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Does ear C sink strength contribute to overcoming photosynthetic acclimation of wheat plants exposed to elevated CO_2?

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Running title: Ear C sink strength under elevated CO_2

Date of submission: 30th Novemeber 2010

Number of tables: 3

Number of figures: 6

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Abstract

Wheat plants (*Triticum durum* Desf., cv. Regallo) were grown in the field to study the effects of contrasting [CO_2] conditions (700 *versus* 370 μmol mol^{-1}) on growth, photosynthetic performance and C management during the post-anthesis period. The aim was to test whether a restricted capacity of sink organs to utilize photosynthates drives a loss of photosynthetic capacity in elevated CO_2. The ambient $^{13}C/^{12}C$ isotopic composition ($\delta^{13}C$) of air CO_2 was changed from -10.2 ‰ in ambient [CO_2] to -23.6 ‰ under elevated [CO_2] between the 7^{th} and the 14^{th} day after anthesis in order to study C assimilation and partitioning between leaves and ears. Elevated [CO_2] had no significant effect on biomass production and grain filling, and caused an accumulation of C compounds in leaves. This was accompanied by an up-regulation of phosphoglycerate mutase and ATP synthase protein content, together with a down-regulation of adenosine diphosphate glucose pyrophosphatase protein. Growth in elevated [CO_2] negatively affected Rubisco and Rubisco activase protein content and induced photosynthetic down-regulation. CO_2 enrichment caused a specific decrease in Rubisco content, together with decreases in the amino acid and total N content of leaves. The C labelling revealed that in flag leaves, part of the C fixed during grain filling was stored as starch and structural C compounds whereas the rest of the labelled C (mainly in the form of soluble sugars) was completely respired 48 h after the end of labelling. Although labelled C was not detected in the $\delta^{13}C$ of ear total organic matter (TOM) and respired CO_2, soluble sugar $\delta^{13}C$ revealed that a small amount of labelled C reached the ear. The $^{12}CO_2$ labelling suggests that during the beginning of post-anthesis the ear did not contribute towards overcoming flag leaf carbohydrate

accumulation, and this had a consequent effect on protein expression and photosynthetic acclimation.

Keywords: C management, elevated CO_2, photosynthetic acclimation, proteomic characterisation, Rubisco, stable isotopes.

Abbreviations: **A_{370}**, photosynthesis determined at 370 μmol mol^{-1} CO_2; **A_{700}**, photosynthesis determined at 700 μmol mol^{-1} CO_2; **ADPG**, ADPglucose; **AGPPase**, adenosine diphosphate glucose pyrophosphatase; **CA**, Carbonic anhydrase; **Ci_{370}**, intercellular [CO_2] determined at 370 μmol mol^{-1} CO_2; **Ci_{700}**, intercellular [CO_2] determined at 700 μmol mol^{-1} CO_2; **DM**, dry matter; **g_m**, mesophyll conductance, **gs_{370}**, stomatal conductance determined at 370 μmol mol^{-1} CO_2; **gs_{700}**, stomatal conductance determined at 700 μmol mol^{-1} CO_2; **IRGA**, infrared gas analyser; **k_{cat}**, overall enzyme catalytic rate; **PAR,** Photosynthetically active radiation; **PDB,** Pee Dee Belemnite; **PGAM**, Phosphoglycerate mutase; **PPFD**, photosynthetic photon flux density; **RuBP**, Ribulose bis-phospahte; **R,** dark respiration; **T_0**, immediately after the end of labelling; **T_1**, 24 h after the end of labelling; **T_2**, 48 h after the end of labelling; **TOM**, total organic matter; **TSP**, total soluble proteins; **TSS**, total soluble sugar; **$\delta^{13}C$**, ^{13}C isotopic composition; $\delta^{13}C_a$, air ^{13}C isotopic composition; $\delta^{13}C_p$, plant ^{13}C isotopic composition; **Δ,** C isotope discrimination; **2-PGA**, 2-phosphoglycerate; **3-PGA**, 3-phosphoglycerate.

Introduction

The global atmospheric concentration of carbon dioxide ($[CO_2]$) has increased from approximately 280 μmol mol^{-1} during the pre-industrial period to 388.5 μmol mol^{-1} in 2010 (Dr. Pieter Tans, NOAA/ESRL, www.esrl.noaa.gov/gmd/ccgg/ trends/) and is expected to reach 700 μmol mol^{-1} by the end of this century (Prentice *et al*., 2001). The primary effects of increased $[CO_2]$ on plants include (i) increased plant biomass and (ii) leaf net photosynthetic rates, and (iii) decreased stomatal conductance (Long *et al.*, 2004; Nowak *et al.*, 2004; Ainsworth and Long 2005). The biochemical basis for the leaf CO_2 assimilation response to increased atmospheric $[CO_2]$ is well established (Farquhar *et al.*, 1980). At concentrations below 600 μmol mol^{-1} CO_2, leaf CO_2 assimilation increases because Rubisco carboxylation is enhanced by increased substrate availability and the suppression of competitive Rubisco oxygenation (Ellsworth *et al.*, 2004). Although the initial stimulation of net photosynthesis associated with elevated $[CO_2]$ is sometimes retained (Davey *et al.*, 2006), some species fail to sustain the initial, maximal stimulation (Aranjuelo *et al.*, 2005a; Martínez-Carrasco *et al.*, 2005; Pérez *et al.*, 2007; Alonso *et al.*, 2009; Gutiérrez *et al.*, 2009), a phenomenon called photosynthetic acclimation or down-regulation.

Stomatal limitations reduce photosynthesis due to depletion of intercellular $[CO_2]$ (C_i) as a result of stomatal closure (Naumburg *et al.*, 2004), i.e. a reduced supply of CO_2 to the photosynthetic apparatus within leaves. Limited mesophyll (g_m) conductance to CO_2 diffusion can also significantly constrain photosynthesis, but the extent of this limitation is still not well known (Evans *et al.*, 2009). Previous studies conducted by Singsaas *et al.*

(2003) and Flexas *et al*. (2007) with a range of plants exposed to different [CO_2] showed that g_m was involved in photosynthetic acclimation. Non-stomatal limitations reduce photosynthesis due to reduced photosynthetic electron transport (Aranjuelo *et al.*, 2008) or decreased Rubisco carboxylation of RuBP (Stitt and Krapp, 1999; Long *et al*., 2004; Aranjuelo *et al.*, 2005a). Decreased Rubisco carboxylation occurs through two basic mechanisms: one that involves C source/sink relationships and a second that involves N allocation. Enhanced leaf C content caused by greater photosynthetic rates in plants exposed to elevated [CO_2] induces repression of the expression of genes coding for photosynthetic proteins, leading to a down-regulation of photosynthetic capacity (Moore *et al*., 1999; Jifon and Wolfe, 2002). At the whole-plant level this occurs when photosynthesis exceeds the capacity of sink organs to utilize photosynthate (Lewis *et al*., 2002; Aranjuelo *et al.*, 2009a). In this sense, a previous study conducted by Ainsworth *et al.* (2004) showed that under elevated [CO_2] conditions, a decrease in carboxylation capacity occurred in a determinate soybean mutant, which was genetically limited in its capacity to add "sinks" for photosynthate, while no acclimation occurred in the wild indeterminate type. Accordingly, when plants exposed to elevated [CO_2] have limitations on increasing C sink strength, plants decrease their photosynthetic activity to balance C source activity and sink capacity (Thomas and Strain, 1991). The second basic mechanism leading to down-regulation is reduced Rubisco content that is caused by non-selective decreases in leaf N content (Ellsworth *et al*., 2004; Aranjuelo *et al*., 2005a) or by reallocation of N within the plant (Nakano *et al*., 1997). In both cases, reduced leaf N decreases Rubisco content.

Leaf carbohydrate accumulation is determined by the C source (photosynthesis) and sink balance (i.e. growth, respiration and partitioning to other organs) (Aranjuelo *et al*.,

2009a). Despite the relevance of C loss through respiration, little attention has been given to this topic in cereals (Araus *et al*., 1993; Bort *et al*., 1996). Previous studies conducted in wheat and other cereals by Araus *et al.* (1993) revealed that dark respiration *(R)* in ears during grain filling ranges from 44-63 % of the gross photosynthesis (net CO_2 assimilation plus dark respiration), 12-20 days after ear emergence. Furthermore, as observed in recent studies (Aranjuelo *et al*., 2009a), the "ability" to respire recently assimilated C may contribute towards preventing carbohydrate build-up and consequently to the avoidance of photosynthetic acclimation. In cereals like wheat, the ear comprises a very important C sink, especially during grain filling (Schnyder, 1993). In wheat, grain filling is sustained by photoassimilates (i) from the flag leaf (Evans *et al.* 1975), (ii) from C fixed by the ear itself (Tambussi *et al*. 2007) and (iii) from C remobilised from the stem internodes that was assimilated before anthesis (Gebbing and Schnyder 1999).

