RESEARCH PAPER

Elevated CO₂ has concurrent effects on leaf and grain metabolism but minimal effects on yield in wheat

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Abstract

While the general effect of CO₂ enrichment on photosynthesis, stomatal conductance, N content, and yield has been documented, there is still some uncertainty as to whether there are interactive effects between CO₂ enrichment and other factors, such as temperature, geographical location, water availability, and cultivar. In addition, the metabolic coordination between leaves and grains, which is crucial for crop responsiveness to elevated CO₂, has never been examined closely. Here, we address these two aspects by multi-level analyses of data from several free-air CO₂ enrichment experiments conducted in five different countries. There was little effect of elevated CO₂ on yield (except in the USA), likely due to photosynthetic capacity acclimation, as reflected by protein profiles. In addition, there was a significant decrease in leaf amino acids (threonine) and macroelements (e.g. K) at elevated CO₂, while other elements, such as Mg or S, increased. Despite the non-significant effect of CO₂ enrichment on yield, grains appeared to be significantly depleted in N (as expected), but also in threonine, the S-containing amino acid methionine, and Mg. Overall, our results suggest a strong detrimental effect of CO₂ enrichment on nutrient availability and remobilization from leaves to grains.

Keywords: Climate change, free-air CO₂ enrichment (FACE), multiple locations, N/C metabolism, physiology, varieties, wheat.
Introduction

Wheat represents 27% of global grain production (FAOSTAT, 2018: http://www.fao.org/faostat/en/#home) and, along with corn and rice, is a major component of the human diet because of its high nutritional value: it is a source of carbohydrates, macroelements and micro (trace) elements, protein, and free amino acids. Bread wheat (Triticum aestivum L.) constitutes up to 95% of global wheat production, while durum wheat (Triticum turgidum ssp. durum) makes up the remainder (He et al., 2013). While bread wheat is cultivated in all parts of the world (Reynolds et al., 2012), durum wheat is mostly grown in the Mediterranean region (Oliveira et al., 2012). Like other crops, wheat cultivation will face serious challenges in the coming decades, with a need to increase production despite challenging environmental conditions caused by climate change. Doubling production by 2050 to meet the anticipated demand is expected to be difficult, since yield would have to increase by 2.4% per year globally whereas the actual rate of increase is only 1.3% per year and, furthermore, ~39% of wheat-cultivating areas have shown no increase in yield in a decade (Ray et al., 2012). Among important questions that must be answered to understand how wheat yield responds to changing climatic conditions is the potential impact of rising atmospheric CO₂.

Atmospheric CO₂ has progressively increased since the beginning of the industrial revolution, and the CO₂ mole fraction is anticipated to double (the predicted mole fraction ranges between 730 and 1020 ppm) by 2100 (Meehl et al., 2010). Since photosynthetic activity in C₃ plants is CO₂-limited, it was predicted that the increase in atmospheric CO₂ would stimulate photosynthesis and thus plant growth (Bowes, 1993). However, crop response to elevated CO₂ varies widely, depending on growth (cultivation) conditions and inter- and intra-specific variability. While a CO₂-driven increase in yield is generally observed, there is substantial variation, with sometimes no yield gain at all (Ainsworth and Long, 2005; Högy and Fangmeier, 2008). This effect mostly comes from photosynthetic acclimation to high CO₂, which in turn favours grain production. In wheat, cultivation under free-air atmospheric CO₂ enrichment (FACE) leads, on average, to a yield enhancement of only 10% (Kimball, 2010, 2016). Photosynthesis acclimates to elevated CO₂ via lower average stomatal conductance (gₛ), lower photosynthetic capacity (maximum carboxylation rate Vₘₐₓ, and/or lower ribulose 1,5-bisphosphate regeneration ability (Miglietta et al., 1996; Adam et al., 2000; Rogers and Humphries, 2000; Wechsung et al., 2000; Zhang et al., 2009). Of course, wheat response to elevated CO₂ also depends on water and N supply.

In general, elevated CO₂ leads to a higher relative increase in wheat grain yield under water-restricted conditions (up to 20%) compared with rain-fed plants (up to 10%), although yield absolute values are, as expected, lower under water restriction (Kimball et al., 1995; Hunsaker et al., 1996; Tubiello et al., 1999). In addition, the extent to which yield is stimulated by elevated CO₂ under low water input depends on the sowing date and whether additional irrigation is implemented under control (well-watered) conditions (O’Leary et al., 2015). Unsurprisingly, minimal effects of elevated CO₂ on photosynthesis and yield are observed under N-limited conditions (Li et al., 2000; Grant et al., 2001; Kimball et al., 2001a; Pacholski et al., 2015; Walker et al., 2017; Manderscheid et al., 2018). Furthermore, elevated CO₂ triggers developmental changes that are detrimental to yield. In particular, wheat cultivated under FACE has a lower shoot-to-root ratio (Wechsung et al., 1995; Pacholski et al., 2015) and perhaps an increased root exudation rate (thereby increasing organic matter deposition in soil and thus soil respiration) (Pendall et al., 2001) and accelerated flag leaf senescence (Zhu et al., 2009). By contrast, elevated CO₂ causes an increase in tiller number (Yang et al., 2007a, b) and has a marginal effect on transpiration, making plants slightly more water-efficient, especially under high N supply (Hunsaker et al., 1996; Erbs et al., 2009; O’Leary et al., 2015; Kimball, 2016; Manderscheid et al., 2018).

