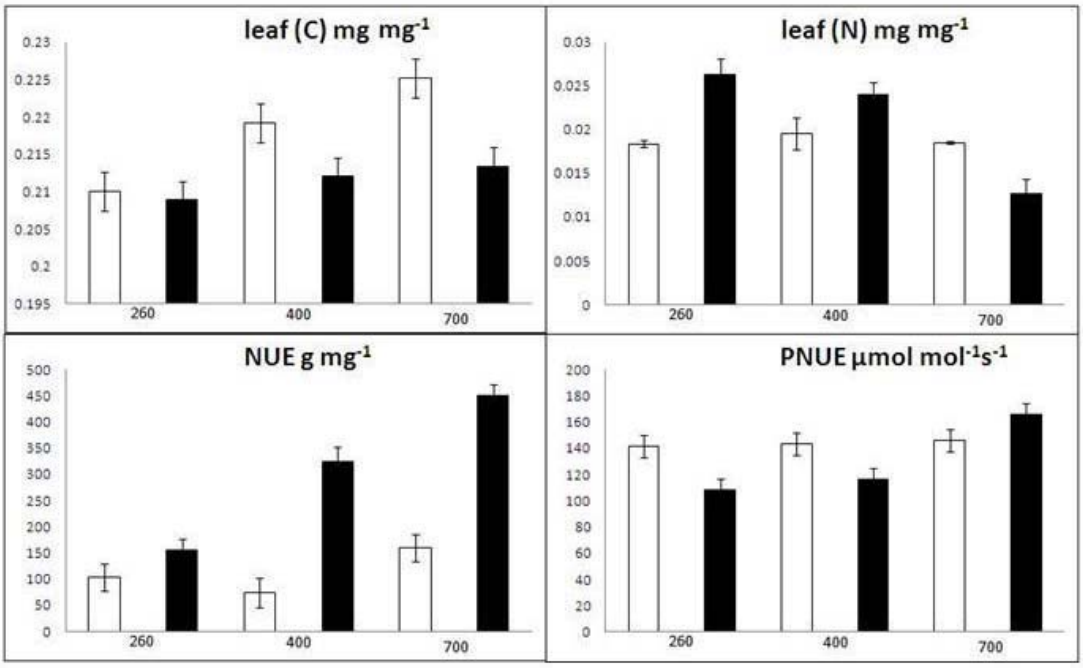

Figure 5: CO_2 effects (260, 400 and 700 ppm) on leaf carbon and nitrogen content (mg mg^{-1}), NUE (g mg^{-1}) and PNUE (µmol $mol^{-1}s^{-1}$) under well-watered (WW, close bars) and mild water-stress (WS, open bars) conditions.

DISCUSSION

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

[CO_2] effects on plants.

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

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

Amthor (2001), in summarising 156 experiments that analysed wheat yield under elevated [CO_2], showed that the CO_2 response ranged from no effect or even negative effects in some studies to several-fold increases in others. Exposure at 700 ppm in the current study slightly decreased biomass production of the spike, stem, flag leaf and other leaves (although differences were not significant); however, there were increases in root biomass (Fig. 1 and Table S1). Aranjuelo et al., (2011), revealed that elevated [CO_2] did not contribute to increased grain filling in wheat plants (Fig 1, Table S1), further indicating the variability of the responses.

An absence of effects on plant biomass, together with the lower spike biomass suggests that under elevated [CO_2], plants were acclimated in terms of photosynthesis and growth parameters. Grain filling may be limited by i) translocation of photoassimilates from source to sink, ii) photosynthetic

activity iii) spike sink capacity and iiii) nitrogen source (Uddling et al., 2008; Bloom et al., 2010; Gutierrez et al., 2013). At elevated [CO_2], plants had lower shoot/root ratios than plants grown at current [CO_2]. This data suggest that plants at elevated [CO_2] invest more photoassimilates in roots than at current or depleted [CO_2]. At elevated [CO_2] plants have a greater source of C and they invest this C into increased root biomass, which gives them greater potential for exploring more soil and thereby increases their uptake of soil moisture and nutrients (Reich et al., 2006; Ghashghaie and Badeck, 2013).

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

Many studies have shown that atmospheric CO_2 has a direct effect on photosynthesis and also on the reduction of assimilation at depleted [CO_2], at optimal temperatures and nutritional conditions C is more limiting as a substrate for the carboxylation reaction in Rubisco in C_3 plants than at current or elevated [CO_2] (Long and Drake, 1992). So, depleted [CO_2] can lead to a reduction in photosynthetic capacity (Sage, 1995). This study of short-term exposure to depleted [CO_2] showed significant decreases in assimilation and V_{cmax}. However, the effect of depleted [CO_2] can be compensated by the acclimation processes of photosynthesis. Similar to our data (Fig. 2, Table S2), increases in the maximum rate of carboxylation V_{cmax}, J_{max}, assimilation and also in the concentration of Rubisco in C_3 plants under long-term exposure to depleted [CO_2] have been described by other authors (Anderson et al., 2001), and this is known as photosynthetic acclimation (up-regulation). Other studies have found evidence of photosynthetic acclimation at depleted [CO_2]. Sage and Reid, (1992) and Cowling and Sage (1998) found that A is limited by Pi regeneration in *Phaselous vulgaris* plants grown at depleted [CO_2]. Allen et al., (1991) and Dippery et al. (1995) suggested that photosynthesis is also enhanced (up-regulation) by increases in partitioning of C to leaves at depleted [CO_2].

On the other hand, when photosynthetic activity was determined in plants at 400 and 700 ppm (Fig. 2, Table S2) it was found that plants grown at elevated [CO_2] had lower photosynthetic capacity than plants grown at current [CO_2] (Zhang et al., 2009; Aranjuelo et al., 2011), suggesting a photosynthetic acclimation at elevated [CO_2] caused by down-regulation. Photosynthetic acclimation has been previously described in wheat plants exposed to elevated [CO_2] (Martínez-

Carrasco et al., 2005; Alonso et al., 2009; Gutiérrez et al., 2009; Aranjuelo et al., 2011). The decrease in photosynthetic capacity under elevated [CO_2] has been attributed to end product inhibition, in which the demand for carbohydrates is insufficient to cope with the enhanced carbohydrate supply (Rogers and Ellsworth, 2002; Ainsworth and Long, 2005; Aranjuelo et al., 2008). Carbohydrate accumulation in leaves, grown at elevated [CO_2], has been shown to stimulate organic acid synthesis (Morcuende et al., 1998; Stitt and Krapp, 1999) and respiratory pathways, irrespective of whether it is a result of sugar-feeding or an inhibition of phloem transport.

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

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

In our study we found evidence that plants showed photosynthetic up-regulation under the depleted [CO_2], because plants modulated the Rubisco content (Table 3). Rubisco content seems to be higher in plants that undergo prolonged exposure to pre-industrial levels of [CO_2] than those grown at current [CO_2] levels (Maherali et al., 2002). Gesch et al., (2000) observed increased Rubisco small

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

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

Water availability effects on plants.

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

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

Besides this, significant differences in pigment contents were also found between water treatments. WS plants showed lower pigment contents than WW plants at depleted and elevated [CO_2], with an overall reduction in chlorophyll content. Similar results have also been reported by other authors (Dalal and Tripathy, 2012), suggesting that Chlorophyll biosynthesis is substantially down-regulated under water-stress. Down-regulation of chlorophyll content could act as a regulatory mechanism in plants to resist drought. Minimisation of light absorption by reduced amounts of chlorophyll would down-regulate the electron transport so as to reduce the ROS production. Decreases in the carotenoid content suggested that the photosynthetic apparatus may be predisposed to photodamage; however, carotenoid increases were observed in mild water-stress under current [CO_2]. Carotenoids prevent destruction of chlorophyll in high light by dissipating excess excitation energy and may also stabilise and photo-protect the lipid phase of the thylakoid membranes (Havaux, 1988). On the other hand, the analysis of Rubisco revealed that in our study mild water-stress treatment affected the expression of Rubisco and we observed decreases from 40 to 60 % of Rbc L and Rbc S. These data corroborate that the plants suffered down expression of Rubisco content with WS stress. Drought and low water availability inhibited N fixation through their effect on biomass production (Aranjuelo et al., 2007). As in our case, the leaf N content decreased in plants kept under mild water-stress and this is related to the Rubisco content, assimilation and also the production of biomass.

[CO_2] effects on plants modulated by water availability.

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

At elevated [CO_2] the autotrophic biomass in WW and mild WS was decreased by 36% and 19%, respectively, with leaves being the most affected organs. However, the root biomass increased 10% and 5% for WW and mild WS plants, respectively, and this is in agreement with previous reports (Amthor, 2001; Uddling et al., 2008; Högy et al., 2009; Aranjuelo et al., 2011). These data suggest

that plants invest more C in roots than shoots at elevated [CO_2] and this effect is modulated by water availability, with a greater effect in WW plants. An explanation for this is that plants under elevated [CO_2] do not need large autotrophic areas to obtain the C and they invest the assimilated C in roots in order to find other nutrients required under the elevated [CO_2] conditions. Further, this effect is increased with higher water availability. However, under depleted [CO_2] the plants kept or even increased their autotrophic organs and decreased roots, suggesting that plants under depleted C invest more in developing the organs that capture more C from the atmosphere. However, mild- WS limits this development. Other authors have also found that plants modify the number of stomata, leaf area, leaf thickness, biomass partitioning and other parameters to adapt to different [CO_2] (Ainsworth and Long 2004; Leakey et al., 2009; Gerhart and Ward, 2010).

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

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

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

Also, lower photosynthetic rates in WW than in WS plants before anthesis and the end of grain filling, and higher rates during grain filling, indicated that photosynthetic down-regulation is

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

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

Changes in pigment contents showed significant differences in the interaction of CO_2xWater availability, indicating that the responses of plants were modified by both factors. Plants under depleted [CO_2] showed decreased pigment contents in both water treatments, however, plants at elevated [CO_2] only showed large decreases in water-stress. These data may indicate the way plants reduce their pigment contents to protect themselves from damage due to drought and [CO_2], minimising the amount of pigment so as to reduce electron transport and ROS production (Dalal and Tripathy, 2012), this effect was higher under depleted [CO_2]. However, the pigment content reduction is maximal at elevated [CO_2] and WS indicating that both treatments together have an enhanced effect with the CO_2 and water availability modulating each other. On the other hand, decreases in pigment content suggest that the combination of mild water-stress at elevated [CO_2] put the greatest constraints on plants, but that the impact of elevated [CO_2] in WW plants was minimum. However, restrictions on C under depleted [CO_2] caused decreases in pigment content in both water treatments suggesting that C restriction at depleted [CO_2] is a more important limitation than water-stress for plants.

The analysis of Rubisco at elevated [CO_2] revealed that the photosynthetic down regulation at elevated [CO_2] was caused by a lower Rubisco content (Theobald et al., 1998; Aranjuelo et al., 2005; Gutierrez et al., 2009) and was modulated by water availability with greater decreases in mild WS plants. The lower photosynthetic rates of plants exposed to 700 ppm may be a consequence of decreases in content of both Rubisco subunits. In our study we observed decreases in Rbc L (25 and 29% for WW and WS respectively) and Rbc S (40 and 50% for WW and WS; Table 4). These data corroborate that plants suffered down-regulation under elevated [CO_2] and with WS stress the

Rubisco content was even more affected, but as we saw before, the photosynthetic acclimation was stronger in WW plants. Specifically, in WW plants there were 103% and 50% more Rbc L and Rbc S subunits, respectively, while in WS plants the Rbc L was only 10% higher and the Rbc S was 12% smaller at depleted than at current [CO_2], suggesting that the drought negatively affected the up-regulation processes at depleted [CO_2]. As we highlighted before, WS also modulated the response of Rubisco content to the down- and up-regulation at elevated and depleted [CO_2].