As revealed by previous studies conducted with plants exposed to elevated [CO_2] conditions (Körner *et al.*, 2005; von Felten *et al.*, 2007; Aranjuelo *et al.*, 2008; 2009a), stable C isotope tracers are a key tool to study C management and its implications in photosynthetic performance. One of the difficulties in analysing the processes of C metabolism (photosynthesis, respiration, allocation and partitioning) is measuring the different processes simultaneously in the same experiment (Amthor, 2001). The lack of studies analysing the loss of photoassimilates by respiration during grain filling underscores the importance of examining this further. Labelling with $^{13}C/^{12}C$ enables the characterisation of assimilated C and its further partitioning into different organs (Nogués *et al*., 2004; Aranjuelo *et al.*, 2009ab). C allocation and partitioning can be studied further

by analysing the isotopic composition of soluble sugars (especially sucrose, glucose, etc.) (Körner *et al.*, 2005; Kodama *et al.*, 2010).

Since ensuring adequate sink strength in crops will be an essential part of maximally exploiting rising [CO_2], the aim of this paper was to determine the role of ears as major C sinks during grain filling and its effect on the leaf C content, photosynthetic acclimation and plant growth of wheat plants exposed to elevated [CO_2] under near field conditions. The significance of C management (photosynthesis, respiration, allocation and partitioning) for grain filling in wheat under elevated [CO_2] was assessed through $^{12}CO_2$ labelling carried out in greenhouses located in the field. $^{12}CO_2$ labelling was conducted at the plant level to gain a better understanding of C management in the whole plant. Furthermore, a biochemical and proteomic characterisation was conducted to extend our knowledge of the effects of elevated [CO_2] on the expression profile of proteins other than the most extensively characterised Rubisco.

Materials and Methods

Experimental design

The experiment was conducted at Muñovela, the experimental farm of the Institute of Natural Resources and Agrobiology of Salamanca, CSIC (Salamanca, Spain). Durum wheat seeds (*Triticum durum* Desf. cv. Regallo) were sown at a rate of 200 kg ha^{-1} and 0.13 m row spacing on 29 October 2007. Before sowing, 60 kg ha^{-1} each of P and K (as P_2O_5 and K_2O, respectively) were added. An application of nitrogen fertiliser [$Ca(NO_3)_2$] as an

aqueous solution was made by hand at 140 kg ha^{-1}, on 15 February 2008. The crop was watered weekly with a drip irrigation system, providing the amount of water required to equal potential evapotranspiration. After seedling emergence, six greenhouses (Aranjuelo *et al.*, 2005b; Pérez *et al.*, 2005; Gutiérrez *et al.*, 2009), based on those described by Rawson *et al.* (1995), were erected over the crop. The greenhouses were 9 m long, 2.2 m wide and 1.7 m high at the ridge. They had rigid polycarbonate walls and a UV-stable polyethylene sheet roof. This material has good transmission of photosynthetically active and UV radiation, adequately mimicking outdoor conditions. Photosynthetically active radiation (PAR) at mid-morning was 1020 ± 187 μmol m^{-2} s^{-1} outdoors, whereas inside the greenhouses the PAR was 825 ± 113 μmol m^{-2} s^{-1}. Three greenhouses were kept at ambient [CO_2] (370 μmol mol^{-1}), while in the other three atmospheric [CO_2] was increased to 700 μmol mol^{-1} (elevated [CO_2]) by injecting pure CO_2 at the two inlet fans during the light hours. CO_2 was not elevated during the night because little or no effect on dark respiration has been reported (Davey *et al.*, 2004). The atmospheric CO_2 concentration inside the greenhouses was continuously monitored at the plant level and regulated by PID controllers (Aranjuelo *et al.*, 2005a). Temperature and humidity were measured with sensors (HMD50; Vaisala, Helsinki, Finland) attached to a computer through analogue-digital convertors (Microlink 751, Biodata Ltd, Manchester, UK). Supplementary Figure S1 shows the temperature and relative humidity inside the greenhouses during the experiment.

To analyse C allocation and partitioning in the plants, during the first week after anthesis and coinciding with the period of largest photoassimilate contribution to grain filling (Schnyder *et al.*, 2003), C labelling was conducted over one week via modification of the

isotopic composition of the air ^{13}C ($\delta^{13}C$). During the C labelling period, the plants exposed to elevated [CO_2] conditions were grown in an environment where the $\delta^{13}C$ of the greenhouses was deliberately modified (-23.6 ± 0.4‰) to distinguish it from the $\delta^{13}C$ of elevated [CO_2] (-20.1 ± 0.4‰) during the previous period. Air $\delta^{13}C$ in the ambient [CO_2] was -10.2 ± 0.4‰. The CO_2 was provided by Air Liquide (Valladolid, Spain). See below for details on air $\delta^{13}C$ collection and measurements. The labelling period lasted for one week starting 7 days after anthesis. All the determinations, with the exception of C labelling derived parameters, were conducted on the last day of the experiment, 14 days after anthesis. Isotopic characterisation data was performed, the day before the beginning of labelling (pre-label period), at the end of 7 days labelling (T_0; two weeks after anthesis) and 24 h (T_1) and 48 h (T_2) later after labelling (during post-labelling period).

Gas exchange and plant growth

Gas exchange of leaves was recorded in the central segment of flag leaves between 3 and 8 h after the start of the photoperiod. Measurements were carried out with an air flow rate of 300 mL min^{-1}, 1500 µmol m^{-2} s^{-1} irradiance, and a 1.6 ± 0.23 kPa vapour pressure deficit, using a 1.7 cm^2-window leaf chamber connected to a portable infrared gas analyser (CIRAS-2, PP Systems, Hitchin, Herts, UK) with differential operation in an open system. Temperature was kept at 25 ºC with the Peltier system of the analyser. Photosynthesis was recorded at 370 and 700 µmol mol^{-1} CO_2.

To determine dry matter accumulation, the number of shoots in 0.5 m of two adjacent rows was counted, five consecutive shoots were harvested from each of the rows and the

dry weight of leaves, stems and ears was recorded after drying in an oven at 60 ºC for 48 h. This allowed the results to be expressed on a ground area basis.

Rubisco protein, amino acids and Rubisco activity

At mid-morning samples consisting of four leaves were harvested and rapidly plunged in situ into liquid nitrogen and then stored at -80 ºC until analysed. The fresh weight, leaf area and chlorophyll content of subsamples of frozen leaves were determined as described (Pérez *et al.*, 2005).This allowed the results to be expressed on a leaf area basis.

Total amino acids were determined spectrophotometrically by the ninhydrin method according to Hare (1977) as described by Morcuende *et al.* (2004). The soluble proteins were extracted and measured spectrophotometrically (Bradford, 1976), and the amount of Rubisco subunits was determined by quantitative electrophoresis followed by densitometry (Pérez *et al.*, 2011).

For Rubisco initial and total activity assays, a NADH-coupled spectrophotometric procedure was followed (Pérez *et al.*, 2005). To estimate the k_{cat}, total Rubisco activity was divided by the number of enzyme active sites, which was obtained by multiplying the number of moles of Rubisco by 8.