In addition to such quantitative changes in crop production and photosynthesis, elevated CO₂ leads to modifications in leaf biochemical composition and grain quality. Under elevated CO₂, wheat leaves invest more C in cellulose and flavonoids (Akin et al., 1995; Peñuelas et al., 2000) and are generally less N-rich, with a higher C/N ratio (Zhu et al., 2009; Aranjuelo et al., 2015) and lower amounts of proteins (or transcripts encoding them) involved in photosynthesis (Calvin cycle enzymes) (Nie et al., 1995; Adam et al., 2000; Zhang et al., 2009; Pandey et al., 2017). Proteomics conducted on leaves sampled during anthesis in wheat cultivated in a CO₂-enriched greenhouse (at 700 µmol mol⁻¹ CO₂) have shown that the Rubisco content did not increase, while there was a slightly higher content of Rubisco activase (Aranjuelo et al., 2015). When grown under FACE, leaves have been found to contain less Rubisco and more mitochondrial malate dehydrogenase (NAD-dependent), suggesting a change in respiratory metabolism (respiratory CO₂ loss) (Pandey et al., 2017). The detrimental effect of CO₂ on the photosynthetic machinery of flag leaves is accompanied by an increase of ear photosynthesis and a faster decline in flag leaf N due to earlier senescence (Sicher and Bunce, 1998; Zhu et al., 2009). Taken as a whole, elevated CO₂ has a major effect on leaf N metabolism, which in turn impacts on remobilization and thus grain filling during maturation.

Unsurprisingly therefore, wheat grains (and flour produced from them) produced at elevated CO₂ have a generally lower nutritional quality, with lower N (and S) content, less protein, and more starch and fibres (Kimball et al., 2001a, b; Högy et al., 2013; Wroblewitz et al., 2013). We have recently shown that when wheat plants are grown in the greenhouse at elevated CO₂ under ample N supply, grains are less N-rich and do not have the same kinetics of gliadin and glutenin accumulation (Soba et al., 2019). In this case, it has been suggested that this effect did not come from deficient N remobilization in leaves but rather a N dilution effect, whereby the quantity (or size) of grains increased while the total available N for remobilization did not change. In addition, under FACE conditions, an increase in the glutenin-to-gliadin ratio has been found (Pandey et al., 2017), as well as a general decrease in all storage proteins except for globulins and albumins (Wieser et al., 2008, but see Verrillo et al., 2017, where globulins also decreased). Metabolic analyses...

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have demonstrated that at elevated CO2, grains contain more secondary metabolites (shikimate and quinate) and free hexoses (fructose and glucose) but less sucrose and alanine, regardless of developmental stage. At maturity, grains are generally depleted in free amino acids, except for the branched-chain amino acids valine, leucine, and isoleucine, which are significantly more abundant (Wroblewitz et al., 2013; Soba et al., 2019). Furthermore, it has been suggested that the metabolism of lysine recycling changes and favours the saccharopine pathway over pimelate (Soba et al., 2019). It is also worth noting that elevated CO2 causes a decline in the content of several microelements in wheat grains, such as Fe and Zn (Wroblewitz et al., 2013; Pandey et al., 2017; Beleggia et al., 2018), further impacting on nutritional quality.

Nevertheless, the effect of elevated CO2 likely depends not only on the cultivation conditions (water and N supply, time of sowing, and temperature), but also on soil properties and the species and cultivar of wheat. For example, wheat lines of different ploidy do not respond similarly to elevated CO2 in FACE experiments, with hexaploid bread wheat being the most responsive in terms of yield (at least in terms of photosynthesis) (Uprety et al., 2009). There is also substantial variability between cultivars, no response at all to significant response to CO2, with little correlation between yield and photosynthesis, but significant correlation with N content and specific leaf area (Thilakarathne et al., 2013, 2018) or transpiration efficiency (Tausz-Posch et al., 2013b; but see also Bourgault et al., 2013). The impact of elevated CO2 on both yield and elemental contents has been found to depend on the cultivar (Fernando et al., 2014; Fares et al., 2016; Houshmandfar et al., 2016; Beleggia et al., 2018). Variability in the response to elevated CO2 between wheat cultivars likely results from differences in N and chlorophyll content, transpiration efficiency, or tillering capacity (Tausz-Posch et al., 2013a; Veres et al., 2017). Modelling has also suggested that variation in the response of wheat lines to elevated CO2 can result from differences in development and allocation (sink/source relationships) (Wolf et al., 2002). In addition, it is probable that the response to elevated CO2 may vary when soil properties change. This aspect is less documented but, in principle, the spatial variability of wheat yield in the field under ambient CO2 has been shown to be related to soil P or organic C content, soil structure, or the proportion of carbonates (Miller et al., 1988; Bhatti et al., 1991; Moulin et al., 1994). Since in wheat there is a relationship between root deposition stimulated by elevated CO2, root-driven acidification, and cation mobility (Cheng et al., 2010), the effect of elevated CO2 could be modulated by soil Na, Ca, and K content and pH.