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

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

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SUPPLEMENTARY MATERIAL

Table S1: CO_2 effects (260, 400 and 700 ppm) on growth parameters under well-watered (WW) and mild water-stress (WS,) conditions before anthesis (T0), the beginning grain filling (T1) and the end of grain filling (T2). Each value represents means of 4 plants.

CO_2	time	Water	NE	LE	NeE	NT	LT	Zadok	Flags	Spikes	Leaves	Stems	Shoots	Roots	Shoot/Root
700	T0	WW	2.0 ±0.6	6.0 ±0.1	11.7 ±0.3	3.3 ±0.3	53.8 ±1.9	60.7 ±0.7	0.5±0.1	2.6±0.5	1.8 ±0.4	5.7 ±0.9	10.5 ±2.0	9.7 ±1.6	1.1 ± 0.3
		WS	1.0 ±0.0	5.2 ±0.2	11.0 ±0.6	1.7 ±0.7	46.4 ±1.4	74.3 ±0.7	0.2±0.0	1.8±0.3	1.0 ±0.2	2.8 ±0.5	5.8 ±0.8	5.9 ±0.2	1.0 ±0.2
	T1	WW	3.0 ±0.0	6.5 ±0.4	14.3 ±0.3	3.0 ±0.0	57.0 ± 0.8	72.0 ±0.0	1.0±0.2	6.0±0.5	2.5 ±0.3	9.7 ±1.0	19.2 ±1.9	16.4 ±2.5	1.2 ±0.1
		WS	2.0 ±0.6	5.3 ±0.4	11.3 ±0.7	2.3 ±0.7	46.6 ±4.1	75.7 ±0.7	0.5±0.1	2.9±0.6	0.9 ±0.2	3.7 ±1.0	8.0 ±1.7	7.1 ±2.1	1.2 ±0.1
	T2	WW	2.3 ±0.3	6.1 ±0.1	13.0 ±1.5	2.3 ±0.3	54.2 ±0.6	81.0 ±1.5	0.6±0.1	5.9±0.5	1.5 ±0.0	7.9 ±0.3	16.0 ±0.9	10.5 ±1.9	1.7 ±0.4
		WS	1.3 ±0.3	5.2 ±0.4	12.3 ±1.3	1.3 ±0.3	43.0 ±2.1	84.3 ±0.7	0.2±0.0	3.3±0.6	0.6 ±0.1	2.4 ± 0.5	6.5 ±1.2	4.4 ±0.2	1.4 ±0.2
400	T0	WW	3.0 ±0.0	6.3 ±0.1	14.0 ±0.0	3.0 ±0.0	55.1 ±2.0	67.3 ±1.5	1.4±0.0	5.3±0.4	4.3 ±0.2	10.3 ±0.5	21.1 ±0.8	22.3 ±2.4	1.0 ±0.1
		WS	1.0 ±0.0	4.1 ±0.2	8.7 ±0.3	1.0 ±0.0	43.3 ±0.7	69.3 ±1.2	0.2±0.0	0.8±0.1	0.6 ±0.2	1.9 ±0.2	3.5 ±0.3	3.2 ±0.3	1.1 ±0.2
	T1	WW	3.0 ±0.0	6.2 ±0.0	15.0 ±0.6	4.3 ±1.3	51.9 ±1.2	70.0 ±1.2	1.7±0.6	5.9±0.5	5.6 ±0.6	13.5 ±2.5	26.7 ±4.1	18.0 ±0.2	1.5 ±0.2
		WS	2.0 ±0.0	4.7 ±0.4	9.7 ±1.5	2.0 ±0.0	45.2 ±1.2	74.7 ±0.3	0.5±0.0	1.9±0.3	0.8 ±0.2	3.2 ±0.2	6.3 ±0.5	4.7 ±0.2	1.3 ±0.1
	T2	WW	3.7 ±0.7	6.3 ±0.1	14.7 ±0.7	4.3 ±0.3	51.7 ±0.2	75.3 ±2.3	1.2±0.1	7.4±0.9	4.1 ±0.2	12.1 ±0.6	24.8 ±1.7	9.5 ±1.2	2.7 ±0.4
		WS	2.7 ±0.3	4.6 ±0.4	9.3 ±1.3	2.7 ±0.3	41.5 ±1.3	82.0 ±1.0	0.5±0.1	3.7±1.0	0.7 ±0.2	3.3 ±0.7	8.2 ±1.8	3.8 ±1.1	2.4 ±0.3
260	T0	WW	1.7 ±0.3	5.6 ±0.2	13.0 ±1.0	2.7 ±0.3	41.3 ±1.7	64.0 ±3.0	0.5±0.1	1.7±0.3	2.6 ±0.6	5.4 ±0.1	10.3 ±1.4	6.1 ±1.1	1.7 ±0.1
		WS	1.0 ±0.0	5.5 ±0.2	11.7 ±1.3	2.0 ±0.0	40.1 ±2.8	69.7 ±1.5	0.4±0.0	1.0±0.2	1.4 ±0.1	2.7 ±0.1	5.6 ±0.2	3.0 ±0.2	1.8 ±0.0
	T1	WW	2.0 ±0.0	5.8 ±0.2	13.0 ±1.2	2.3 ±0.3	45.0 ±2.8	69.7 ±2.3	0.7±0.1	2.4±0.4	3.6 ±0.4	6.0 ±0.5	12.7 ±0.9	8.8 ±1.3	1.5 ±0.1
		WS	1.0 ±0.0	5.2 ±0.4	10.3 ±1.9	1.7 ±0.3	39.0 ±1.8	73.0 ±1.0	0.3±0.0	1.0±0.2	1.3 ±0.1	3.0 ±0.4	5.7 ±0.7	2.4 ±0.7	2.6 ±0.4
	T2	WW	2.7 ±0.7	6.2 ±0.2	15.0 ±0.6	3.7 ±0.3	48.5 ±3.0	75.7 ±2.3	1.1±0.3	4.8±0.6	3.7 ±0.4	9.5 ±0.8	19.0 ±2.0	8.8 ±1.3	2.2 ±0.1
		WS	2.7 ±0.3	4.5 ±0.1	9.3 ±0.3	2.7 ±0.3	37.4 ±1.3	80.7 ±0.7	0.7±0.1	2.2±0.4	4.2 ±3.2	3.2 ±0.2	10.3 ±3.7	3.1 ±0.3	3.2 ±0.9
CO_2			0.121	0.139	0.379	0.422	**	0.06	**	**	*	*	**	***	*
Water			***	***	**	***	**	*	***	***	***	***	**	***	0.165
CO_2 x water			0.121	**	*	0.203	*	**	***	**	**	**	**	***	*

Table S2. Summary of the ANOVA results for the effects of CO_2 (260, 400 and 700 ppm) under well-watered (WW) and mild water-stress (WS) conditions on assimilation (A), conductance (gs), $V_{c.max}$, J_{max}, A_{sat}, F_v/F_m, F'_v/F'_m, Φ_{PSII}, q_P, q_N, NPQ, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and total soluble protein content (TSPC).

	A	gs	$V_{c.max}$	J_{max}	A_{sat}	F_v/F_m	F'_v/F'_m	Φ_{PSII}	q_P	q_N	NPQ	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoids	TSPC
CO_2	0.83	0.89	0.48	0.57	0.08	*	0.45	0.77	0.13	0.90	0.12	***	***	***	***	***
Water	0.17	0.59	0.39	0.75	0.27	*	*	*	0.11	0.16	0.49	***	***	***	***	***
CO_2 x water	*	*	0.28	0.09	0.53	*	*	*	*	0.77	0.35	***	***	***	***	***

General Discussion

En la discusión general de esta Tesis se pretenden discutir los resultados de una forma integrada:

- El estudio del efecto de bajas [CO_2] y su modulación por diferentes factores ambientales sobre el crecimiento de las plantas, su desarrollo y resistencia a estreses ambientales como es la sequía, nos dará las claves para entender los cambios que se producirán en las plantas frente a los futuros incrementos de [CO_2] en la atmósfera y mayor sequía asociada a la disminución de disponibilidad de agua y al calentamiento global (Ainsworth y Rogers, 2007). Esto nos permitirá entender las respuestas del trigo a nivel fisiológico tanto a los cambios en la [CO_2] en el ambiente como a la modulación del efecto de la sequía moderada a distintos niveles de CO_2.
- Destacar los procesos de aclimatación de la planta, sobre todo a nivel de la fotosíntesis y del crecimiento. Señalar que, por medio del citado objetivo, se han identificado nuevas estrategias de las plantas para aclimatar su fotosíntesis o crecimiento a los cambios de [CO_2] ambientales, existiendo diferentes explicaciones para el fenómeno de la aclimatación al CO_2. Por ejemplo, a elevada [CO_2] hay un inicial incremento de la asimilación de CO_2 en forma de carbohidratos, pero la limitación de un sumidero donde acumular los carbohidratos producidos hace que la planta disminuya la asimilación de CO_2. Otra explicación puede ser la progresiva limitación de N en hoja, ya que la planta acumula carbohidratos en la parte aérea más rápido que la adquisición de N y el nivel de N en hoja disminuye afectando a la cantidad de proteínas y produciendo la aclimatación fotosintética (Bloom y col., 2010). Estos procesos tienen un efecto fisiológico en el contenido de proteínas y enzimas, pero también en la calidad de los alimentos. En esta Tesis se han estudiado algunas de las estrategias de las plantas para poder compensar los efectos negativos del cambio climático, lo cual resulta fundamental para futuros estudios, pero también para el desarrollo de la agricultura.
- Los estudios en ambientes controlados como los de esta Tesis resultan muy relevantes para comprender los mecanismos y adaptaciones que se producen en la planta, ya que algunos de estos descubrimientos podrán aplicarse al estudio de ecosistemas afectados por el cambio climático en el futuro.
- Mostrar la aplicación del uso de isótopos estables para trazar y seguir su movimiento a través de la planta bajo diferentes [CO_2] y estreses ambientales.
- Sintetizar los principales resultados obtenidos a través de los diferentes capítulos en relación a los objetivos y discutirlos según la literatura actual existente.
- Sugerir potenciales y futuras líneas de investigación acordes a los resultados obtenidos.

1. Bases fisiológicas en la variación del CO_2 ambiental.

Las plantas han desarrollado una amplia flexibilidad de respuestas fisiológicas a las variaciones ambientales. En los siguientes apartados, se pretende resumir los resultados derivados de la presente Tesis en los cuales se caracterizaron los cambios fisiológicos mediante la aclimatación en la fotosíntesis para adaptarse a la variación en la [CO_2] ambiental.

1.1. Cambios fisiológicos asociados a la aclimatación fotosintética a baja [CO_2].

En esta sección nos centraremos en las respuestas de las plantas a baja [CO_2] atmosférico y discutiremos cómo las respuestas de las variaciones de las [CO_2] en el pasado, pueden ser significativas para la adaptación de las plantas a futuros ambientes con elevadas [CO_2].