Soluble sugar and starch content analyses

For sugar extraction, plant samples were lyophilised and then ground to a fine powder (<10 μm). About 50 mg of the fine powder were suspended in 1 mL of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany), mixed, and then centrifuged at 12,000 g for 5 min at 5 ºC. After centrifugation, the supernatant was used for sugar quantification, whereas the pellet was stored at -80 ºC for further starch analyses. The supernatant was heated during 3 min at 100 ºC and afterward the solution was put on ice for 3 min. The supernatant containing the total soluble sugar (TSS) fraction was centrifuged at 12,000 g for 5 min at 5 ºC (Nogués *et al.*, 2004). The supernatant was used for quantification of the individual sugars. Soluble sugar samples were purified using a solid phase extraction pre-column (Oasis MCX 3cc, Waters). Sugar content was analysed using a Waters 600 high performance liquid chromatograph (HPLC, Waters Millipore Corp., Milford, MA, USA). The HPLC refractive index detector (Waters 2414) was set at 37 ºC. Samples were eluted from the columns at 85 ºC (connected in series Aminex HPX-87P and Aminex HPX-87C, 300 mm x 7.8 mm, BioRad) with water at 0.6 mL min^{-1} flow rate and a total run time of 45 min. Sucrose, glucose and fructans were collected and transferred to tin capsules for isotope analysis. The use of the purification pre-columns, together with the two Aminex columns connected in series enabled the separation of sucrose, glucose and fructans, avoiding possible contamination problems raised by Richter *et al.* (2009). Furthermore, as an additional precaution, initial and final phases of peaks were discarded when collecting the peaks. As mentioned by Richter *et al.* (2009), there is no method that enables analysis of purified starch $\delta^{13}C$. Following one of the protocols described in the study conducted by Ritcher *et al.* (2009), we analysed the $\delta^{13}C$ of the HCl-hydrolysable C (HCl-C) that is mainly composed by starch. Therefore, as suggested we use the HCl-C as a reference for starch C isotopic composition. $\delta^{13}C$ of individual sugars and HCl-C was

analysed by isotope ratio mass spectrometry (Delta C, Finnigan Mat, Bremen, Germany) as described by Nogués *et al.* (2008).

C isotope composition ($\delta^{13}C$) of carbohydrates, total organic matter (TOM), together with C and N analyses

Flag leaf and ear samples were collected (T_0, T_1 and T_2) and dried at 60 ºC for 48 h and then ground; 1.5 mg samples were used for total organic matter (TOM) analyses, and 4 biological replicates were analysed for each sample. Determinations of cabohydrates and TOM C, $\delta^{13}C$ and N were conducted at the Serveis Cientifico-Tècnics, University of Barcelona (Barcelona, Spain) using an elemental analyser (EA1108, Series 1, Carbo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen Germany) operating in continuous flow mode.

Closed system for dark respiration sampling

Flag leaves and ears were placed separately in a gas analysis chamber to collect dark-respired CO_2 and analyse $\delta^{13}C$. The chamber was connected in parallel to the sample air hose of a LI-COR 6400 (LI-COR, Lincoln, Nebraska, USA) (Aranjuelo *et al.*, 2009a).

To accumulate CO_2 for the $\delta^{13}C$ analyses, respiration samples of flag leaves and ears were collected separately in the chamber described above. The gas analysis chamber was first flushed with CO_2-free air to ensure that only the CO_2 respired in the chamber was accumulated. The CO_2 concentration inside the chamber was measured by the LI-COR

6400. When the CO_2 inside reached the 300 μmol mol^{-1} concentration value, CO_2 samples were collected and analysed as described (Aranjuelo *et al.*, 2009a).

$\delta^{13}C$ measurements corresponding to each greenhouse and plant respiration (flag leaf and ear respiration), air samples were analysed by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) at the Serveis Cientifico-Tecnics of the University of Barcelona (as previously described by Nogués *et al*., 2008).

$^{13}C/^{12}C$ ratios of air samples and plant materials were expressed in δ notation:

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

where R_{sample} refers to plant material and $R_{standard}$ to Pee Dee Belemnite (PDB) calcium carbonate.

C isotope discrimination (Δ) of leaf and ear TOM was calculated as described by Farquhar *et al.* (1989):

$$\Delta = \frac{\delta_a - \delta_p}{\delta_p + 1}$$

where δ_a and δ_p denote air ($\delta^{13}C_a$) and plant ($\delta^{13}C_p$) isotopic composition, respectively.

Proteomic characterisation

Four biological replicates of flag leaf samples (200 mg fresh weight) were ground in a mortar using liquid nitrogen and re-suspended in 2 mL of cold acetone containing 10% TCA. After centrifugation at 16,000 *g* for 3 min at 4 °C, the supernatant was discarded and the pellet was rinsed with methanol, acetone, and phenol solutions as previously described by Wang *et al*. (2003). The pellet was stored at –20 °C or immediately re-suspended in 200 µL of R2D2 rehydratation buffer The total soluble protein (TSP) concentration was determined by the method of Bradford (Bradford, 1976) using BSA as standard. For Two-Dimensional Electrophoresis, we followed the protocol detailed in Aranjuelo *et al*. (2011).

After staining, the images of the two-dimensional gels were acquired with the ProXPRESS 2D proteomic Imaging System and analysed using Phoretix 2-D Expression Software v2004 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Gels from four independent biological replicates were used and the analysis of gels was performed as previously described by Aranjuelo *et al.* (2011). Molecular mass (Mr) and isoelectric point (pI) were each calculated using Samespots software calibrated with commercial molecular mass standards (precision protein standards prestained; Bio-Rad) run in a separate marker lane on the 2-DE gel. ANOVA ($P < 0.05$) was performed using MiniTAB to compare the relative abundance of the total volume of all detected spots for each gel.

For the protein identification by ESI-LC MS/MS, excised spots were washed several times with water and dried for a few minutes. Trypsin digestion was performed overnight with a dedicated automated system (MultiPROBE II, PerkinElmer). The gel fragments were subsequently incubated twice for 15 min in a H_2O/CH_3CN solution to allow extraction of peptides from the gel pieces. Peptide extracts were then dried and dissolved in starting

buffer for chromatographic elution, which consisted of 3 % CH_3CN and 0.1 % HCOOH in water. Peptides were enriched and separated using a lab-on-a-chip technology (Agilent, Massy, France) and fragmented using an on-line XCT mass spectrometer (Agilent). The fragmentation data were interpreted using the Data Analysis program (version 3.4, Bruker Daltonic, Billerica, USA). For protein identification, tandem mass spectrometry peak lists were extracted and compared with the protein database using the MASCOT Daemon (version 2.1.3; Matrix Science, London, UK) search engine as previously described by Desclos *et al.* (2009). Once the proteins were identified, we proceeded to their presumed biological function according to Bevan *et al*. (1998). The authors would like to clarify that apparent discrepancies concerning Rubisco concentration decreases observed by SDS-PAGE and not by the 2-DE were explained by saturation of the silver staining of Rubisco in the 2-DE methodology, due to its abundance.

Statistical analyses

Data was processed by one-factor analysis of variance (ANOVA). Means ± standard errors (SE) were calculated, and when the *F*-ratio was significant, least significant differences were evaluated by the LSD test using the statistical software package SPSS 12.0 (SPSS Inc., Chicago, IL, USA). The results were accepted as significant at $P < 0.05$. All values shown in the figures and tables are means ± SE.

Results

Growth in elevated [CO_2] had no effect on leaf and total biomass; however, ear DM marginally decreased (P= 0.093) in these treatments (Table 1). Furthermore, no significant differences were observed in the ear DM / total DM ratio. At the respective CO_2 growth conditions, flag leaf photosynthesis was higher in elevated than ambient CO_2 plants, although the difference was not significant (Table 1). However, when photosynthesis was determined at a common concentration of 370 or 700 μmol m^{-2} s^{-1} (A_{370} and A_{700} respectively) the results revealed that plants grown under elevated [CO_2] had lower photosynthetic rates. Intercellular [CO_2] (C_i) and stomatal conductance values (g_s) determined at the same [CO_2] (g_{350} *versus* g_{700} and Ci_{370} *versus* Ci_{700} respectively), showed that limitations in C_i were not the cause of the lower photosynthetic capacity of plants exposed to elevated [CO_2] (Table 1).

The leaf carbohydrate determinations (Fig. 1) showed that although glucose and sucrose were not affected by [CO_2], starch (marginally) and fructan concentration increased in plants exposed to 700 μmol mol^{-1}. In ears, no significant differences were detected in any of the analysed carbohydrates. As shown in Figure 2, N content decreased in leaves exposed to elevated [CO_2], whereas no significant differences were detected in ears. The C/N ratio showed an increase in flag leaves and no significant difference in ears in response to elevated [CO_2]. Leaf N, Rubisco and amino acid content decreased in elevated [CO_2] (Figure 3). Although total soluble protein (TSP) content was not significantly affected by [CO_2], the percentage of Rubisco in TSP decreased in elevated [CO_2]. Total Rubisco activity (Fig. 4) was decreased by elevated [CO_2] while initial Rubisco activity was not

significantly affected, because Rubisco activation increased. The k_{cat} of Rubisco (Fig. 4) was significantly lower in elevated [CO_2] than in ambient [CO_2].