Taken as a whole, whenever there are differences among growth conditions and cultivars, it is difficult to understand the physiological mechanisms underlying the response to elevated CO2. In fact, it could well be that FACE experiments conducted separately in different countries with different cultivars are not comparable, not only because soil and climate conditions vary but also because the response to CO2 is cultivar-specific. To tackle this issue, we conducted a targeted metabolic analysis of samples collected during FACE experiments (at 550 µmol mol−1 CO2) conducted in different countries (USA, Australia, Germany, China, and Italy) with different species (T. aestivum and T. turgidum ssp. durum) and cultivars (Table 1; Supplementary Fig. S1). We carried out elemental analyses (macroelements and some microelements), quantitation of amino acids and sugars, and targeted protein analysis in leaves and grains. Our objective was to assess whether a significant effect of elevated CO2 could be observed in both leaves and grains across countries and wheat lines and, by doing so, to look at the potential effect of leaf-to-grain nutrient remobilization on CO2 responsiveness. Our data could also be exploited further by exploring the potential relationship between yield (treated as a quantitative response variable) and grain biochemical composition. Our working hypothesis was that (i) detrimental effects of elevated CO2 on photosynthesis and N assimilation were associated with significant changes in protein and amino acid composition in leaves, and thus (ii) the amino acid composition of grains was affected, reflecting alteration of both the provision and recycling of nitrogenous compounds; and (iii) there might be a relationship between grain metabolic features and yield.

Materials and methods

Plant material and experimental design

The experiment was conducted with durum wheat (cultivars Miradoux, Duramant, Claudio, and Smneto) and bread wheat (cultivars Jantz, Kite, Norin, Triumph, Milan, and PRL). The study involved five FACE facilities located in Stuttgart-Hohenheim (Germany), Fiorenzuola (Italy), Changping-Beijing (China), Horsham (Australia), and Beltsville (USA), in which plants were exposed to ambient (400 ppm) and elevated (550 ppm) CO2 concentrations. FACE facilities included three to five rings (for each CO2 concentration condition) with a diameter of 12–14 m each, or (in Stuttgart-Hohenheim) 2×2 m square plots. In all cases, CO2 enrichment was performed from sunrise to sunset (in Australia and Italy) or 24 h (a day in China, Germany, and the USA) and throughout the entire growing season. The principle of operation and the performance of the FACE systems used here have been described previously (Mollah et al., 2009; Fangmeier et al., 2016; Fares et al., 2016). When plants reached the stem elongation (Z31) and anthesis halfway (Z65) stages (BBCH), sampling and gas-exchange analyses were carried out in the last expanded leaves (Z31) or flag leaves (Z65). Collected leaf samples were immediately frozen in liquid N2 and stored at −80 °C for later analyses. Finally, at maturity stage (Z90), grain samples were collected for agronomic and metabolic analyses.

Gas exchange determinations

Analyses were carried out in healthy expanded flag leaves grown under ambient or elevated CO2 conditions. The light-saturated rate of CO2 assimilation (A_s), stomatal conductance (g_s), and intercellular CO2 concentration (c_i) were estimated at a photosynthetic photon flux density of 1500–1600 µmol m−2 s−1 using equations developed by von Caemmerer and Farquhar (1981). The vapour pressure deficit was 1.5 kPa. CO2 response curves were constructed by a series of measurements whereby photosynthesis was determined first under standard conditions (400 µmol mol−1), then at low CO2, then back to standard conditions, and then at high CO2: overall, the sequence of CO2 concentrations in the reference channel was 400, 200, 100, 50, 200, 400, 600, 750, and 950 µmol mol−1. Estimations of the maximum carboxylation velocity of Rubisco (V_{max}) and the maximum electron transport rate contributing to ribulose 1,5-bisphosphate regeneration (I_{max}) (measurements made in Germany) were made using the method of Harley et al. (1992).
Mineral composition

C and N concentration (％) analyses were determined using an elemental analyzer (EA1108; Carlo Erba Strumentazione, Milan, Italy). Macronutrient and macronutrient concentrations were determined by inductively coupled plasma/optical emission spectrometry (ICP/OES, iCAP 6500 Duo, Thermo Fisher Scientific, Waltham, MA, USA).