En los capítulos 1, 2 y 3, se ha comprobado cómo las plantas modifican un gran número de funciones fisiológicas, niveles de proteínas, distribución de C o desarrollo de partes vegetativas para adaptarse a una [CO_2] preindustrial (Figura 1). Nuestro trabajo se ha enfocado en la respuesta de las plantas (una variedad moderna y otra antigua, capítulos 1 y 2) sometidas a un prolongado efecto de baja [CO_2], durante todo el ciclo de vida de la planta (capítulos 1, 2 y 3) y su modulación con estrés hídrico moderado (capítulo 3).

Sin embargo, muchos estudios han observado que, una corta exposición a baja [CO_2], tiene un efecto directo e inmediato en variedades modernas, limitando la fotosíntesis (Tissue y Lewis, 2012), con una subsecuente reducción en la producción de biomasa (Lewis y col., 2010), reproducción (Dippery y col., 1995), y supervivencia (Ward y Kelly, 2004). No obstante, señalar que los mencionados estudios no reflejan las respuestas adaptativas a una prolongada exposición a baja [CO_2]. Según Tissue y Lewis, (2012), como la duración de esta baja [CO_2] ha sido muy larga (más de 20 000 años) y únicamente ha habido un incremento en estas concentraciones en los últimos 150 años, la presión selectiva ha sido mucho más fuerte y prolongada a baja que a elevada [CO_2].

Como en este caso, y a nivel de toda la planta, la adaptación a baja [CO_2] puede incluir una mayor distribución de C en la biomasa de las hojas a costa de la biomasa de las raíces (Sage y Cowling, 1999; Ghashghaie y Badeck, 2014). Así, en los capítulos 1, 2 y 3, se ha podido observar un incremento de la biomasa aérea y una disminución de la biomasa de las raíces. Además, se ha observado que el ratio parte aérea/parte subterránea (en inglés *shoot/root*) se incrementa cuando disminuye el CO_2 atmosférico porque las plantas incrementan la parte fotosintética y el área de captación de C para compensar la menor disponibilidad de CO_2 en el ambiente.

En las plantas C_3, la baja [CO_2] afecta a las tasas netas de fotosíntesis mediante la reducción de la tasa de carboxilación de Rubisco resultante de las limitaciones de sustrato y por medio de mayores tasas de fotorespiración (Gerhart y Ward, 2010). A lo largo de la Tesis hemos estudiado las curvas *A*-ci ya que permiten entender la respuesta de la Rubisco a las diferentes tratamientos de baja y alta [CO_2] (Sage, 1994; Overdieck, 1989; Sage y Reid, 1992). Resaltar que, debido a que esta enzima ha sido altamente conservada a través del tiempo (Griffiths, 2006), tales mediciones pueden tener el potencial de ser extrapoladas a plantas en condiciones glaciales. En los capítulos 1, 2 y 3 comprobamos los factores que favorecen la aclimatación fotosintética a [CO_2] preindustrial, así en los capítulos 1 y 3, demostramos que puede haber una mayor proporción de Rubisco en hoja para compensar los efectos limitantes de las bajas [CO_2]. Esto ha sido observado por otros estudios (Maherali y col., 2000), donde tras una prolongada exposición a baja [CO_2] incrementó el nivel de Rubisco en hoja, lo que indicó una mayor tasa de fotosíntesis, además de aumentar también el contenido total de proteínas solubles en hojas (TSPC).

Además, Zhu y col., (2004) modeló los efectos de la variación en la especificidad de la Rubisco en la fotosíntesis y mostró que la velocidad catalítica de la Rubisco en plantas C_3 actuales es óptima a niveles de CO_2 de aproximadamente 200 ppm. Podemos decir que la evolución de la Rubisco no ha seguido el ritmo del aumento de CO_2 lo que sugiere una baja adaptación de la misma a mayores niveles de CO_2 y una buena adaptación a niveles preindustriales (Leakey y Lau, 2012). Sin embargo, el crecimiento en las plantas C_3 se ha visto reducido a baja [CO_2], pues las plantas responden incrementando la conductancia estomática para dar mayor acceso al CO_2, lo que permite una regulación a la alta (en inglés *up-regulation*) de la fotosíntesis con incrementos de la V_{cmax}, J_{max} y *A* (Anderson y col., 2001; Gerhart y Ward, 2010), al igual que ocurre en nuestro caso.

Como en este estudio, una mayor inversión de C hacia las hojas y menor hacia las raíces puede resultar en un mayor incremento de la tasa de fotosíntesis a baja [CO_2] (Allen y col., 1991), siempre que no haya ningún otro estrés como, por ejemplo, la carencia de N. Además, en la aclimatación del crecimiento a baja [CO_2] se han podido observar incrementos en el contenido de N y también en el PNUE (Pinto y col., 2014).

Otro de los puntos interesantes de la presente Tesis ha sido el estudio del efecto de las diferentes [CO_2] en la fluorescencia de la clorofila (capítulos 1 y 3). Así, a baja [CO_2], se ha podido observar que la planta ajusta el transporte electrónico de las membranas tilacoidales con la carboxilación (Nogués y Baker, 2000). Pero además, también se observó una disminución en F_v/F_m, Φ_{PSII} y q_P; y un aumento de NPQ y q_N. Estos datos indican una disminución de la eficiencia

fotosintética que se pudo deber por un lado, al ajuste del transporte electrónico, pero también pudo deberse a un posible daño en el PSII debido al aumento en el transporte de electrones hacia los procesos foto-respiratorios, los cuales pudieron haber incrementado las especies reactivas de oxígeno (ROS). Sin embargo, el aumento de la disipación de energía en forma de calor indicaba procesos de protección del fotosistema, ya que se desviaría el transporte de electrones al ciclo de las xantofilas donde hay disipación de energía en forma de calor (Demming-Adams, 2003). En este caso fue la variedad antigua la que disminuyó menos su eficiencia fotosintética, lo que nos indicaría que estaban mejor adaptadas a las bajas [CO_2].

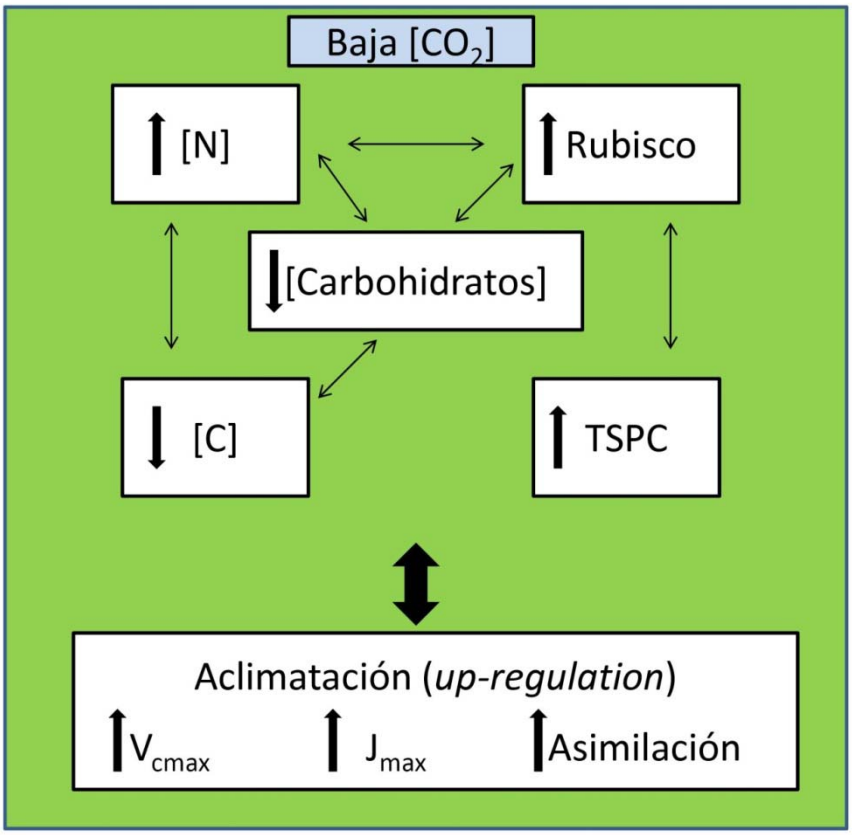

Figura 1: Representación de algunos cambios producidos tras el proceso de aclimatación fotosintética en la hoja de trigo duro sometida a baja [CO_2]. Los datos corresponden a los capítulos 1, 2 y 3. TSPC: Concentración total de proteínas solubles; V_{cmax}: Máxima velocidad de carboxilación de la Rubisco; J_{max}: Transporte máximo de electrones al PSII.

1.2. Cambios fisiológicos asociados a la aclimatación fotosintética a elevada [CO_2].

En esta sección nos enfocamos en las respuestas de las plantas a elevada [CO_2] atmosférico y discutimos cómo las respuestas de las plantas pueden ser significativas para entender la adaptación de las mismas a futuras y elevadas [CO_2].

Las plantas modifican un gran número de funciones, proteínas, fotosíntesis, respiración, distribución de C o desarrollo de partes vegetativas entre otros para adaptarse a una [CO_2] futura como podemos observar en los capítulos 1, 2 y 3 (Figura 2).

La aclimatación de la fotosíntesis es comúnmente definida como una disminución de la V_{cmax} y J_{max} (Long y col., 2004). Sin embargo, también se estimula la asimilación de C, aunque esta varía dependiendo de las condiciones ambientales (Nowak y col., 2004). Los factores que determinan la estimulación de asimilación de C en plantas C_3 crecidas a elevada [CO_2] pueden ser variables. Así, la asimilación de C es limitada por el CO_2 interno (c_i) a elevada [CO_2] (Ainsworth y Rogers, 2007). Las curvas de asimilación (A-c_i) indican que, llegado un cierto grado de aumento de c_i, el aumento de la A está limitada por el transporte electrónico que contribuye a la capacidad de regeneración de la Rubisco (J_{max}) (Long y Bernacchi, 2003). Además del ambiente, factores experimentales o genéticos, pueden también limitar el desarrollo de los sumideros y producir una mayor aclimatación en la planta y reducir la estimulación de asimilación a elevada [CO_2] (Long y col., 2004; Ainsworth y Rogers, 2007; Leakey y col., 2009) como ha ocurrido en nuestro caso en los capítulos 1 y 2 entre la variedad tradicional y moderna (diferencias genéticas) y en el capítulo 3 con la sequía (diferencias ambientales).

Algunos estudios han confirmado, como en nuestro caso, que la insuficiente capacidad para generar sumideros de C (debido a diferencias genéticas o ambientales) puede dar lugar a una excesiva fuente de C para las plantas, incluso aunque se haya producido la aclimatación (Aranjuelo y col., 2009, 2013). Una de las respuestas generales de muchas hojas sometidas a elevada [CO_2] es un incremento de carbohidratos en las mismas, las cuales acumulan altos niveles de azúcares y almidón que inhiben el rendimiento fotosintético y que favorecen la aclimatación de la fotosíntesis (Moore y col., 1997; Stitt y Krapp, 1999; Long y col., 2004; Ainsworth y Rogers, 2007).

Algunos experimentos tanto de enriquecimiento de CO_2 en condiciones de campo (tipo *FACE, Free-Air Carbon dioxide Enrichment)* como en cámara de crecimiento, han apoyado la evidencia de la capacidad de la fotosíntesis para aclimatarse a elevada [CO_2] en plantas C_3 y, tal y como acabamos de poner de manifiesto, esta aclimatación varía con el tipo de planta y las condiciones ambientales. Además, la ganancia de C es marcadamente mayor para estas plantas crecidas a elevada [CO_2] (Ainsworth y Rogers, 2007; Leakey y col., 2009).