After 7 days of labelling (during labelling period), the $\delta^{13}C$ in leaf total organic matter (TOM) was -39.92 ‰. This value was constant during post-labelling period, 24 and 48 hours (-40.22 and -40.08 ‰ respectively) after the end of labelling (Fig. 5). Interestingly, the analyses of leaf respired CO_2 also revealed that in elevated [CO_2], the (T_0) $\delta^{13}C$ was lower in labelled than non-labelled plants (-34.10 and -30.72 ‰ respectively) immediately after the labelling. However such depletion decreased to -32.36 ‰ by 24 hours and to -31.12 ‰ by 48 hours after the end of labelling (Fig. 5). For ears of labelled and non-labelled plants in elevated [CO_2], the similar $\delta^{13}C$ in TOM (-36.90 and -37.75 ‰, respectively) and in respired CO_2 (-33.51 and -33.66 ‰, respectively) suggests that pre-labelled C was present in ears (Fig. 5). In both flag leaves and ears, the $\delta^{13}C$ of sucrose and fructans were similar in labelled and non-labelled plants exposed to 700 μmol mol^{-1} (Fig. 5). However, for leaf glucose immediately after the end of labelling (T_0), $\delta^{13}C$ changed from -33.43 ‰ in non labelled plants to -35.97 ‰ in labelled plants. Twenty-four (T_1) and 48 (T_2) hours later, the corresponding $\delta^{13}C$ values were -35.49 ‰ and -30.98 ‰ (Fig. 5). Immediately after labelling, the $\delta^{13}C$ of glucose in ears was similar to that of pre-labelled plants in elevated [CO_2] (-32.37 and -31.69 ‰, respectively). As shown in Fig. 5, such values were depleted to -34.03 ‰ at T1 and to -31.66 ‰ at T2. Figure 5 also shows that $\delta^{13}C$ of leaf starch in elevated [CO_2] conditions was -38.01 ‰ in labelled plants and -35.81 ‰ in non-labelled plants. Such values were maintained at T_1 and T_2. However, Fig. 5 also

shows that starch in ears of labelled (-34.93 ‰) and non labelled (-34.20 ‰) plants had a similar $\delta^{13}C$.

The effect of elevated CO_2 on the leaf protein pattern in wheat plants was studied using 2-DE (Fig. 6). Our protocol enabled the identification of 14 proteins that differed in their expression under ambient and elevated CO_2 conditions (Tables 2 and 3). Eight of these proteins were up-regulated under elevated CO_2 conditions (Table 2), with the remaining 6 being down-regulated (Table 3). These proteins were classified in different groups according to their presumed biological function. The up-regulated proteins were classified into 6 groups: metabolism processes (1 protein identified), energy processes (1 protein identified), transporters (1 protein identified), disease/defence processes (1 protein identified), proteins with unclear classification (2 proteins identified) and unclassified proteins (2 proteins identified). Among the down-regulated proteins, energy processes (2 proteins identified), disease/defence (1 protein identified) and unclassified proteins (3 proteins identified) were detected. The roles of these proteins are discussed in the following section with regard to the changes in physiological traits in response to elevated CO_2 conditions.

Discussion

A review of wheat performance under elevated [CO_2] in 156 experiments (Amthor, 2001) has shown CO_2 responses ranging from no effect or a negative one in some studies to several-fold increases in others. As shown in Table 1, exposure to 700 μmol mol^{-1} CO_2

marginally decreased ear DM during the post-anthesis period (P= 0.093) and no effect was observed in total DM and ear DM / total DM. This revealed that elevated [CO_2] did not contribute to increased grain filling, which is in agreement with previous reports (Högy *et al*, 2009; Amthor, 2001; Uddling *et al.*, 2008). Our results were corroborated in the supplementary harvest conducted at the grain maturity stage (see supplementary Table). Absence of effects on total DM, together with the lower ear DM suggest that under elevated [CO_2] exposure, the plants invested a larger amount of photoassimilates in the development of vegetative biomass rather than in grain filling. Grain filling may be limited by (i) translocation of photoassimilates from source to sink, (ii) photosynthetic activity and (iii) ear sink capacity (Uddling *et al.*, 2008). Evans *et al.* (1970) showed that assimilate movement from leaves to ears in wheat was not limited by phloem stem transport. Photosynthesis (measured at the respective growth conditions) was increased by elevated [CO_2] (Table 1). However, when photosynthetic activity was determined in all plant treatments at 370 and 700 μmol mol^{-1} [CO_2] (Table 1) it was found that plants grown in elevated [CO_2] had lower photosynthetic capacity than plants grown in ambient [CO_2] (Zhang *et al.*, 2009). Photosynthetic acclimation has been previously described in wheat plants exposed to elevated [CO_2] in greenhouses located in the field (Martínez-Carrasco *et al.*, 2005; Alonso *et al.*, 2009; Gutiérrez *et al.*, 2009). Although exposure to elevated [CO_2] decreased stomatal conductance (*gs*), similar (Ci_{700}) or even higher (Ci_{370}) intercellular CO_2 concentrations (C_i) in elevated [CO_2] than ambient [CO_2] ruled out stomatal closure as the main cause of the reduction in photosynthetic capacity in elevated [CO_2]. Carbonic anhydrase (CA), a protein that catalyses the reversible conversion of CO_2 to HCO_3^-, has been recognised as an important enzyme that is closely associated with photosynthesis

(Sasaki *et al.*, 1998; De Lucia *et al.*, 2003; Evans *et al.* 2009). CA, together with aquaporins, has been described (De Lucia *et al.*, 2003) as a fast responding biochemical processes that regulates mesophyll conductance. We have found a 198% increase in this enzyme in elevated [CO_2] relative to control leaves that could partly compensate for the closure of stomata, thus ensuring the supply of CO_2 to the chloroplasts.

The SDS-PAGE densitometric analysis revealed that the photosynthetic down-regulation in elevated [CO_2] was caused by a lower Rubisco protein content (Fig. 3) (Theobald *et al.*, 1998; Aranjuelo *et al.*, 2005a). This decrease was not detected by proteomic analysis due to saturation of the silver staining. Moreover, the proteomic characterisation showed a decrease in Rubisco activase content in plants exposed to 700 μmol mol^{-1} CO_2 (Table 3). Rubisco activase is essential for the maintenance of Rubisco catalytic activity because it promotes the removal of tightly bound inhibitors from the catalytic sites (Robinson and Portis, 1989; Parry *et al.*, 2008). The lower photosynthetic rates of plants exposed to 700 μmol mol^{-1} [CO_2] (Table 3) may be a consequence of both decreased Rubisco protein and increased binding of inhibitors to Rubisco active sites, which is consistent with the decreased k_{cat} of the enzyme in elevated [CO_2] found in this (Fig. 4) and previous studies (Pérez *et al.*, 2005; 2007).

Lack of significant differences in total soluble protein (TSP) content, and the decrease of Rubisco as a fraction of TSP (Fig. 3) revealed that the diminished Rubisco concentration was caused by a specific inhibition of this protein in leaves exposed to elevated [CO_2] (Pérez *et al.*, 2007). According to Zhu *et al.* (2009) and Fangmeier *et al.* (2000), in flag

leaves of wheat exposed to elevated [CO_2] there is an increase in protease activity that enables the remobilisation of N. In agreement with this finding, the lower amino acid level in flag leaves (Table 2) under elevated [CO_2] suggests that the flag leaf Rubisco-derived N was reallocated to the ear, an organ with high N sink capacity. Furthermore, according to Theobald *et al.* (1998), in elevated [CO_2] there is a greater reduction in Rubisco than in other photosynthetic components (ATP synthase, etc.). The up-regulation of proteins involved in RuBP regeneration, like ATP synthase (β subunit), ruled out limitations on RuBP regeneration as the cause of diminished carboxylation in elevated [CO_2], and suggests a rebalancing away from carboxylation to RuBP-regeneration capacity (Theobald *et al.*, 1998).