C Isotope discrimination

Carbon isotope composition was determined using an elemental analyzer (EA1108; Carlo Erba Strumentazione, Milan, Italy) coupled to an isotope mass spectrometer (Delta C; Finnigan MAT, Bremen, Germany). Values were expressed as delta values $\delta^{13}C = (R_{\text{sample}}/R_{\text{standard}})^{-1} - 1$ and expressed in ‰, where $R_{\text{standard}}$ is the $^{13}\text{C}$-to-$^{12}\text{C}$ ratio of the international standard (V-PDB). The carbon isotope discrimination ($\delta$) was calculated as follows:

$$\delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

where $R_{\text{sample}}$ is the isotope ratio in atmospheric CO2. The addition of (industrial) CO2 in the course of the FACE experiments was such that $\delta^{13}C_{\text{CO2}}$ was lower than the natural $^{13}\text{C}$ abundance in the atmosphere (near –8‰). The $\delta^{13}C$ value of the added CO2 was known (for the experiment in Germany) or calculated from mass balance, taking advantage of the known contribution of added CO2 to total CO2: $c_{\text{CO2}} = (550 – 400)/550 = 27.2\%$. In fact, if the isotope fractionation under ambient CO2 and under FACE conditions was the same, then the proportion of CO2 coming from the added CO2 could be calculated as $c_{\text{CO2}} = (\delta_{\text{OM,amb}} - \delta_{\text{OM,FACE}})/(\delta_{\text{CO2,amb}} - \delta_{\text{CO2,added}})$, where OM refers to organic matter and amb refers to ambient conditions, and $\delta^{13}C$ values are abbreviated to $\delta$ to simplify the notation. $\delta_{\text{OM,FACE}}$ is the $^{13}\text{C}$ value of organic matter under FACE conditions if the fractionation had not changed compared with ambient conditions. In practice, the isotope fractionation could have varied, and thus (neglecting denominators): $\delta_{\text{OM,FACE}} = \delta_{\text{OM,amb}} + \Delta_{\text{FACE}}$, where $\Delta_{\text{FACE}}$ and $\Delta_{\text{amb}}$ are the net photosynthetic fractionation under FACE and ambient conditions, respectively. These equations can be combined to give $c_{\text{OM}} = (\delta_{\text{OM,amb}} - \delta_{\text{OM,FACE}})/(\delta_{\text{CO2,amb}} - \delta_{\text{CO2,added}}) - (\Delta_{\text{FACE}} - \Delta_{\text{amb}})/\Delta_{\text{FACE}}$. The right term is relatively small since added industrial CO2 was naturally $^{13}\text{C}$ depleted and thus $\delta_{\text{CO2,amb}} - \delta_{\text{CO2,added}}$ is much larger than $\Delta_{\text{FACE}} - \Delta_{\text{amb}}$. That is, by neglecting the right term, $\delta_{\text{CO2,added}}$ can be estimated (and therefore $\Delta_{\text{FACE}}$ with equation 1) by imposing $c_{\text{OM}} = 0.272$. Note that such an approximation was not critical, since even under the assumption ($\Delta_{\text{FACE}} - \Delta_{\text{amb}})/\Delta_{\text{FACE}}$ represented 0.10 (i.e. 40% error in $c_{\text{OM}}$); this would ultimately cause an error of only 1% in $\Delta_{\text{FACE}}$.

Abscisic acid content

Extraction, purification, and quantification of abscisic acid (ABA) were carried out as described by Torres et al. (2018), using a high-resolution mass spectrometry (HPLC-ESI-HRMS) system, with some modifications: freeze-dried material (15 mg) was used instead of frozen powdered material (0.1 g), and the residue obtained after final evaporation (SpeedVac) was redissolved in 0.25 ml methanol instead of 0.5 ml.

Soluble sugar and starch content

Sucrose, glucose, and fructose content were determined using a Beckman P/ACE5000 capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA), following the method of Cabreroiz et al. (2001). Starch content in the pellet was determined according to Ether and Livingston (2004).

Amino acid content

Amino acids were derivatized at 22–25 °C for 12–16 h with 1 mM fluorescein isothiocyanate dissolved in 20 mM acetone/borate, pH 10. The content of single amino acids was determined by capillary electrophoresis in a Beckman–Coulter PA-800 system.