Como ya hemos mencionado, cabe destacar lo relevante de la capacidad de desarrollar sumideros de C en las plantas ya que va a determinar también el grado de aclimatación. Como

pudimos ver en los capítulos 1 y 2, aquellas plantas seleccionadas para desarrollar partes reproductivas mayores (sumideros de C), pudieron evitar en mayor grado la aclimatación a elevada [CO_2]; en cambio, aquellas que tenían más reducida capacidad de desarrollar sumideros en las partes reproductivas sufrieron más acusadamente la aclimatación de la fotosíntesis. Todo ello se traduce en un incremento de carbohidratos en las hojas y una mayor regulación a la baja (en inglés *down-regulation*) de la fotosíntesis (Aranjuelo y col., 2013).

A nivel de toda la planta, la adaptación a elevada [CO_2] puede incluir una mayor distribución de C en la biomasa de las raíces ya que la fuente de C, es mayor y las plantas pueden invertir más en el crecimiento de las raíces, que además tendrán un mayor área de búsqueda de agua y nutrientes (Ghashghaie y Badeck, 2014). Por lo tanto, y como ocurre a lo largo de esta Tesis, el ratio parte aérea/parte subterránea (en inglés *shoot/root)* disminuye conforme las plantas se desarrollan a mayor CO_2 atmosférico.

Otro aspecto que influye en la aclimatación de la fotosíntesis a elevada [CO_2] es la cantidad de nitrógeno asimilada en la hoja y la eficiencia en el uso del nitrógeno. A lo largo de la Tesis hemos podido observar los cambios en el [N] a futuras [CO_2] y en la eficiencia en el uso del nitrógeno en plantas de trigo a elevada [CO_2]. Al igual que otros investigadores, en esta Tesis también se ha observado una disminución en el contenido de N en la hoja, una marcada disminución de la concentración de la Rubisco y de las proteínas a elevada [CO_2]. La Rubisco generalmente representa entre un 20 a 30 % de N en la hoja (Sage y Pearcy, 1987), aunque en algunos casos la inversión del N en la misma puede llegar hasta un 50% (Spreitzer y Salvucci, 2002). Así, es importante hacer coincidir la mayor oferta de C a elevada [CO_2] con un óptimo contenido en N para evitar la disminución en la asimilación, puesto que hay evidencias claras de la relación entre la cantidad de N y la aclimatación (Leakey y col., 2009). En estudios hechos con diferentes tratamientos de N, los investigadores pudieron observar que las plantas que crecen con poco N, por lo general acumulan más carbohidratos foliares y exhiben una mayor aclimatación del contenido de Rubisco que las cultivadas con una mayor [N] (Farage y col., 1998, Ainsworth y col., 2003; Ainsworth y Long, 2005). Existen estudios de plantas con una gran capacidad de absorción de N, como por ejemplo el álamo que en ausencia de otros estreses, como sequía o nutrientes, pueden evitar la limitación del sumidero y de este modo minimizar la pérdida asociada de Rubisco cuando crecen a elevada [CO_2] (Davey y col., 2006). Las leguminosas, tienen el potencial para responder a una elevada [CO_2], ya que sus bacterias fijadoras de nitrógeno proporcionan un gran sumidero de C (el exceso de C puede ser utilizado para la asimilación de N permitiéndoles al mismo tiempo evitar la limitación del sumidero y de aumentar su suministro de N; Rogers y col., 2006; Sanz-Sáez y col.,

2010). Esto se ha traducido en una mejora de la productividad de las leguminosas a elevada [CO_2] en comparación con las plantas no leguminosas (aumento del contenido de N foliar, la fotosíntesis y, en algunos casos, la productividad). En plantas no leguminosas, un aporte externo de N podría favorecer que se evite la aclimatación de la fotosíntesis y de la Rubisco, esto ha sido objeto de estudio y se ha visto que plantas crecidas a elevadas condiciones de N en suelo reducen menos su *A* y V_{cmax} que plantas crecidas a baja disponibilidad de N (Ainsworth y Long, 2005).

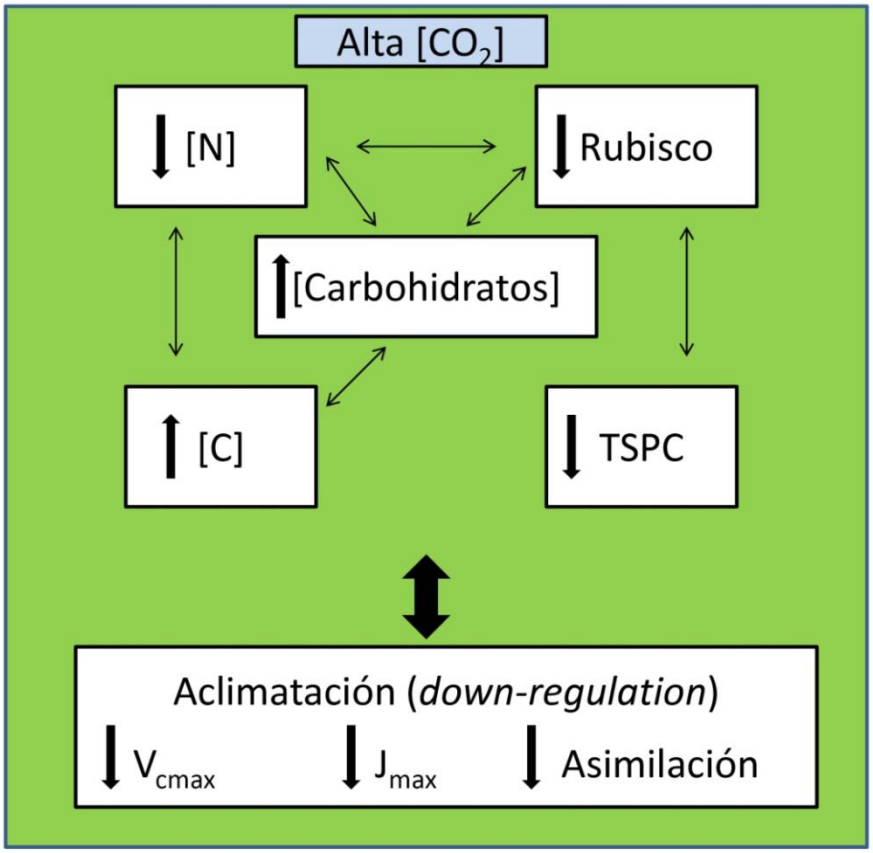

Figura 2: Representación de algunos cambios producidos tras el proceso de aclimatación fotosintética en la hoja de trigo duro sometida a elevada [CO_2]. Los datos corresponden a los capítulos 1, 2 y 3. TSPC: Concentración total de proteínas solubles; V_{cmax}: Velocidad de carboxilación máxima de la Rubisco; J_{max}: Transporte máximo de electrones al PSII.

Otros investigadores han propuesto que la disminución de N foliar, se debe a una disminución de la transpiración en las plantas sometidas a elevada [CO_2], como por ejemplo Tezara y col., (2002), que indicaron cómo la conductancia estomática se ve reducida. La transpiración puede facilitar la toma de N gracias a un aumento de flujo de la solución del suelo hacia la raíz y la ascensión y asimilación en la hoja (Del Pozo y col., 2007). Otros estudios también sugieren que la elevada [CO_2] inhibe la asimilación de NO_3^- de las partes aéreas del trigo, que a su vez inhiben la absorción de NO_3^- por las raíces (Bloom y col., 2010) y da una explicación de la aclimatación de la

fotosíntesis que se podría extrapolar a los datos de esta Tesis. Ésta se basa en que las plantas acumulan carbohidratos más rápidamente de lo que pueden asimilar el N, lo que implica una disminución en el contenido de N en hoja que, conjuntamente con la acumulación de carbohidratos, producen una disminución de Rubisco y otras proteínas. En recientes estudios con trigo, este mismo autor propone que debido a un mayor crecimiento de las plantas a elevada [CO_2] y a la acumulación de carbohidratos en hojas se observa una disminución de la cantidad de Rubisco y de otras proteínas, pero también hay un proceso de dilución en sus tejidos. Además, estos autores mencionan la mayor acumulación de C exudado en raíces, debido a la elevada [CO_2], como otro factor que inhibe la absorción de N, lo que provoca mayores limitaciones de N en la planta (Bloom y col., 2014).

Por otro lado, en las medidas de fluorescencia de la clorofila, las plantas mostraron una disminución en la eficiencia fotoquímica a elevada [CO_2], aunque la actividad del PSII resultó menos afectada a elevada que a baja [CO_2]. Como se ha dicho antes, las plantas disminuyeron el metabolismo de C mediante la *down-regulation*, con lo que disminuyó la demanda de electrones al PSII que fueron hacia otros procesos (*quenchings*). El F_v/F_m no disminuyó, con lo que no se observó daño en el PSII a elevada [CO_2] debido a la aclimatación en la asimilación del C y del contenido de la Rubisco; por un lado las plantas ajustaron a la baja el transporte de electrones junto con la carboxilación, y a su vez, también por una mayor disipación de energía en forma de calor. Las plantas de trigo a elevadas [CO_2] disiparon más energía en forma de calor mediante el ciclo de las xantofilas que las plantas a bajas [CO_2], lo cual puede prevenir incrementos del transporte de electrones a la fotorespiración (Taub y col., 2000; Hymus y col., 2001).

Otro de los intereses de esta Tesis era observar los cambios efectuados a nivel de la respiración de la planta (capítulos 1 y 2) y su relación con el cambio climático, debido a su importancia en el balance del ciclo del carbono. A escala global, el flujo de carbón de la respiración de las plantas es 5-6 veces mayor que las emisiones derivadas de actividades humanas (Canadell y col., 2007). Aunque el potencial del incremento de la [CO_2] atmosférica para alterar la respiración de las plantas ha sido muy discutido, aún no se conoce muy bien el efecto sobre la respiración (González-Meler y col., 2004; Leakey y col., 2009).Algunos estudios en plantas, han descrito disminuciones de la respiración a la oscuridad debido a un incremento en las [CO_2] (Wang y Curtis, 2002; Drake y col. 1999). Sin embargo, otros estudios han observado situaciones con diferentes resultados sin alteraciones entre los distintos tratamientos o incluso hasta con pequeños incrementos (Davey y col., 2004). En los capítulos 1, 2 y 3 pudimos observar incrementos en la tasa de respiración al

aumentar la [CO_2] atmosférica. Sin embargo no se encontraron diferencias significativas en la mayoría de los casos indicándonos una aclimatación de la tasa de respiración.

1.3. Variación de la eficiencia en el uso de agua a diferentes [CO_2].

La eficiencia en el uso de agua (en inglés *water use efficiency*, WUE) puede indicar el potencial de las plantas y las hojas para evitar los estreses (Farquar y Sharkey, 1982). Para ver el potencial de nuestras plantas de trigo frente a las diferentes [CO_2], medimos el WUE que es fuertemente dependiente del CO_2 atmosférico. Como en nuestro caso el WUE disminuye a baja [CO_2] (Cowling y Sykes, 1999). Además, Polley y col., (1993; 1995) observaron que *Triticum aestivum* y otras C_3, redujeron el WUE en condiciones de baja [CO_2]. Esta disminución en el WUE está relacionada con la capacidad de las plantas para regular la conductancia estomática (g_s) de acuerdo a la cantidad de CO_2 en la atmósfera. Así, plantas sometidas a baja [CO_2] abren más los estomas que en condiciones actuales, lo cual nos sugiere que las plantas fueron menos conservativas con el agua en el pasado, como muestran nuestros datos, indicando que las plantas crecidas a baja [CO_2] necesitan abrir más los estomas para asimilar más CO_2 de la atmosfera que las plantas a niveles de [CO_2] actuales.