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). The accumulation of fructans and starch in flag leaves in elevated CO_2 (Fig. 1) was associated with decreases of Rubisco (Fig. 2) and Rubisco activase (Table 3), and may be causal in down-regulation of photosynthetic capacity (Moore *et al.*, 1999; Jifon and Wolfe, 2002). As shown in Tables 2 and 3, the proteomic characterisation provided relevant information concerning the possible involvement of altered protein levels in carbon metabolism in elevated CO_2. Our study revealed that phosphoglycerate mutase (PGAM) content increased by 627.13 % in plants grown in elevated [CO_2] (Table 2). PGAM catalyses the intercoversion of 3-phosphoglycerate (3-PGA) to 2-phosphoglycerate (2-PGA) (Batz *et al.*, 1992), and its increase could lead to

enhanced glycolysis. Carbohydrate accumulation in leaves, irrespective of whether it is a result of sugar-feeding or an inhibition of phloem transport or growth in elevated [CO_2], has been shown to stimulate organic acid synthesis (Morcuende *et al.*, 1998; Stitt and Krapp, 1999) and respiratory pathways, leading to a decrease in the levels of 3-PGA (Morcuende *et al.*, 1996; Morcuende *et al.*, 1997) and increased formation of ATP (Stitt and Krapp, 1999). Furthermore, the proteomic characterisation also revealed a 79% decrease of adenosine diphosphate glucose pyrophosphatase (AGPPase) in elevated [CO_2] (Table 3). AGPPase catalyses the hydrolytic conversion of ADPglucose (ADPG), the universal glucosyl donor for starch biosynthesis, to AMP and G1P (Rodriguez-López *et al.*, 2000). Although starch and fructan accumulation in leaves in elevated [CO_2] may be accounted for by the observed decrease in leaf nitrogen content, since nitrate is known to repress AGP pyrophosphorylase (Scheible *et al.*, 1997) and at least one enzyme of fructan synthesis (Morcuende *et al.*, 2004), the decrease in AGPPase protein can contribute to the observed starch build-up in elevated [CO_2]. The fact that this protein is inhibited by ATP content (Emes *et al.*, 2003), and that the ATP synthase β subunit increased under elevated [CO_2], points to a tight control of starch build-up in leaves. The up-regulation of PGAM and down-regulation of AGPPase show an altered protein pattern that can enhance C utilisation for storage and energy in elevated [CO_2].

Carbohydrate build-up in leaves is determined by the plant's ability to develop new sinks (e.g. new vegetative or reproductive structures, enhanced respiratory rates), or to expand the storage capacity or growth rate of existing sinks (Lewis *et al.*, 2002). Although respiration processes require an investment of a large quantity of photoassimilates (Amthor,

2001; Aranjuelo *et al.*, 2009b), little attention has been given to this topic (especially in ears) in C balance studies analysing grain filling in cereals. Leaf respired $\delta^{13}C$ (Fig. 5) was depleted immediately after $^{12}CO_2$ labelling, and 24 h (T_1) and 48 h (T_2) later, showing that these plants were respiring, in part, C assimilated during the labelling period. However the fact that 48 h later (T_2) the $\delta^{13}C$ was similar to the values obtained before labelling (E) suggests that two days after labelling the leaves had respired almost all the labelled respiratory substrates. The determination of $\delta^{13}C$ in the various carbohydrates (Fig. 5), suggested that these leaves were respiring the labelled total soluble sugar (TSS) and especially glucose. This point is reinforced by the fact that 48 h after the end of labelling, pre-labelled C was present among glucose C, which is similar to the observation for leaf respiration $\delta^{13}C$. Opposite to the observation for leaf respiration and soluble sugar, the $\delta^{13}C$ of total organic matter (TOM) of flag leaves remained constant even 48 hours after the end of labelling. Such results could be explained by part of the labelled C being partitioned to structural and storage compounds. While fructan $\delta^{13}C$ did not contribute detectable labelled C in flag leaves, the $\delta^{13}C$ depletion in starch (-35.81 ‰ in non-labelled plants *versus* -37.93 ‰ in labelled plants) revealed that part of the labelled C present in TOM was accounted for by C accumulation in starch. It is very likely that because pre-labelled C was present in soluble sugars 48 h after the labelling, most of the remaining labelled C consisted of structural C compounds. The fact that TOM was more depleted than starch (-39.86 and -37.93 ‰ respectively) confirmed this point.

As mentioned above, leaf carbohydrate in wheat is also determined by ear C sink strength. Our data revealed that although exposure to 700 μmol mol^{-1} CO_2 did not modify

sucrose and glucose concentrations in ears, fructan and starch concentrations tended to increase. During grain filling, the strong C demand by wheat ears is met with ear photosynthesis and respiration (Tambussi *et al.*, 2007), together with translocation of C from flag leaves and stem internodes (Gebbing *et al.*, 1999; Aranjuelo *et al.*, 2009b). Absence of differences in the $\delta^{13}C$ in ear TOM and respired CO_2 between labelled and non-labelled plants (Fig. 5) confirmed that exposure to elevated [CO_2] did not increase ear filling during the beginning of anthesis, which is in agreement with the data on ear DM/ total DM ratios (Table 1). Even if the ear TOM was not labelled, the fact that sucrose and glucose $\delta^{13}C$ was depleted (Fig. 5) highlighted that a small fraction of labelled C reached the ear. Apparent discrepancies in TOM and sugar $\delta^{13}C$ could be explained by the fact that glucose and sucrose concentrations represent a small fraction of ear C, and therefore labelled C was diluted in TOM that was almost totally composed by non-labelled C (see Fig. 2). Although the photosynthetic activity of ears should not be ignored (Tambussi *et al.*, 2007; Zhu *et al.*, 2009), the fact that glucose, total soluble sugar and especially sucrose $\delta^{13}C$ depletion were more marked at 24 and 48 hours after labelling, indicates that this labelled C originated in flag leaves.

In summary this study suggested that the absence of elevated [CO_2] effects on biomass production, and especially ear grain filling, reflected the inability of these wheat plants to increase C sink strength. Absence of elevated [CO_2] effects on biomass production of plants with larger photosynthetic rates caused a leaf carbohydrate build-up. Such an increase induced photosynthetic acclimation, as reflected by the lower carboxylation capacity of treatments exposed to 700 μmol mol^{-1}. The $\delta^{13}C$ determinations conducted

during the post-anthesis period showed that in flag leaves, under elevated [CO_2], part of the newly assimilated C was allocated to storage compounds and that another part of labelled C (mainly soluble sugars) was totally respired 48 h after the end of labelling. In ears, the differences in the $\delta^{13}C$ data revealed that although no changes were detected in ear total organic matter (TOM), a small amount of C reached the ears in the form of soluble sugars. Proteomic characterisation showed that in these plants the changes in protein content enhanced C storage and glycolysis. Furthermore, the protein characterisation also revealed that photosynthetic acclimation was caused by a decrease in Rubisco protein content and in the capacity to release Rubisco tight-binding inhibitors. The decreases in leaf N, Rubisco and amino acid content suggest that under elevated [CO_2] there was a reallocation of leaf N to ears during grain filling. The ear DM, together with the ear isotopic and biochemical determinations revealed that two weeks after anthesis, ears of wheat plants exposed to elevated [CO_2] did not contribute to an increase in C sink strength. Therefore, such plants were incapable of overcoming leaf photoassimilate accumulation, with a consequent alteration in leaf N and protein content that caused the photosynthetic down-regulation.

Acknowledgements

The technical cooperation of AL Verdejo and MA Boyero in gas exchange and growth measurements, and in analyses of Rubisco protein, amino acids and total nitrogen content is acknowledged. We thank the staff of Muñovela experimental farm for technical assistance in crop husbandry. The assistance of Pilar Teixidor during the isotopic analyse is greatly acknowledged. This work has been funded by the Spanish National Research and Development Programme-European Regional Development Fund ERDF (Projects AGL2009-13539-C02-01, AGL2006-13541-C02-02 and CGL2009-13079-C02-02).

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Table 1

Effect of [CO_2] during growth on wheat total, flag leaf, ear, and ear DM/total DM, together with photosynthesis, stomatal conductance (gs) and intercellular CO_2 (Ci) determined at 370 (A_{370}, gs_{370} and Ci_{370} respectively) and 700 (A_{700}, gs_{700} and Ci_{700} respectively) μmol mol^{-1} [CO_2] 14 days after anthesis. Each value represents the mean ± SE (n= 6). The different letters indicate significant differences (P<0.05) among treatments and genotypes as determined by ANOVA test.