Protein samples

Samples were previously quantified by microBCA analysis (Pierce) and similar amounts (5 µg per sample) were individually dissolved in 8 M urea, 25 mM ammonium bicarbonate, reduced with DTT, and alkylated with iodoacetamide, according to a method described by López-Ferrera et al. (2004). Digested samples were diluted with 0.2% trifluoroacetic acid in water and subjected to multiple reaction monitoring analysis using a 1D Plus nanoLC Ultra system (Eksigent, Dublin, CA, USA) interfaced to a Sciex 3500 QTRAP triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA) equipped with a nano-electrospray ionization source and controlled by Analyst v.1.5.2 software (ABSciex). Tryptic-digested samples were loaded online on a C18 PepMap 300 µm internal diameter × 5 mm trapping column (5 µm, 100 Å, Thermo Scientific) and separated using a BioSphere C18 75 µm internal diameter × 150 mm capillary column (3 µm, 120 Å, Nanoseparations). A list of 84 transitions (usually 3–4 per peptide, with a preference toward higher-mass y series ions), corresponding to 21 unique peptides selected for 10 different proteins, was monitored. Skyline software determined automatically the collision energy values for the candidate peptides according to MacLean et al. (2010).

Statistical analysis

To explore the effect of CO2, univariate statistics were conducted with a two-way ANOVA, with one factor representing ‘conditions’ (time of sampling, country, and species/cultivar) while the second factor was CO2. It was not possible to carry out a three-way analysis because not all species/cultivars were represented in all countries. Thus, here, the two-way ANOVA was conducted with the factor ‘conditions’ having 12 possible qualitative values (combinations time–country–cultivar) and the factor ‘CO2’ having two values (ambient or elevated). Unless otherwise stated, statistical significance was accepted when $P<0.05$. Multivariate statistics were carried out using orthogonal projection on latent structure (OPLS; with Simca®, Umetrics), using CO2 as the predicted Y variable (while ‘conditions’ are embedded into orthogonal dimensions), and metabolic features (elemental contents, metabolites, and proteins) as predicting X variables. Before running the OPLS, a principal component analysis was conducted to check the presence of outliers (samples outside the Hotelling’s ellipse). The performance of the OPLS was assessed using the correlation coefficient between predicted and observed Y (R2), the cross-validated correlation coefficient (Q2), the Q2 intercept of the permutation test (which was checked to be negative), and the P-value of testing the OPLS model against a random-error model (i.e. average ±error) via a χ2 test (this P-value is referred to as PXY). Univariate and multivariate analyses were combined using −log(P-value) (univariate) plotted against the OPLS loading (P Loading) in a volcano plot. To explore the relationship between yield and grain composition, we conducted (i) an OPLS using yield as the predicted Y variable (with the same parameters of performance as described above) and (ii) a univariate analysis by linear regression (done in R). Linear regression was done without and with variable elimination. Without variable elimination, each variable was correlated with yield separately and the associated P-value is reported. To carry out variable elimination, we used a multiple linear regression. It was carried out via sampling subsets (regsubsets) of eight variables (304 iterations) and a correlation plot was generated to select the eight most correlated variables overall. Then, a multiple linear regression model (1n) comprising only these eight variables was generated and the P-value for each variable was calculated.

Results

Photosynthetic parameters

The photosynthetic activity of flag leaves was assessed using measurements of gas exchange at various times of the development cycle (shown as BBCH stage numbers), at the CO2 mole fraction ($\epsilon$) used during growth (Fig. 1). Overall, there
was little variation in the photosynthetic rate, which was ~22 µmol m⁻² s⁻¹ regardless of CO₂, with the following properties (Fig. 1C): (i) in China, the USA, and Australia, there was a significantly higher photosynthetic rate at elevated CO₂, and (ii) in Australia, plants generally had lower photosynthetic rates compared with other countries. These effects were unrelated to stomatal conductance (Fig. 1B), which was roughly constant at 0.25 mol m⁻² s⁻¹; however, CO₂ had a significant depressing effect in plants grown in Italy. As a result, there was little variation in the intercellular-to-external CO₂ ratio (c_i/c_a) (Fig. 1A), which was always ~0.75, except in Italy (c_i/c_a <0.7) and in Australia (c_i/c_a = 0.7) at elevated CO₂. The natural carbon isotope abundance (δ¹³C) in raw leaf matter was measured, and ‘average’ c_i/c_a (i.e. across the leaf lifespan) was estimated using the simplified equation of Farquhar et al. (1982) (Fig. 1D, E).

The ¹²C/¹³C fractionation (raw matter versus source CO₂) was within 20–22‰, showing that average c_i/c_a was always ~0.7. However, the isotope fractionation was significantly lower (and therefore so was average c_i/c_a) in Germany, Italy, and Australia at elevated CO₂, suggesting a long-term decrease in stomatal (or internal) conductance and/or an increase in photosynthetic capacity at elevated CO₂. In addition, there were no significant changes either between CO₂ conditions or among countries in ABA content (data not shown).