También hemos podido observar que la WUE es mayor en plantas a elevada [CO_2] (Aranjuelo y col., 2011). Los datos experimentales indicaron que la WUE incrementó desde bajos a elevados niveles de CO_2 atmosférico (Gerhart y col., 2010). Como dijimos anteriormente, el WUE está relacionado con la capacidad de las plantas para regular la conductancia estomática dependiendo de la cantidad de CO_2 en la atmósfera. Las plantas a elevada [CO_2] cierran más los estomas y su conductancia es menor, por lo tanto, estas plantas son más conservativas en términos del uso de agua que en condiciones actuales como muestran nuestros datos.

1.4. Consecuencias de las adaptaciones de baja [CO_2] a un nuevo escenario de cambio climático.

Un aspecto importante de esta investigación es conocer si las adaptaciones a baja [CO_2] están limitando significativamente la respuesta de las plantas a elevada [CO_2].

Como hemos podido observar a lo largo de la Tesis, si se asigna demasiado carbono para su almacenamiento o para la producción de carbohidratos en hoja en lugar del crecimiento, se impiden el metabolismo de los fotoasimilados en las tasas exigidas para explotar una mayor disponibilidad del CO_2 (Aranjuelo y col., 2011; Gutiérrez y col. 2013). Así, se ha observado como la planta modula su tasa fotosintética a diferentes niveles de CO_2 para adaptarla a sus necesidades. Por

ejemplo, la *down-regulation* de la fotosíntesis puede ser consecuencia de la selección de las plantas a la baja [CO_2] de una época anterior.

Estas limitaciones no tienen que ser necesariamente estresantes, pero pueden impedir que las plantas aprovechen el aumento del potencial de producción que confiere un aumento de CO_2. Por lo tanto, podrían ser una desventaja en un entorno competitivo (Sage y Coleman, 2001).

2. Respuestas del trigo al estrés hídrico

Las plantas han desarrollado una amplia flexibilidad de respuestas fisiológicas a las variaciones ambientales como es la sequía. En este apartado se pretende resumir los resultados derivados de la presente Tesis en los que se caracterizaron los cambios fisiológicos mediante aclimatación en la fotosíntesis para adaptarse a la variación en la [CO_2] de plantas sometidas a un estrés hídrico moderado, ya que la sequía es uno de los factores ambientales que afectan más negativamente a la productividad y al establecimiento de las plantas sobre todo en ambientes mediterráneos (Araus y col., 2002). Además se espera que, con los diferentes escenarios predichos por los modelos presentados por el Panel Intergubernamental sobre el Cambio Climático (en inglés IPCC), se produzca una reducción en las precipitaciones y un incremento en la temperatura y evapotranspiración que también afectará negativamente a las plantas (Alley, 2007).

Como se ha visto en el capítulo 3, frente a una reducción en la disponibilidad de agua, las plantas respondieron con el cierre de estomas y las tasas de carboxilación disminuyeron. Es bien conocido que la conductancia estomática de la hoja se ve afectada por la sequía y las plantas cierran sus estomas originando una reducción de c_i, y aumento en el $\delta^{13}C$ (Farquhar y col., 1989; Scheidegger y col., 2000; Aranjuelo y col., 2009). Aunque una gran parte de la reducción de la tasa de fotosíntesis bajo el estrés hídrico es atribuida al cierre estomático, otra parte de esta reducción también ha sido atribuida a efectos directos de deshidratación en las reacciones bioquímicas de la fotosíntesis (Sharkey y Seeman, 1989; Flexas y Medrano, 2002). Sin embargo, la limitación del crecimiento de las plantas bajo estrés hídrico es también debido a la reducción en el balance del carbono, el cual a su vez es dependiente del balance de la fotosíntesis y la respiración, los cuales están íntimamente ligados (Nunes-Nesi y col., 2005). Normalmente, más de la mitad del carbono asimilado en fotosíntesis es utilizado en procesos respiratorios necesarios para el crecimiento y mantenimiento de la planta, pero este balance puede cambiar con el estrés hídrico. (Flexas y col., 2006).

Las restricciones a la difusión del CO_2 en hoja son habituales durante el estrés hídrico debido al cierre de estomas (como comentamos con anterioridad), el cual limita la fuente de carbono, cambiando el balance de carbono en la planta y el sustrato para la Rubisco. Por tanto, se produce una disminución tanto de la fotosíntesis como de la transpiración (Reich y col., 2006). Además, tal y como se ha podido observar en el capítulo 3, ha habido una disminución de la fluorescencia de las clorofilas con el estrés hídrico moderado, con disminuciones en F_v/F_m, Φ_{PSII} y q_P, indicando que la sequía disminuye la eficiencia fotoquímica de la hoja (con lo que el exceso de energía podría ocasionar daños en los centros de reacción del PSII).

Las respuestas fotosintéticas al estrés de agua comentadas en los párrafos anteriores, pueden ser moduladas por otros factores como el CO_2 o la luz, e incluso la planta puede aclimatar su fotosíntesis a la sequía, como ha ocurrido en nuestro caso (capítulo 3). El nivel de sequía que las plantas sufrieron en nuestro estudio se mantuvo durante todo el ciclo de vida de las plantas, se puede considerar como moderado. Así, las plantas pudieron mejorar su relación hídrica con la fotosíntesis y las disminuciones en V_{cmax}, J_{max} o en la A no fueron tan elevadas como podrían haber sido sin aclimatación fotosintética a una [CO_2] atmosférica actual.

Esta respuesta fisiología del estrés hídrico moderado con la [CO_2] afecta en primer lugar al grado de apertura del estoma. Las plantas a elevada [CO_2] controlan sus estomas para regular la cantidad de agua que es transpirada. La mayor función del estoma es maximizar la tasa a la cual el CO_2 puede difundir a la hoja para ser usado en la fotosíntesis mientras que minimiza la pérdida de agua (Leakey y col., 2009).

Como en la presente Tesis, existen diversos estudios que han observado disminuciones de g_s a elevada [CO_2] tanto en FACE como en cámaras de crecimiento (Medlyn y col., 2001; Ainsworth y Rogers, 2007; Aranjuelo y col., 2013). Además, asociados a la disminución de gs también se han observado disminuciones en la evapotranspiración en plantas de trigo a elevada [CO_2] (Leakey y col., 2009). Este hecho favorece la aclimatación de la fotosíntesis a la sequía moderada, con incrementos de la asimilación frente a plantas que no sufren aclimatación a la sequía moderada (Flexas y col., 2006) y también con respecto a las plantas bien regadas (Reich y col., 2006; Leakey y col., 2009), que se traduce en una ganancia de C. Otro factor a tener en cuenta es la aclimatación de la fotosíntesis a una elevada [CO_2] que conlleva (como se mencionó al principio de la discusión) una *down-regulation* de la fotosíntesis. Concluimos, por tanto, que las plantas bien regadas a elevada [CO_2] producen mayor cantidad de carbohidratos que las plantas en sequía moderada y, por ello, sufren más aclimatación de la fotosíntesis. Así, podemos decir que existe una mayor

estimulación de la productividad a elevada [CO_2] en sequía frente a regadío. Mencionar finalmente que existen diversos estudios que avalan estos resultados en sequía y elevada [CO_2] (Dukes y col., 2005; Reich y col., 2006).

En el caso de baja [CO_2], podemos decir que las plantas de trigo en condiciones bien regadas abren más los estomas para permitir la entrada de más CO_2 y además con la *up-regulation* de la fotosíntesis transpiran más agua sufriendo más los efectos de la sequía. En nuestros experimentos pudimos observar que las plantas se ven afectadas más severamente por la suma de los dos estreses (baja disponibilidad de C y de agua) que en las plantas bien regadas y con elevada [CO_2], ya que debido a lo comentado anteriormente, a baja [CO_2] la planta abre los estomas para coger el CO_2 pero a su vez pierden más agua.

3. Uso de los isótopos estables como marcadores de los metabolismos del C y N.

En este apartado se pretende resumir los resultados derivados de la presente tesis en donde se caracterizaron y siguieron los cambios en los isótopos estables de ^{13}C y ^{15}N en plantas sometidas a la variación en la [CO_2] en el ambiente. A lo largo de la Tesis y con el objetivo de estudiar los flujos de C y N en los diferentes órganos y en el $^{13}CO_2$ respirado, se ha utilizado esta técnica realizando un doble marcaje con ^{13}C y ^{15}N y el posterior análisis de $\delta^{13}C$ y $\delta^{15}N$.

3.1. Ventajas de la técnica.

Los isotopos estables son trazadores no radiactivos, en condiciones *in vivo*, y son integradoras de las respuestas de las plantas a estreses bióticos o abióticos (Dawson y col., 2002). A diferencia del empleo de los isótopos estables en su abundancia natural, el marcaje con isótopos estables enriquecidos (^{13}C y ^{15}N) permite monitorizar los flujos de C y N a lo largo de las diferentes vías metabólicas (Roscher y col. 2000) disminuyendo los problemas de fraccionamiento natural de estos compuestos (Dawson y col., 2002).

El doble marcaje que se realizó en el capítulo 2 en los tres niveles de CO_2 y en condiciones controladas fue homogéneo a lo largo del experimento como evidenciaron los valores de $\delta^{13}C$ y $\delta^{15}N$ a nivel de la fuente y de la materia orgánica total. Es por ello que, la repetitividad de esta técnica de marcaje para estudiar los flujos de C y N a través de la planta, la hace idónea para alcanzar nuestro objetivo. Asimismo, se realizó el análisis de C y N en los diferentes órganos y en el CO_2 respirado por la planta mediante la EA-IRMS y la GC-C-IRMS, por lo tanto, podemos concluir que los isótopos estables son una herramienta eficaz que permite estudiar los flujos *in vivo* de C y N sin alterar los procesos fisiológicos o metabólicos. Además, los isotopos estables tienen el potencial

para proporcionar una visión única de los procesos fisiológicos y de las interacciones entre las plantas y el medio ambiente (Cernusak y col., 2013).

3.2. Estudio del doble marcaje con ^{13}C y ^{15}N.

Carbono-13

En este trabajo se ha utilizado el carbono, que es el elemento más abundante de la biosfera y un componente esencial de los seres vivos. En la biosfera coexisten de forma natural diferentes isótopos de C, aunque en este trabajo se han empleado los isotopos estables ^{12}C (98.99%) y ^{13}C (1.1%).

Los factores ambientales modificaron la composición isotópica de los tejidos de las plantas a través de su influencia de la conductancia de la hoja y la tasa de fotosíntesis. Los cambios en los niveles de intensidad de luz, la [CO_2] y el estado hídrico de la planta pueden reflejar variaciones en el $\delta^{13}C$. Es por ello por lo que para entender la relación entre la variación ambiental y la composición isotópica se ha llevado a cabo el estudio de los isótopos estables en la presente Tesis (Ehleringer y Vogel, 1993).

En general, las hojas están más empobrecidas en ^{13}C que el ambiente debido a la discriminación fotosintética durante la asimilación del CO_2 y post-fotosintética (Badeck y col., 2005), habiéndose observado también diferencias en ^{13}C entre las hojas con otros órganos al igual que podemos ver en el capítulo 2 de esta Tesis (Badeck y col., 2009; Cernusak y col., 2009; Ghashghaie y Badeck, 2014).