Parameters	**Ambient CO_2**				**Elevated CO_2**			
				Plant growth data				
Total DM (g m^{-2})	2287.1	±	510.4	a	1871.2	±	171.9	a
Flag leaf DM (g m^{-2})	92.3	±	15.3	a	73.3	±	152	a
Ear DM (g m^{-2})	662.0	±	240.0	a	426.7	±	46.9	a (F=0.093)
Ear DM/Total DM	0.29	±	0.09	a	0.23	±	0.01	a
				Gas exchange data				
A_{370} (μmol m^{-2} s^{-1})	14.59	±	5.5	a	3.77	±	0.61	b
A_{700} (μmol m^{-2} s^{-1})	33.7	±	6.0	a	21.6	±	4.4	b
gs_{370} (mmol m^{-2} s^{-1})	146.9	±	62.3	a	51.8	±	4.73	b
gs_{700} (mmol m^{-2} s^{-1})	184.3	±	46.4	a	124.0	±	35.7	b
Ci_{370} (mol mol^{-1})	153.6	±	12.7	b	277.0	±	9.90	a
Ci_{700} (mol mol^{-1})	324.5		17.6	a	345.9		78.7	a

Table 2. Annotation of elevated CO_2 **up-regulated** spots identified in silver stained two-dimensional electrophoresis gels of leaves collected 14 days after anthesis. A total of 125 µg of total proteins was loaded on an 18-cm gel strip forming an immobilized linear pH gradient from 4 to 7. Second dimension electrophoresis (SDS1258 PAGE) was carried out on 12 % polyacrylamide (w/v) gels (20 × 20 cm) (for details see ‘‘Material and Methods’’).

Spot no.	Spot % volume variations	pI/Mr	PM	SC (%)	Score ($p < 0.05$ corresponding to score > 51)	Protein name / Organism /NCBI accession no.
01. Metabolism						
104	198.03	6.25/32.01	7	28	249	Chloroplastic carbonic anhydrase gi|729003
02. Energy						
7	627.13	5.51/62.91	2	11	55	Phosphoglycerate mutase / gi|32400802
07. Transporters						
45	314.21	4.05/54.82	3	6	98	ATP synthase β subunit / gi|3850920
11. Disease/defence						
116	164.06	6.48/24.09	1	6	53	Manganese superoxide dismutase / gi|1621627
12. Unclear classification						
105	188.61	8.67/81.43	2	3	53	Putative blue light receptor / gi|20797092
123	139.12	5.35/42.85	3	3	54	SNF2 superfamily protein / gi|159466410
13. Unclassified						
79	244.27	4.42/23.86	2	2	63	Predicted protein / gi|226460198
943	195.90	4.43/24.77	4	18	122	Hypothetical protein / gi|1076722

Table 3. Annotation of elevated CO_2 **down-regulated** spots identified in silver stained two-dimensional electrophoresis gels of leaves collected 14 days after anthesis. A total of 125 µg of total proteins was loaded on an 18-cm gel strip forming an immobilized linear pH gradient from 4 to 7. Second dimension electrophores is (SDS1258 PAGE) was carried out on 12 % polyacrylamide (w/v) gels (20 × 20 cm) (for details see ‘‘Material and Methods’’).

Spot no.	Spot % volume variations	pI/Mr	PM	SC (%)	Score ($p < 0.05$ corresponding to score > 51)	Protein name / Organism /NCBI accession no.
02. Energy						
114	60.59	6.21/48.39	2	8	112	Ribulose-bisphosphate carboxylase activase / gi|100614
2090	79.12	5.81/23.97	2	5	65	Adenosine diphosphate glucose pyrophosphatase / gi|13160411
11. Disease/defence						
2496	87.75	4.91/85.64	2	3	81	Cytosolic heat shock protein 90 / gi|32765549
13. Unclassified						
61	34.66	4.95/24.65	2	9	92	Hypothetical protein / gi|1076722
68	37.85	5.20/38.94	1	91	66	Unknown protein 18 / gi|205830697
734	28.14	5.64/44.58	2	10	103	Hypothetical protein / gi|125602085

Figure Legends

Figure 1. Elevated [CO_2] effect on wheat flag leaf and ear glucose, sucrose, fructan and starch content 14 days after anthesis. Open bars correspond to plants grown under ambient CO_2 (ca. 370 µmol mol^{-1}) and closed bars to those grown under elevated CO_2 (ca. 700 µmol mol^{-1}). Each value represents the mean ± SE (n= 4). The different symbols indicate non significant differences (ns), significant differences $P<0.05$ (*) and $P<0.01$ (**) between treatments as determined by LSD.

Figure 2. Elevated [CO_2] effect on wheat flag leaf and ear N content and C/N ratio 14 days after anthesis. Open bars correspond to plants grown under ambient CO_2 (ca. 370 µmol mol^{-1}) and closed bars to those grown under elevated CO_2 (ca. 700 µmol mol^{-1}). Otherwise as in Figure 1.

Figure 3. Elevated [CO_2] effect on wheat flag leaf N, total soluble protein (TSP), Rubisco and amino acid content and Rubisco as a percentage of TSP 14 days after anthesis. Otherwise as in Figure 1.

Figure 4. Elevated [CO_2] effect on wheat flag leaf total Rubisco activity, Rubisco activation and Rubisco k_{cat} 14 days after anthesis. Open bars correspond to plants grown under ambient CO_2 (ca. 370 µmol mol^{-1}) and closed bars to those grown under elevated CO_2 (ca. 700 µmol mol^{-1}). Otherwise as in Figure 1.

Figure 5. Elevated [CO_2] effect on wheat flag leaf and ear ^{13}C isotopic composition ($\delta^{13}C$) in total organic matter (TOM), respired CO_2, (DR CO_2), total soluble sugars (TSS), glucose (Glu), sucrose (suc), fructans (Fru) and starch (HCl-C). A an E stand for ambient and elevated [CO_2], respectively, before labelling (pre-labelling period). T_0, refers to the end of labelling (labelling period; 14 days after anthesis), whereas T_1, T_2, refer to 24 h and 48 h after the end of labelling (post-labelling period), respectively**.** Otherwise as in Figure 1.

Figure 6. Silver-stained two-dimensional gel electrophoresis of proteins extracted from wheat leaves grown under ambient and elevated conditions 14 days after anthesis. In the first dimension, 125 mg of total protein was loaded on a 18 cm IEF strip with a linear gradient of pH 4–7. The second dimension was conducted in 12% polyacrylamide (w/v) gels (20 × 20 cm) (for details see ‘‘Materials and Methods’’). The gel image analyses was conducted with Progenesis SameSpots software v3.0 and the subsequent mass spectrometry analyses identified up to 14 proteins (marked by arrows) with significantly different expression in elevated [CO_2].