Leaf metabolism

The contents of amino acids, sugars, macro- and microelements, and some target proteins were analyzed in flag leaves (contents were expressed relative to dry weight). A statistical analysis combining multivariate (OPLS) and univariate (two-way ANOVA) statistics was performed and results showing the effect of CO₂ are represented as a volcano plot in Fig. 2A. In both the OPLS and ANOVA, two factors were considered: CO₂ and ‘conditions’ (comprising country, species/cultivar, and growth stage). Growth at elevated CO₂ led to an increased content of several elements (Mo, Mg, Ca, Cr, Ti, Fe, and S) and a decrease in K. Several enzymes [superoxide dismutase, Rubisco (large subunit), carbonic anhydrase, pyruvate dehydrogenase, and nitrate reductase] had a lower content at elevated CO₂, and so had threonine. There was a tendency to have more fructose and less sucrose at elevated CO₂ (significant changes in ANOVA) but OPLS loadings were rather small, showing that the change in sugar composition was not a strong marker of growth at elevated CO₂. When expressed as a percentage of total amino acids and proteins, glycine and glycine decarboxylase (GDH subunit h) were lower (P<0.001) under elevated CO₂ (Fig. 2B), likely reflecting lower photorespiration activity. When examined separately for each country, the fructose-to-sucrose ratio appeared to be significantly different in Germany and Italy (Fig. 2C), and threonine content appeared to be significantly different in Italy and China (Fig. 2D), showing that the effect of elevated CO₂ was country-specific (i.e. it depended on environmental local conditions). This phenomenon was clearly visible in the results of the two-way ANOVA, where several features appeared to be significant for the CO₂ × conditions effect (Supplementary Fig. S2). For example, under elevated CO₂, there was a notably lower content of amino acids in Australia, nearly opposite effects on starch content in the USA and Australia, and a strong decrease in the content of several microelements in China. Nevertheless, the patterns of mineral elements were the same across all countries and CO₂ conditions. In fact, there was as expected an antagonism between major cations, with Ca + Na or Mg being negatively correlated with K (Fig. 2E). However, there was little difference in this correlation between ambient and elevated CO₂. In other words, the significant decrease in K and increase in Ca and Mg at elevated CO₂ (Fig. 2A) followed the same cation balance relationship as under ambient CO₂.
Grain metabolism

The contents of free amino acids, macro- and microelements, and total proteins and starch were quantified in mature grains (relative to dry weight). As in leaves, an analysis combining univariate (ANOVA) and multivariate (OPLS) statistics was performed and presented as a volcano plot (Fig. 3A). Interestingly, no component appeared to be significantly increased at elevated CO₂. By contrast, there were significant decreases in several elements (N, Mg, and Pb), amino acids (methionine, threonine, alanine, and tyrosine), and the glutamate derivative γ-aminobutyrate (GABA). Although nitrogen content (%N) was found to be lower, there was no significant change in protein content. Such a decrease in N content could be explained by changes in the concentration of many N-containing compounds (e.g., free amino acids, polyamines, or insoluble proteins not accounted for in our assay), which in total sum up to a larger and significant change in overall %N. When plotted together, %N and carbon content (%C) showed considerable scattering across countries (Fig. 3B), with a difference greater than 1.5% in %N between samples. The effect of elevated CO₂ on alanine and threonine appeared to be condition-specific, with significant differences between conditions found in the USA and Italy (Fig. 3C, D). More generally, the two-way ANOVA showed that amino acids, %N, and Pb were significant for the CO₂ × conditions effect (Supplementary Fig. S3). Elevated CO₂ did not lead to significant changes in major cations (K, Ca, Na, and Mg) (Fig. 3E). In fact, Ca + Na and Mg were negatively correlated with K, reflecting cation balance, and the correlation was not changed by elevated CO₂; that is, the lower Mg content at elevated CO₂ (Fig. 3A) was not associated with a change in the Mg/K balance in grains.

Relationships with yield

There were considerable differences in grain yield between countries, with low values in the USA (≈200 g grain m⁻²) and
The difference in yield between countries was unsurprising and resulted from seasonal differences, as well as differences in climatic conditions, fertilization (Table 1), and soil composition (Supplementary Fig. S1), or sowing density (which ranged within 120–350 m$^{-2}$). To gain insight into the metabolic determinants of yield, we conducted both a multivariate OPLS analysis using yield as a predicted quantitative Y variable, and a univariate analysis by linear regression. The OPLS analysis generated a good statistical model ($R^2=0.75$) with good robustness ($Q^2=0.67$) and very high significance ($P_{CV-ANOVA}=10^{-16}$).