La composición isotópica del C de las hojas en plantas C_3 está relacionada con la presión parcial de CO_2 intercelular y la externa o atmosférica (c_i/c_a) en los órganos fotosintéticos y viene afectada por la conductancia estomática (Farquhar y col., 1982). El cierre estomático, debido a algunos estreses como la sequía, conlleva una reducción de la concentración interna del CO_2 (c_i), que incrementan los valores del $\delta^{13}C$ en los fotoasimilados de la planta y la consecuente disminución de la discriminación isotópica del ^{13}C.

Otra hipótesis que explica los valores de $\delta^{13}C$ más negativos en la MOT de este trabajo es que el CO_2 respirado por las hojas en la oscuridad está enriquecido en ^{13}C comparado con la MOT (Ghashghaie y col., 2003). Esto lo hemos podido observar en nuestro experimento, sobre todo después del marcaje con valores de $\delta^{13}C$ respirado más enriquecidos en ^{13}C. Además existe un fraccionamiento entre las hojas y la raíz debida a los procesos de discriminación posfotosintéticos y

de transporte que hace que la hoja esté más empobrecida en ^{13}C que el resto de los órganos (Capitulo 2; Badeck y col., 2005).

Antes del marcaje isotópico, el CO_2 respirado en las hojas y el tallo de las especies C_3 es, en muchos casos, enriquecido en ^{13}C. Aunque el CO_2 respirado por la raíz en herbáceas C_3 está normalmente empobrecido comparado con la MOT porque en las reacciones de decarboxilación del metabolismo respiratorio, en el ciclo de las pentosas fosfato se libera el C que está en posición 1 y que normalmente es el isótopo ligero (Bathelier y col., 2009). Sin embargo, en nuestro caso no hay diferencias significativas en abundancia natural. Ghashghaie y Badeck, (2014) han resumido como el CO_2 respirado en la raíz está normalmente empobrecido comparado con la MOT en muchas herbáceas C_3, pero depende también del momento de recogida de la muestra y de la especie, presentando la mayor parte de especies C_3 arbóreas y algunas herbáceas valores de ^{13}C respirado más enriquecidos que en MOT (esto podría explicarse por la utilización de diferentes sustratos respiratorios). Sin embargo, la raíz asimila poco CO_2 (solamente a través de la PEPc) y, por lo tanto, la mayoría del carbono enriquecido que le llega desde zonas autotróficas se produjo a través del tallo.

En el capítulo 2 se ha observado cómo el tallo, tanto en la MOT como en el $^{13}CO_2$ respirado, está también muy enriquecido en ^{13}C después del marcaje debido en gran parte a sus diferentes funciones. Los tallos de las plantas conectan las hojas con las raíces o los frutos y transportan el agua y los nutrientes desde la raíz a las hojas, o los asimilados en la fotosíntesis desde las hojas hacia la raíz, los frutos o zonas de crecimiento. Además, los tallos también contienen tejidos fotosintéticamente activos, es decir fijan el ^{13}C directamente y lo incorporan en sus tejidos pudiendo almacenar reservas y fotoasimilados que están marcados con los isótopos estables (Cernusak y col., 2001; Teskey y col., 2008; Ghashghaie y Badeck, 2014).

Nitrógeno-15

En este estudio también se ha utilizado el ^{15}N como isótopo estable ya que el nitrógeno se encuentra en la naturaleza en forma orgánica e inorgánica y la variación de la abundancia natural en la composición isotópica del N ($\delta^{15}N$) en plantas terrestres, puede aportar información valiosa sobre la absorción del nitrógeno por las plantas y el ciclo del N en el ecosistema. El análisis de $\delta^{15}N$ puede ser utilizado como vía de información sobre las diversas fuentes de N utilizadas por los diferentes organismos, además de reflejar diferentes niveles de fertilización nitrogenada y de variaciones en la disponibilidad hídrica (Lopes y Araus, 2006; Yousfi y col., 2010). Como en el

capítulo 2, también existen numerosos trabajos que utilizan el marcaje de N enriquecido en ^{15}N para medir la transferencia de N entre órganos, plantas y el ecosistema (Robinson, 2001).

En esta Tesis, se ha utilizado el $\delta^{15}N$ para estudiar el contenido total de N en los diferentes tejidos (capítulos 1 y 3) y el estudio de la interacción y los flujos *in vivo* de C y N sin alterar procesos fisiológicos o metabólicos (capítulo 2). Esto es muy importante ya que el nitrógeno como nutriente puede afectar al crecimiento, la eficiencia en el uso del agua, la productividad de los cultivos o los procesos de aclimatación al CO_2, sobretodo en cereales (Lopes y Araus, 2006) y en ambientes mediterráneos, donde la disponibilidad de N y de agua son limitantes (Passioura y col., 2002).

3.3. Isotopos estables (^{13}C y ^{15}N) con diferentes concentraciones atmosféricas de CO_2

En los años 60, Park y Epstein (1960) crecieron plantas de tomate a dos niveles de CO_2 y observaron que, las plantas crecidas a elevados niveles de CO_2, tenían $\delta^{13}C$ más negativo que las plantas crecidas a menor concentración de CO_2. Similar a nuestros resultados (capítulo 2), otros científicos también lo han mostrado que en plantas C_3, tanto herbáceas (Beerling y Woodward, 1995; Polley y col., 1993) como arbóreas (Beerling, 1997; Picon y col., 2006) sobre un gran rango de niveles de CO_2. En plantas C_3, el CO_2 es normalmente limitante de la fotosíntesis, así que, por un lado, incrementos en la concentración de CO_2 atmosférica podrían aumentar la tasa fotosintética, pero también la eficiencia en el uso del agua debido al cierre de estomas como vimos con anterioridad.

Los resultados experimentales indican que esta reducción en la conductancia estomática no limita la fotosíntesis, por tanto los valores de $\delta^{13}C$ son más negativos cuando las concentraciones de CO_2 aumentan (Beerling y Woodward, 1995; Polley y col., 1993).

Evans (2001), resumió los mecanismos fisiológicos que influencian la composición isotópica del ^{15}N en las plantas, como son la absorción de N desde la solución del suelo en forma de NH_4^+ y NO_3^-, transformación del NH_4^+ (tóxico) en NO_3^-, la translocación al lugar de asimilación y la asimilación de ese N inorgánico en forma orgánica.

Cada uno de estos pasos conlleva procesos de fraccionamiento isotópico, lo que la hace una herramienta muy buena como trazador de N tanto de la fuente, como en los sumideros de N, así como de cambios ambientales o diferencias entre genotipos. La composición isotópica del ^{15}N ha servido en esta Tesis para ver cómo influencian los genotipos o los estreses como la sequía a la

adquisición de N en la planta y su modulación con el CO_2. Muchos estudios (Makino, 2000; Evans, 2001; Bloom y col., 2010; 2014) han demostrado que elevadas concentraciones de CO_2 atmosféricas inhiben la asimilación de nitrato en la hoja en las plantas C_3. Es por ello que las plantas acumulan menos N orgánico y por tanto pueden crecer menos a elevado que a normal CO_2 (Bloom y col., 2010) como ocurrió en el capítulo 3 de la Tesis. Todo ello sucede, porque elevadas [CO_2] inhiben la asimilación de NO_3^- en las hojas (Lekshmy y col., 2013). También, ha sido reportado que el $\delta^{15}N$ está más empobrecido en ^{15}N a elevado que a actual concentración de CO_2 (Bloom y col., 2014) indicando que, la asimilación de nitrato en hoja es menor a elevada [CO_2] como ocurre en el capítulo 2. Asimismo, la señal isotópica en hojas depende de la fuente y también del transporte desde la raíz a través del tallo; por ejemplo, si la asimilación de nitrato en la raíz es más rápida a elevada [CO_2], la discriminación isotópica enriquecerá la raíz en ^{15}N y éste se transportará a la hoja que estará más enriquecida en ^{15}N.

Como ha ocurrido en nuestros estudios, a elevada [CO_2], encontramos menores concentraciones de N en la hoja, pero también en el grano de trigo, lo cual ha sido reportado anteriormente (Erbs y col., 2010). Todo ello conlleva una disminución de la cantidad de proteína disponible en la hoja y el grano, lo cual, a su vez, repercute negativamente en la calidad de los cultivos y del alimento destinado al consumo humano a elevada [CO_2]. Cabe destacar que lo que se ha observado en algunos casos es que el elevado CO_2 puede aumentar el índice de cosecha, pero la disminución de N y de proteína sugiere una peor calidad de estos alimentos.

4. Perspectivas de futuro.

Uno de los puntos más importantes a estudiar en el futuro es la respuesta múltiple de las plantas a las diferentes [CO_2] y otros estreses abióticos asociados (principalmente el aumento de temperatura, la salinidad y los nutrientes) (Tissue y Lewis, 2012). Es importante que se aborden los efectos de las interacciones entre [CO_2] y otros estreses abióticos ya que las condiciones ambientales varían y los efectos de la selección pueden ser más fuertes bajo el efecto simultáneo de varios estreses, lo que hace esta línea de investigación interesante para un futuro próximo. Además, potenciales estreses como la temperatura, la salinidad o la sequía incrementarán simultáneamente con la [CO_2], haciendo imprescindibles tales estudios para identificar efectos evolutivos a futuros ambientes (Leakey y Lau, 2012). Por lo tanto, dada la importancia de los efectos interactivos de varios factores ambientales sobre la respuesta de las plantas a las [CO_2], sugerimos que la futura investigación se centre en múltiples factores (por ejemplo, interacción de baja y elevada [CO_2] con la temperatura, agua o nutrientes).

Asimismo, futuras investigaciones deberían de hacerse en un mayor número de especies de plantas C_3, y sobretodo C_4 a fin de confirmar que a baja [CO_2] existe una *up-regulation* y a elevada [CO_2] existe una *down-regulation* de la fotosíntesis. Los experimentos deberán hacerse a través de diferentes grupos funcionales de plantas, tal y como comentamos con anterioridad, en un esfuerzo para determinar en última instancia, la respuesta de los ecosistemas al futuro cambio climático. Además sería interesante mantener estos estudios durante varias generaciones de plantas para poder observar si estos cambios y adaptaciones se mantendrán en el futuro, generación tras generación.

Por otro lado, habrá de investigarse si la identificación de los rasgos de adaptación probables a través de modelos o el conocimiento de las respuestas fisiológicas, fenológicas y morfológicas pueden conducir a un mayor crecimiento de las plantas en el futuro (Sage y Coleman, 2001).Si como comentábamos al principio de la discusión, las adaptaciones durante milenios a las bajas [CO_2] implican importantes limitaciones para las plantas en la actualidad, como por ejemplo en la velocidad catalítica de la Rubisco y en el crecimiento, entonces hay un gran margen para la selección y la bioingeniería en agricultura para eliminar las limitaciones naturales, creando así nuevos genotipos con mayores ventajas para explotar las condiciones de elevada [CO_2]. Un objetivo obvio para la manipulación es el alto nivel de la Rubisco en relación con otros componentes fotosintéticos. En general, las plantas C_3 (y el trigo en particular), invierten 20-30% de nitrógeno foliar en Rubisco, lo que representa un costo innecesariamente elevado en entornos de elevada [CO_2] (Mitchell y col., 2000; Makino, 2011). Reducir el contenido de Rubisco disminuiría la asimilación de N por esta proteína que podría ser utilizado en otros procesos que se ven limitados por el N como se demostró en el arroz, en el que la reducción de Rubisco promovió un aumento del 15% en la tasa de fotosíntesis a niveles elevados de CO_2, en gran parte debido a la mayor disponibilidad de N para mejorar la capacidad de regeneración de RuBP (Makino y col., 2000). También en el mismo estudio se observó que la disminución de la Rubisco hacía mejorar la NUE en las plantas.