Figure 1

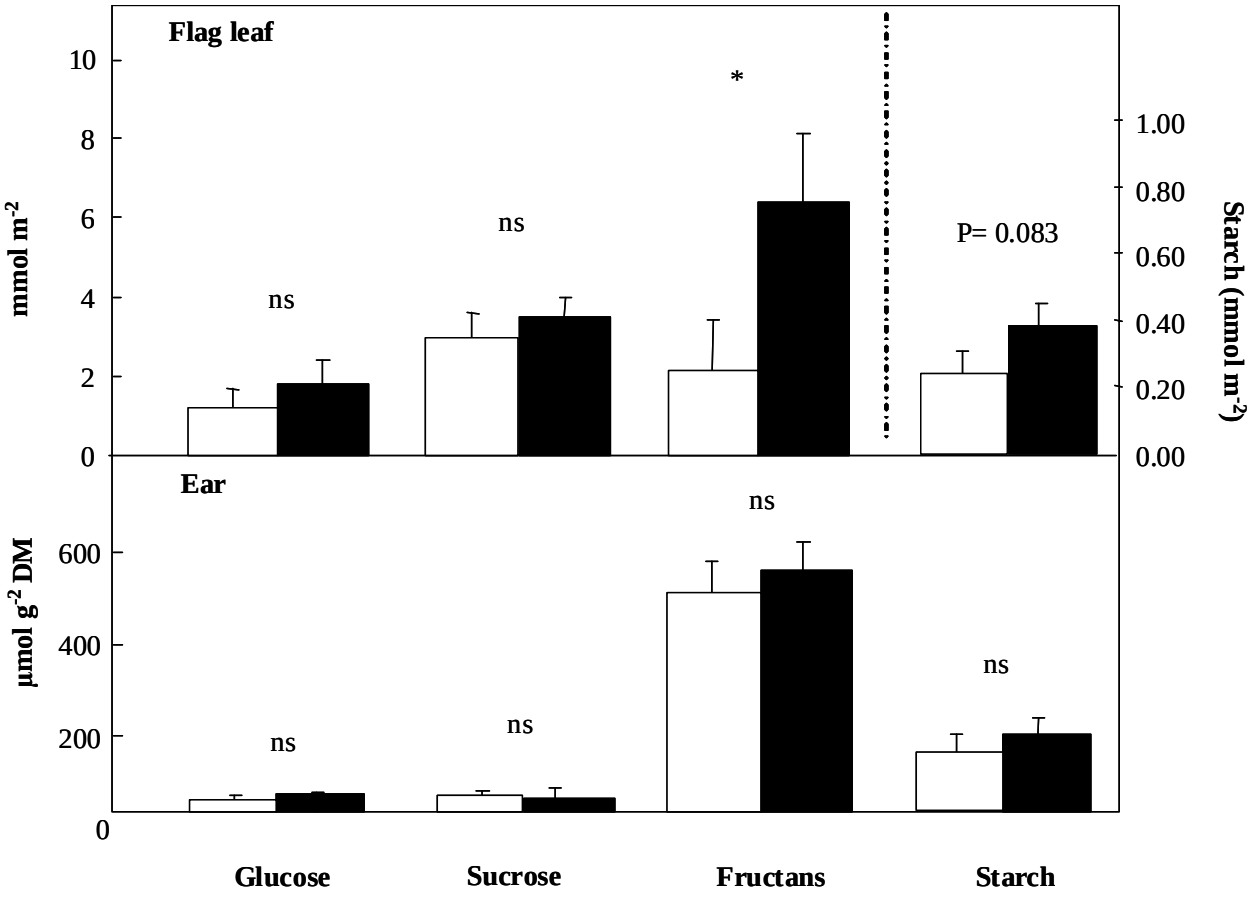

Flag leaf
Ear
mmol m-2
µmol g-2 DM
Starch (mmol m-2)
10
8
6
4
2
0
1.00
0.80
0.60
0.40
0.20
0.00
600
400
200
0
*
ns
P= 0.083
Glucose
Sucrose
Fructans
Starch

Figure 2

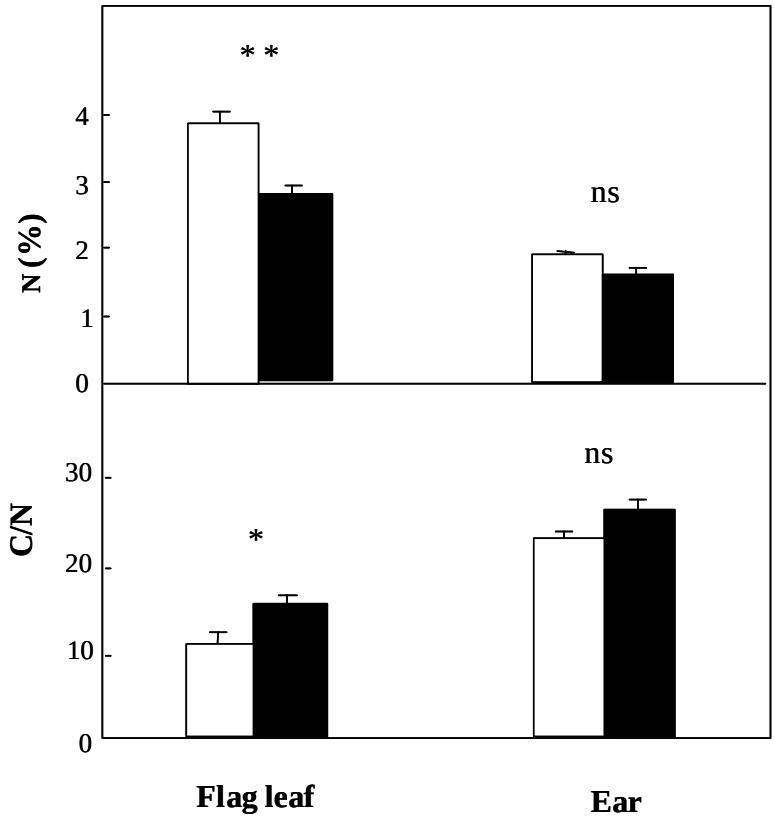


Figure 3

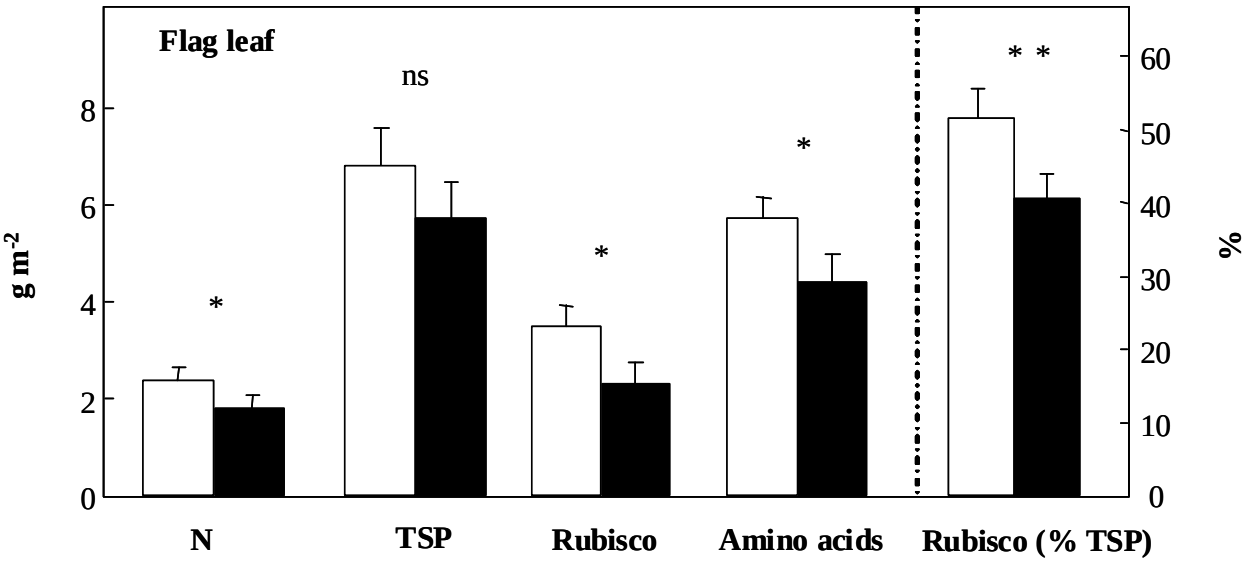

Flag leaf
ns
*
*
*
* *
g m-2
%
0
2
4
6
8
0
10
20
30
40
50
60
N
TSP
Rubisco
Amino acids
Rubisco (% TSP)

Figure 4

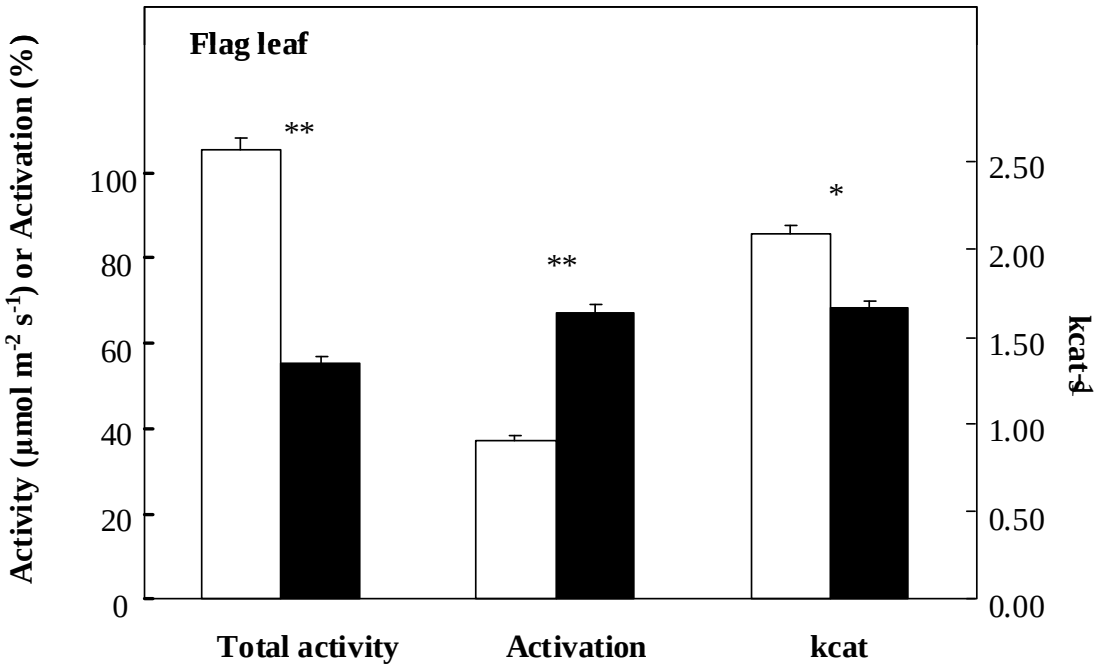

Flag leaf
Activity (µmol m-2 s-1) or Activation (%)
kcat-1
0
20
40
60
80
100
0.00
0.50
1.00
1.50
2.00
2.50
**
**
*
Total activity
Activation
kcat