When observed and predicted yield were plotted together, it was clear that the relationship was driven partly by differences between countries (Fig. 4B) and not at all by the CO2 treatment (Fig. 4C). Accordingly, there were no significant CO2 effects, except in the USA (Fig. 4A). The best drivers of yield were investigated using volcano plots. Two volcano plots are shown here: the first (Fig. 4D) is associated with an analysis that disregards ‘conditions’ (countries and species/cultivars), while the second (Fig. 4E) incorporates ‘conditions’ as a variable. In Fig. 4D, P-values are associated with a variable-by-variable linear regression or with a multiple linear regression including variable elimination (where variables that are not best correlated with yield are discarded) (Supplementary Fig. S4). Regardless of country and cultivar, the yield appeared to be significantly and positively related to micro- and macroelements (Fe, Al, K, Mg, Cr, and N) and negatively related to starch, threonine, Rb, and %C. After variable elimination upon multiple linear regression, only six features were significantly related to yield: Mg, glutamine, and asparagine (positively related), and Rb, valine, and %C (negatively related). Among these features, some appeared to be significant because of the confounding factor of country of origin (Fig. 4B, C). When this effect was removed by incorporating ‘conditions’ as a variable, the two most significant drivers were glutamine (increased) and valine (decreased) (Fig. 4E). Tyrosine and isoleucine were found to be significant ($P<0.05$) but their loading value was close to zero, showing that their impact (in multivariate analysis) was numerically very small. When plotted separately, there was a positive relationship between yield and the glutamine-to-valine ratio in grains (Fig. 4F).
Discussion

Here, we used five FACE sites in different countries, with different wheat cultivars/species, to investigate the metabolic effects of elevated CO₂ in leaves and grains. Our results show that elevated CO₂ (i) had a limited effect on photosynthesis rate and grain yield, reflecting photosynthetic acclimation; (ii) caused a decline in proteins involved in photosynthesis, photorespiration, or N assimilation in leaves; and (iii) altered grain quality, with lower contents of amino acids and mineral nutrients. In addition, a quantitative analysis of yield suggested that high yield values correlate with higher glutamine, K, and Mg contents and lower valine content.

Growth at elevated CO₂ had a limited impact on crop yield

Crop yield and photosynthetic responsiveness to elevated CO₂ depend considerably on surrounding (local) environmental conditions. Here, we found that with the exception of the FACE site in the USA, grain yield was not significantly affected by elevated CO₂. In practice, changes in wheat yield in response to high CO₂ are determined by the cultivar(s), fertilization protocols, and interactions with other environmental factors such as water availability and temperature. The absence of an effect of CO₂ was probably linked to the lack of significant changes in assimilation (mechanisms are further discussed below). Accordingly, several previous studies have shown that the initial stimulation by CO₂ can be compensated for by acclimation (reviewed in Ainsworth and Rogers, 2007). The meta-analyses carried out by Galmés et al. (2013) showed that under elevated CO₂ conditions, Rubisco content is the primary driver in the regulation of Rubisco activity and, consequently, photosynthetic activity. Within this context, the absence of an effect of elevated CO₂ on crop yield could be linked to limitations in N assimilation and altered leaf C sink/source balance (Ainsworth and Rogers, 2007). Photosynthetic performance is believed to be affected by two key factors: the CO₂ concentration in the chloroplast (c) and the carboxylation capacity, which is linked to leaf N content. Here, the absence of significant differences in stomatal conductance (accompanied by the lack of an effect on leaf ABA content) suggests that stomatal limitation of CO₂ diffusion was a minor component of acclimation under our conditions. The lower content of nitrate reductase (and other proteins) suggests an inhibitory effect of elevated CO₂ on N metabolism, as previously observed in wheat leaves (Jauregui et al., 2015). The inhibition of nitrate assimilation under elevated CO₂ in wheat has been suggested to reflect lower electron allocation to nitrite reductase in the chloroplast and/or the inhibition of the GS/GOGAT cycle driven by photorespiration (Bloom et al., 2014). Here, the lower contents of ferredoxin, chlorophyll binding proteins, and glycine decarboxylase under elevated CO₂ would be consistent with a down-regulation of N assimilation.

Fig. 4. Yield analysis. (A) Yield (in g grains m⁻²) of the different cultivars and countries. Asterisks indicate significant differences (P < 0.05) between control (grey) and elevated (blue) CO₂. (B, C) Relationship between observed yield and yield predicted using the OPLS model (comprising country+cultivar as a qualitative X variable), differentiating countries (B) or CO₂ levels (C) (R² = 0.75). (D) Volcano plot [-log(P-value) versus OPLS loading pcorr] showing the importance of variables for statistical analysis: P-values from separate linear regressions (each variable taken separately) (white circles), or P-values from variable elimination and linear model (black circles) against OPLS loadings (both univariate and OPLS models without country+cultivar as a qualitative X variable). (E) P-values from variable elimination and linear model against OPLS loadings (both univariate and OPLS models with country+cultivar as a qualitative X variable). In D and E, the horizontal dashed line indicates the P-value threshold of 0.05, and arrowheads indicate valine (Val) and glutamine (Gln). (F) Relationship observed yield and the glutamine-to-valine ratio (log scales). The solid line represents the linear regression (R² = 0.37). (This figure is available in colour at JXB online.)
Besides the effect of CO2 (or lack thereof), our study shows important differences in yield between locations and cultivars. The lowest values were found in the USA, where sowing density (120 m$^{-2}$), precipitation, and cumulated day·°C values were low (Table 1), and seedling mortality was pronounced in the year of the FACE experiment due to bad weather. The high yield values observed in China were probably explained by higher water availability. We note that the FACE site in the USA, where elevated CO2 had a significant effect on yield, had the highest N fertilization rate (250 kg ha$^{-1}$) despite low plant sowing density and low cumulated days·°C; this suggests that perhaps the two most important factors for CO2 responsiveness were available N and intercepted light (minimal shading) under our conditions. Differences between locations could also have originated from the contrasting behaviour of the two species of wheat studied, with bread wheat exhibiting generally higher yield values than durum wheat. This species effect comes from the fact that durum wheat is more water conservative and thus more suitable to grow in stressful environments, while bread wheat is believed to have a higher yield potential (Slafer et al., 1996; Marti and Slafer, 2014).