La disminución de proteínas y N en plantas a elevada [CO_2] sugieren que la calidad de los alimentos puede verse alterada negativamente. Resultaría de gran importancia indagar y estudiar en mayor profundidad el efecto del cambio climático en la calidad de los alimentos vegetales, sobre todo en alimentos de primera necesidad como es el trigo y cómo se ven afectadas sus cualidades para la producción de pan, sémola o pasta.

Aunque los estudios en ambientes controlados como los de esta Tesis son muy importantes para entender los mecanismos y adaptaciones que se producen en la planta, sin embargo estos estudios

han de ser trasladados a condiciones de campo mediante experimentos a gran escala (aunque muy difíciles de realizar en el caso de bajas [CO_2]), y a través de la creación de modelos que predigan la adaptación y evolución de los ecosistemas a un futuro cambio climático, lo que nos dará una gran información sobre las adaptaciones fenotípicas pero también en el ámbito evolutivo de las plantas (Wingate y col., 2010; Leakey y Lau, 2012).

Conclusions:

1. The different atmospheric [CO_2] affected the development and physiology of durum wheat plants: at pre-industrial [CO_2] plants suffered photosynthetic acclimation (up-regulation), while at future [CO_2], plants suffered photosynthetic down-regulation.
2. Photosynthetic up-regulation led to increases on leaf protein content, mainly increasing Rubisco, and leaf N content; while photosynthetic down-regulation led to decreases in total protein content, mainly affecting Rubisco, and leaf N content.
3. Different genotypes of durum wheat modulated the acclimatory photosynthetic response to the different [CO_2]. Old genotypes suffered major photosynthetic acclimation at pre-industrial [CO_2] and modern wheat genotype at future [CO_2].
4. The sink/source ratio of C in the plant is modulate the plant response to the different [CO_2]: the modern variety showed higher capacity to increase the sink of C or to create new sinks during the grain filling. In this phenological stage, the plant is able to avoid the photosynthetic down regulation at future [CO_2].
5. The water availability modulates the photosynthetic acclimation in plants. Plants sudjected to mild water-stress suffer less photosynthetic down-regulation at future [CO_2] and less photosynthetic up-regulation at pre-industrial [CO_2].
6. Stomatal conductance is modulated by atmospheric CO_2 availability. At future [CO_2] plants showed more closed stomata than at pre-industrial [CO_2], showing a better capacity to resist the drought and a higher WUE.
7. Plants at pre-industrial [CO_2] have less discrimination against ^{13}C than plants at current [CO_2] because the availability of CO_2 in the atmosphere is lower. Plants at future [CO_2] have more discrimination against ^{13}C than plants at current [CO_2] because the availability of CO_2 in the atmosphere is higher.
8. Plants under drought have lower discrimination against ^{13}C than well watered plants. This effect of discrimination against ^{13}C is minor when the [CO_2] in the atmosphere is higher.

Summary:

Desde el último periodo glacial hasta la Revolución Industrial, la [CO_2] atmosférica se mantuvo constante entorno a los 200-260 ppm de CO_2 durante miles de años (Gerhart y Ward, 2010). Sin embargo, desde el inicio de la Revolución Industrial la [CO_2] atmosférica está aumentando rápida y continuamente debido a las actividades humanas como son el consumo de fuentes de energía fósil (carbono y petróleo), la deforestación, el aumento de la población humana, o los cambios de hábitos alimenticios (por ejemplo un mayor consumo de carne) entre otros factores (Pardo y col., 2012).

La [CO_2] atmosférica preindustrial ha tenido una importancia crucial en el desarrollo de las plantas actuales. Por un lado, la baja [CO_2] ha influenciado la producción primaria (producción de materia orgánica por parte de los organismos autótrofos) y ésta a su vez, debe haber influenciado toda la biota terrestre. Por otro lado, las plantas se han adaptado y desarrollado en bajas [CO_2] durante milenios, estas adaptaciones podrían estar interfiriendo en las respuestas de las plantas a los niveles actuales y a futuros enriquecimientos de CO_2 atmosférico. Estas circunstancias podrían haber dejado un legado evolutivo que podría afectar al rendimiento de las plantas en el futuro (Sage y Coleman, 2001).

La [CO_2] atmosférica actual se ha situado prácticamente a 400 ppm (exactamente a 398 ppm según NOAA-ESRL, 2014). Este incremento en la [CO_2] atmosférica y otros gases de efecto invernadero están afectando el clima, tanto local como global, y están asociados a cambios en la temperatura y en los regímenes hídricos (Smith y col., 2012). A este efecto a nivel global se le denomina “Cambio Climático” y conlleva riesgos para la población humana y los ecosistemas naturales con grandes impactos en la salud, la economía, el tipo de vida, etc, debido a la interacción de los eventos climáticos (sequías, inundaciones, incrementos del nivel del mar) con las poblaciones humanas y los ecosistemas (IPCC, 2014).

El informe IPCC de 2014 da predicciones utilizando modelos que indican un aumento de la [CO_2] atmosférica de hasta 985 ± 95 ppm para el final de este siglo. Debido a este incremento y acumulación de CO_2 y otros gases de efecto invernadero (GHG) en la atmosfera, se esperan, por un lado, incrementos de temperatura de entre 4 a 5 grados en algunas zonas del planeta y, por otro lado, más frecuentes y prolongados periodos de sequías.

Uno de los temas importantes de investigación relacionado con la variación de la [CO_2] atmosférica es la aclimatación de la fotosíntesis de las plantas a los diferentes niveles de CO_2. La aclimatación fotosintética es el proceso de ajuste fisiológico de las plantas a una determinada [CO_2].

Las plantas pueden experimentar incrementos de la fotosíntesis en el caso de bajas [CO_2] (regulación a la alta o *up-regulation*), y disminuciones de la fotosíntesis a altas [CO_2] (regulación a la baja o *down-regulation*), a través de ajustes en la maquinaria fotosintética (Nogués y Azcón-Bieto, 2013). Sin embargo, el grado de aclimatación fotosintética de las diferentes especies, puede variar dependiendo de otros factores tanto intrínsecos como ambientales (bióticos o abióticos) como, por ejemplo, la relación fuente de carbono y sumidero de carbono, enfermedades, estado nutricional de la planta o estreses como por ejemplo la sequía y la temperatura.

En las últimas dos décadas, muchos estudios (Sage, 1995; Sage y Coleman, 2001; Pagani y col., 1999; Ayub y col., 2014) han examinado el efecto de las bajas [CO_2] en la fotosíntesis y la productividad de las plantas. En plantas sometidas a bajas [CO_2] se observó una reducción inicial de la fotosíntesis y de la biomasa. Sin embargo, tras una prolongada exposición a bajas [CO_2] las plantas ajustaron su maquinaria fisiológica y sufrieron una regulación a la alta de la fotosíntesis. Esta aclimatación de la fotosíntesis ha sido mostrada en otros estudios (Sage y Reid, 1992; Sage, 1994; Cowling y Sage, 1998; Anderson y col., 2001) y conlleva cambios en la planta tanto fisiológicos, como de expresión de enzimas, proteínas o nutrientes, pero también anatómicos. Las plantas sometidas a baja [CO_2] atmosférica y en condiciones ideales de crecimiento, han sufrido una regulación a la alta de la fotosíntesis, mayor conductancia estomática (se mantienen abiertos los estomas para permitir una mayor entrada de CO_2). A su vez se produce un incremento en el nivel de proteínas en hoja como por ejemplo la Rubisco, un aumento del contenido de N en hoja, una mayor inversión de C en la parte aérea (para tener más superficie de captación de CO_2) y una menor inversión de C en la raíz.

Una de las razones de la importancia de los estudios a baja [CO_2] es que nos ayuda a entender la evolución de las plantas en respuesta a las variaciones en las [CO_2] en la atmósfera a lo largo del tiempo (Ward y Strain, 1997; Ward y col., 2000; Gerhart y Ward, 2010). Además, la influencia de las bajas [CO_2] ha sido muy larga (durante milenios) y debe de haber influenciado en el desarrollo y evolución de la planta en todos los niveles, pero también en el ecosistema. Así, entender la adaptación de las plantas a bajas [CO_2] y como se han vuelto a adaptar a las [CO_2] actuales, nos podrán ayudar a entender cómo evolucionarán en el futuro con el incremento constante del nivel de CO_2 atmósferico.

Por otro lado, la exposición de la planta a una elevada [CO_2] inicialmente estimula el crecimiento de ésta e incrementa la tasa de fotosíntesis, en algunos casos más del 40% (Ainsworth y Rogers, 2007). Sin embargo, una exposición prolongada de la planta a una elevada [CO_2] hace que

haya un ajuste de la maquinaria fotosintética, con una regulación a la baja de ésta. Esto implica una serie de cambios tanto anatómicos, como fisiológicos, moleculares o bioquímicos entre otros. Debido a la estimulación inicial de la fotosíntesis, hay una mayor producción de carbohidratos en la hoja, cuando la producción de carbohidratos es mayor a la demanda de la planta, éstos comienzan a acumularse en la hoja y se produce la regulación de la Rubisco (Sage y Coleman, 2001; Aranjuelo y col., 2009, 2014). Además, se observa un exceso del contenido de Rubisco y la planta la reduce para disminuir la producción de carbohidratos. Esta reducción de la Rubisco va acompañada de reducciones de otras proteínas y componentes fotosintéticos tales como las clorofilas, proteínas del fotosistema II, ATP sintasa o la anhidrasa carbónica (Moore y col., 1999). Además hay otros cambios asociados a la elevada [CO_2] como es la disminución del contenido de N en hoja, o un aumento de la producción de la raíz en comparación con niveles menores de CO_2 en la atmósfera (lo que incrementa la captación de nutrientes o agua del suelo al tener una mayor zona de exploración).

Las plantas que han crecido a elevadas [CO_2] controlan mejor la perdida de agua a través de los estomas, ya que hay una reducción de la conductancia estomática. Las plantas reducen la pérdida de agua a través de los estomas, y esto, se traduce en una mayor eficiencia en el uso de agua (EUA) como resultado de la estimulación de la fotosíntesis y de la reducción de la conductancia estomática (Urban, 2003; Leakey y col, 2009).

Los isótopos estables son átomos de un elemento con el mismo número de protones pero diferente número de neutrones. Desde que en los años 50 se comenzaron a utilizar los isótopos estables en Ciencias Biológicas como trazadores e indicadores, se han ido consolidando como una importante herramientas de trabajo en investigación, sobre todo en los últimos años. Algunos autores (Farquhar y Sharkey, 1982; Farquhar y Richards, 1984) establecieron un modelo detallado del uso del ^{13}C en la hoja, y desde entonces, se inició el uso de isótopos estables en fisiología vegetal. Dicha técnica se ha desarrollado y extendido en investigación como una herramienta muy útil para estudiar los flujos de C, N, S, H y O a través de la planta en los diferentes órganos y vías metabólicas.

El carbono es el elemento más abundante de la biosfera y participa en muchas reacciones biológicas, incluyendo la fotosíntesis o el metabolismo de las plantas. Las medidas de la composición isotópica del carbono ($\delta^{13}C$) en compuestos orgánicos e inorgánicos es muy útil para estudiar los procesos que controlan el ciclo del C en plantas, pero también en la atmosfera y en los ecosistemas.