Figure 5

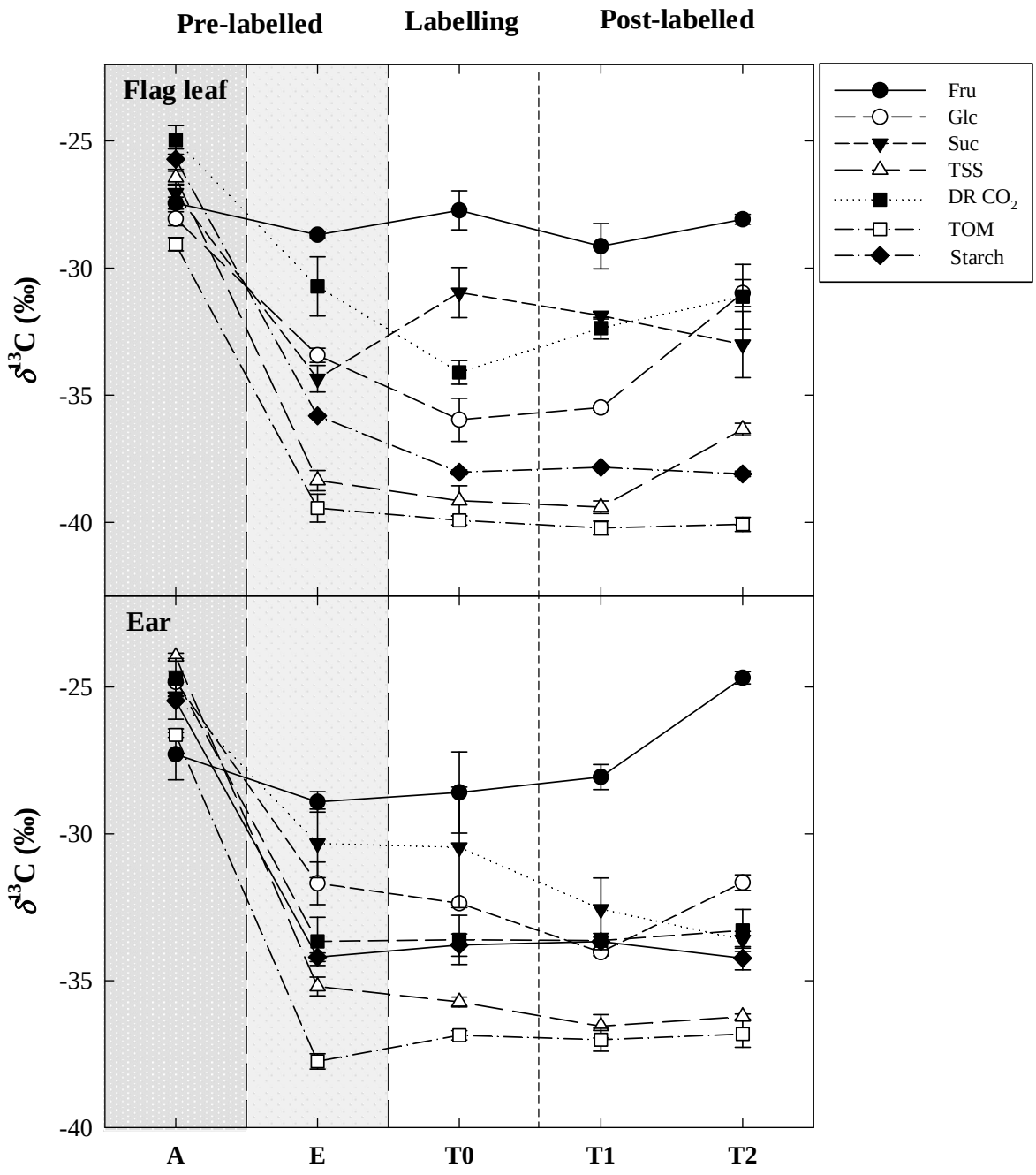
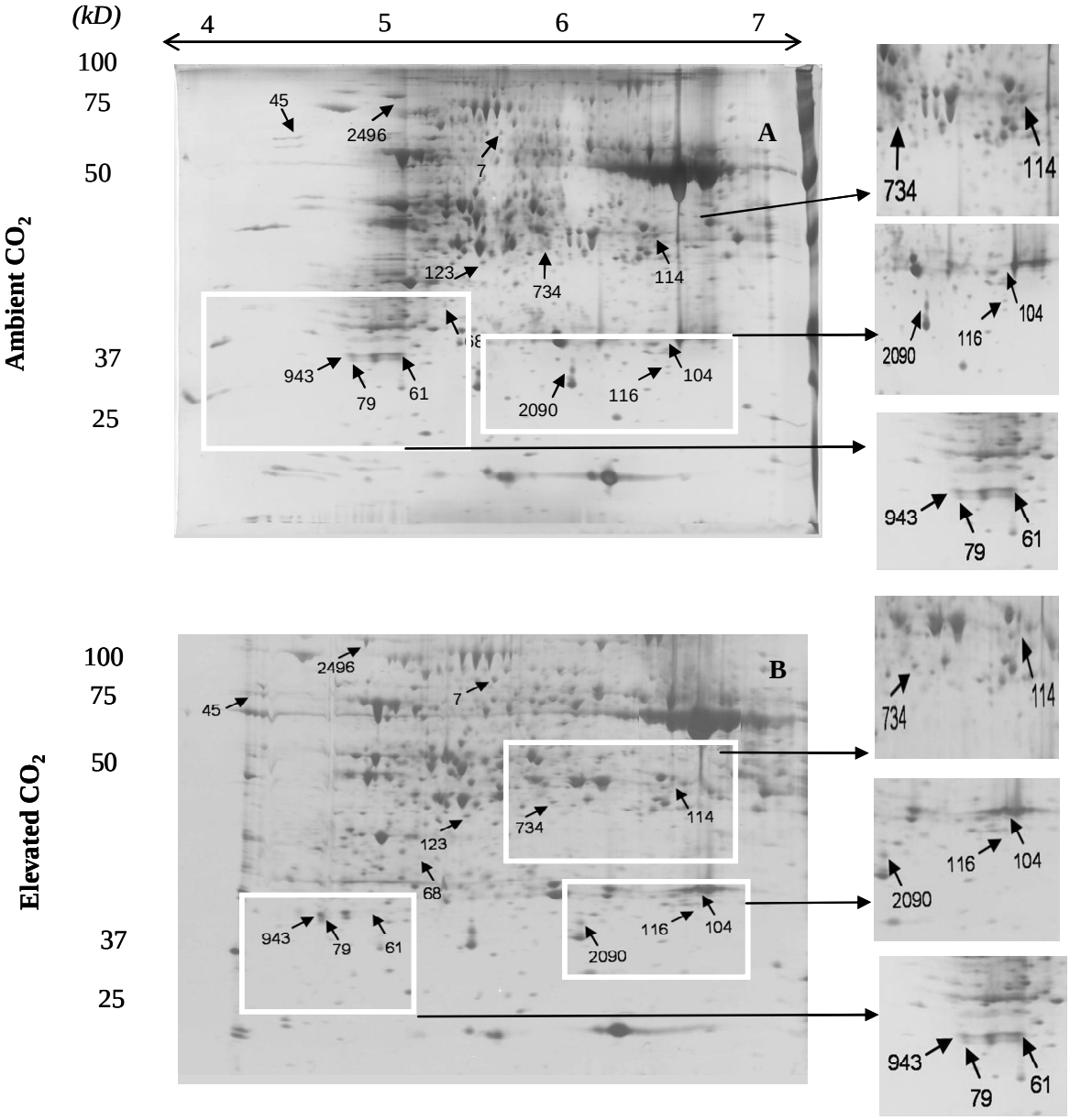

Pre-labelled
Labelling
Post-labelled
Flag leaf
Ear
δ13C (‰)
-25
-30
-35
-40
A
E
T0
T1
T2
Fru
Glc
Suc
TSS
DR CO2
TOM
Starch

Figure 6


(kD)
4
5
6
7
100
75
50
37
25
Ambient CO_2
45
2496
7
A
123
734
114
943
79
61
2090
116
104
Elevated CO_2
B
68