La principal fuente de carbono para las plantas es la atmósfera. A través de diversos procesos físicos y bioquímicos se producen fraccionamientos isotópicos que originan variaciones en el ratio $^{13}C/^{12}C$ en las plantas. La proporción de ^{13}C en los tejidos vegetales es menor que en la atmósfera debido a la discriminación del isótopo más pesado (^{13}C) frente al más ligero (^{12}C), tanto en el proceso de difusión del CO_2 a través de los estomas como posteriormente en el proceso de carboxilación de la enzima Rubisco, o en los procesos de discriminación post-fotosintéticos como el transporte a través de la planta o la respiración.

Además, hay distintos factores ambientales que pueden modificar la composición isotópica del carbono de las plantas a través de su influencia en la conductancia estomática, en la tasa de la fotosíntesis, o en ambos a la vez. Cambios en la intensidad de luz, el estado hídrico de la planta o los niveles de CO_2 de la atmósfera son claramente reflejados en la composición isotópica del carbono en la planta.

El nitrógeno es un elemento que se encuentra principalmente en forma de gas (N_2) en la tierra (78%). Sin embargo, las plantas obtienen la mayor parte del N directamente del ambiente a través de las raíces. Las plantas absorben NO^{3-} y NH^{4+} del suelo, que normalmente se trata del isotopo más abundante que es ^{14}N. Sin embargo, estudios con el isotopo más pesado ^{15}N son cada vez más usados en investigación como trazadores del ciclo del N, tanto en las plantas, como en los ecosistemas (Robinson, 2001).

La composición isotópica del N en los tejidos de la planta, están determinados por la fuente externa de N y los mecanismos fisiológicos de éstas. Las plantas pueden discriminar el isotopo ^{15}N más pesado en los diferentes procesos de absorción, asimilación, transporte y distribución del N a través de la planta. Las variaciones de la composición isotópica del N dentro de la planta pueden ser causadas por diferencias en la asimilación por las raíces o a través de las micorrizas, perdidas de N y reabsorción entre otros factores. Además, factores ambientales como la sequía o la [CO_2] atmosférica puede hace variar la forma de absorción del N y su composición isotópica. Por ello, es importante conocer el rol de la planta en la absorción del N del suelo, tanto inorgánico como orgánico, el rol de las micorrizas y las transformaciones internas de la planta (Evans, 2001; Yoneyama y col., 2003).

En esta Tesis se muestra cómo las plantas de trigo sometidas a una [CO_2] preindustrial proporcionan información sobre el comportamiento y la adaptación del trigo al futuro incremento de [CO_2]. Además futuras [CO_2] atmosféricas estarán asociadas a un aumento en las temperaturas y

los periodos de sequía, modificando el clima y causando un daño grave para los ecosistemas, el desarrollo económico y la salud humana (IPCC 2014). Asimismo se han realizado diferentes estudios de la importancia de estas adaptaciones fisiológicas a fin de evaluar la respuesta de dos variedades de trigo duro (capítulos 1 y 2) en condiciones de cambio climático a [CO_2] futuras y preindustriales (capítulos 1, 2 y 3) y modulados por sequía moderada (capítulo 3).

A continuación se resumen cada uno de los capítulos de esta Tesis:

Capítulo 1:

Título: EFFECTS OF PRE-INDUSTRIAL, CURRENT AND FUTURE CO_2 LEVELS IN TRADITIONAL AND MODERN WHEAT GENOTYPES y publicado en la revista *Journal of Plant Physiology*.

El trigo es un cereal que se cultiva a nivel mundial y es, hoy en día, uno de los alimentos más importantes para la población humana. La calidad y productividad de este cultivo se ve fuertemente afectada por las condiciones ambientales, sobre todo durante el desarrollo y el llenado del grano. Nosotros hemos analizado dos genotipos de trigo duro, Blanqueta que es una variedad antigua o tradicional y Sula que es una variedad moderna y cultivada actualmente de forma extensiva en algunas regiones de España e Italia. Estas dos variedades han sido cultivados en tres cámaras de crecimiento de plantas y a tres niveles de CO_2 atmosférico diferentes: preindustrial (260 ppm), actual (400 ppm) y futuro (700 ppm).

El crecimiento de la planta, la asimilación de carbono, la fluorescencia de la clorofila o la respuestas a nivel de reproducción fueron analizadas durante tres momentos fenológicos del desarrollo de las plantas: el crecimiento vegetativo, la antesis y el llenado de grano con el objetivo de estudiar las adaptaciones de los dos genotipos a las diferentes [CO_2] y la capacidad de las plantas de desarrollar nuevos sumideros y su rol durante el proceso de aclimatación fotosintética. Las plantas sufrieron procesos de aclimatación fotosintética, tanto a [CO_2] pre-industrial en un proceso denominado aclimatación a la alta de la fotosíntesis (*up-regulation*), como a futura [CO_2] en un proceso denominado regulación a la baja de la fotosíntesis (*down-regulation*).

Sin embargo, nuestro genotipo moderno (Sula) evita la aclimatación a la baja de la fotosíntesis creando nuevos sumideros de carbono a futura [CO_2] (por ejemplo la espiga durante el llenado de grano). En cambio, el genotipo tradicional (Blanqueta) no tiene esa capacidad de desarrollar nuevos sumideros de carbono y no puede evitar la regulación a la baja de la fotosíntesis.

En este capítulo se ha mostrado el rol esencial de la espiga como nuevo sumidero para evitar la regulación a la baja de la fotosíntesis a futura [CO_2]. Las respuestas de crecimiento de las plantas a futuras [CO_2] dependerán de la habilidad de las plantas de desarrollar nuevos sumideros o expandir los ya existentes.

Capítulo 2:

Título: C AND N ALLOCATION AND PARTITIONING IN TRADITIONAL AND MODERN WHEAT GENOTYPES UNDER PRE-INDUSTRIAL AND FUTURE CO_2 CONDITIONS y publicado en la revista *Plant Biology*.

La respuesta de las plantas al cambio climático es uno de los principales factores que determinaran los balances de C tanto a nivel de planta como de ecosistema. Es fundamental entender la relación entre la fuente (CO_2 atmosférico) y las plantas como sumidero de C, y su relación con otros factores como el cambio climático. Se realizó un marcaje simultáneamente con ^{13}C y ^{15}N, en dos genotipos de trigo duro, Blanqueta que es una variedad antigua o tradicional y Sula que es una variedad moderna, con el objetivo de seguir el marcaje del ^{13}C y ^{15}N, su distribución y asignación en los diferentes órganos de la planta.

El marcaje "puntual" de aproximadamente tres días, se ha llevado a cabo en tres cámaras de crecimiento de plantas con $^{13}CO_2$ (aplicado como gas en la atmósfera de la cámara de crecimiento) y $^{15}NH_4$-$^{15}NO_3$ (aplicado en la solución nutritiva). Las plantas crecieron desde semilla y durante todo su ciclo de vida en unas cámaras de crecimiento de ambiente controlado a tres niveles de CO_2 atmosférico: preindustrial (260 ppm), actual (400 ppm) y futuro (700 ppm).

Las plantas sufrieron procesos de aclimatación fotosintética a las diferentes [CO_2]. A [CO_2] preindustrial hubo una regulación a la alta de la fotosíntesis (*up-regulation*), y a futura [CO_2] una regulación a la baja de la fotosíntesis (*down-regulation*). Además el ^{13}C del marcaje reveló que a [CO_2] preindustriales las plantas invierten más C en la parte aérea de la planta, mientras que a futuras [CO_2] las plantas hacen una mayor inversión en la parte subterránea porque la fuente de C (CO_2 de la atmósfera) es mayor que a [CO_2] preindustriales. El genotipo moderno invirtió más C en espigas que el genotipo tradicional, el cual invirtió más C en las partes aéreas no reproductivas. El ^{15}N del marcaje, reveló que el genotipo moderno se adaptó mejor y asimiló más N a futura [CO_2], mientras que el genotipo tradicional fue más eficiente en la asimilación y uso de N a [CO_2] preindustriales.

Capítulo 3:

Título: EFFECTS OF WATER STRESS IN WHEAT PLANTS GROWN UNDER DEPLETED, CURRENT AND ELEVATED CO_2 LEVELS y ha sido enviado a la revista *Environmental and Experimental Botany*.

El estrés hídrico ha sido identificado como uno de los mayores problemas que afectan durante el desarrollo del trigo en la región mediterránea. La fotosíntesis, fluorescencia de la clorofila y parámetros de crecimiento y biomasa de plantas de trigo duro (*Triticum turgidum,* L. var. *durum* variedad Sula) fue comparada a tres niveles diferentes de CO_2 atmosférico: baja [CO_2] (260 ppm), actual [CO_2] (400 ppm) y elevada [CO_2] (700 ppm) y dos tratamientos de disponibilidad de agua: plantas bien regadas (100% de agua de capacidad de maceta, WW) y plantas sometidas a un estrés hídrico moderado (60% de agua de capacidad de maceta, WS). Las plantas crecieron durante todo su ciclo de vida en cámaras de crecimiento y fueron analizadas durante tres momentos del desarrollo fenológico (antes de antesis, durante antesis y al final de llenado de grano).

Se demostró que la respuesta de las plantas al estrés hídrico en los distintos niveles de CO_2 ha sido diferente. Las plantas se aclimataron a la baja [CO_2] y la fotosíntesis sufrió regulación a la alta. A baja [CO_2] y sequía las plantas se aclimataron y redujeron su biomasa y el Índice de Cosecha (HI), especialmente cuando ambos factores se combinaron.

Las plantas sufrieron procesos de aclimatación fotosintética a elevada [CO_2]. Que hicieron disminuir la fotosíntesis y el crecimiento de la planta. Asociado a este proceso de regulación a la baja de la fotosíntesis, las plantas redujeron el contenido de N en hoja el contenido de proteínas totales y de la Rubisco en particular. Sin embargo las plantas sometidas a estés hídrico moderado y elevada [CO_2] sufrieron menor regulación a la baja de la fotosíntesis que las plantas bien regadas, por lo que estas últimas sufrieron mayor grado de aclimatación y una menor biomasa.

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Effects of pre-industrial, current and future [CO_2] in traditional and modern wheat genotypes

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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 [CO_2]. 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 [CO_2] (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 [CO_2]. Moreover, we have demonstrated that at future [CO_2] the growth response will depend on the ability of plants to develop new sinks or expand existing ones.

Introduction

Global atmospheric [CO_2] 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 CO_2. These models have shown that [CO_2] were 30–50% lower than currently (between 180 and 260 μmol mol^{-1}) and that atmospheric [CO_2] 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 [CO_2] have been produced at an alarming rate and currently, [CO_2] stand at around 398 μmol mol^{-1} (NOAA-ESRL, 2014). Increases in atmospheric [CO_2] 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 [CO_2] 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 CO_2 levels. Knowledge of these adaptations may help us to better understand how plants will respond to future increases in CO_2 levels (Prentice et al., 2001; Sage and Coleman, 2001; Nogués and Azcón-Bieto, 2013).

Specifically, photosynthesis in C_3 plants is usually affected by changes in [CO_2]. Moreover, there is a wide variation of responses to these changes in different species such as the acclimation of photosynthesis to different atmospheric [CO_2] 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 CO_2, where photosynthesis can either decrease (down-regulation) in response to high [CO_2] or increase (up-regulation) in response to low [CO_2] through adjustments made to the photosynthetic machinery (Sage, 1994; Anderson et al., 2001; Nogués and Azcón-Bieto, 2013).

Abbreviations: A_{sat}, light and CO_2-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; NtS, 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-photoquenical 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_{cmax}, maximum carboxylation velocity of Rubisco.

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