**Photosynthetic effects of elevated CO2**

Growth at elevated CO2 generally leads to a down-regulation of some photosynthetic parameters, such as carboxylation efficiency, maximal carboxylation velocity, and CO2 conductance (see Introduction). Here, photosynthesis increased by a modest but significant amount at elevated CO2 (in the USA, China, and Australia) or did not increase at all (in Italy and Germany). In Italy, the lack of a stimulating effect of CO2 mole fraction was associated with a decrease in stomatal conductance (Fig. 1B). It is also possible that internal conductance decreased under elevated CO2, and this could contribute to explaining why the $^{12}$C/$^{13}$C isotope fractionation did not increase much (with apparent $\delta^{13}$C staying at ~0.7 despite the increase in CO2 mole fraction) (Fig. 1E). In addition, there was probably a decrease in carboxylation capacity, as suggested by the significantly lower Rubisco content in leaves (Fig. 2), and $A/\delta^{13}$C response curves for wheat grown in Germany suggest a lower $V_{c,max}$ (non-significant) and $J_{max}$ ($P<0.05$) (Supplementary Fig. S5). More generally, elevated CO2 led to a down-regulation of the photosynthetic and photorespiratory machinery, with less carbonic anhydrase, glycine decarboxylase, and superoxide dismutase, an enzyme of redox metabolism (as reported previously in Aranjuelo et al., 2015). As a result, the lower content of major enzymes such as Rubisco was accompanied by a decline in %N in some countries (the USA, China, and Australia) but not others. The origin of this effect of elevated CO2 could be lower biosynthesis of proteins or an increase in protein degradation, for example, earlier remobilization of proteins to facilitate export of N from leaves to developing grains (Sicher and Bunce, 1998; Zhu et al., 2009). Earlier remobilization seems unlikely, since leaves were sampled at stage Z31 (stem elongation with first node above tillering node) or Z65 (anthesis, 50% of anthers mature), that is, before the onset of remobilization. Therefore, it is probable that elevated CO2 down-regulated N metabolism, causing a general decrease in the biosynthesis of

| Table 1. Growth conditions associated with the CO2 enrichment experiments in this study |
|-----------------------------------|----------------------------------|-------------------|----------------|----------------|-----------------|----------------|----------------|
| Site | General information | Climatic conditions during growth | Fertilization | Cumulated d·°C | Nitrogen (kg N ha$^{-1}$) | Element P2O5-K2O | Cumulated time window | Species and cultivars |
|------|---------------------|-----------------------------|----------------|----------------|----------------|-----------------|-----------------|----------------|
| USA | Beltsville | 16/10/15–7/6/16 | T. durum | 10-10-10 | 10-10-10 | 4.2 | 25.9 | 981 |
| Italy | Fiorenzuola | 9/11/15-11/7/16 | T. aestivum | 15-15-15 | 15-15-15 | 10.3 | 27.8 | 1774 |
| Germany | Hohenheim | 5/4/16-20/6/16 | T. durum | 10-5-5 | 10-5-5 | 2.6 | 24.8 | 1679 |
| China | Beijing | 6/10/15-20/6/16 | T. durum | 18-18-18 | 18-18-18 | 2.6 | 24.8 | 1679 |
| Australia | Horsham | 1/06/16-21/12/16 | T. aestivum | 10-1-1 | 10-1-1 | 9.0 | 23.1 | 2394 |

Climatic conditions are further documented in Supplementary Fig. S1. Cumulated d·°C, cumulated temperature during cultivation period (in days·°C); Ptot, total precipitation; $T_{av,min}$, average minimum temperature; $T_{av,max}$, average maximum temperature; $T_{av}$, average daily temperature; $P_{tot}$, total precipitation; $T_{av,min}$, average minimum temperature.
major proteins and thus potentially photosynthesis. Despite the
change in Rubisco content, the net effect on photosynthesis
also depends on other enzyme activities, because net assimila-
tion is not highly sensitive to Rubisco content. For example,
other enzymes, such as Rubisco activase, are crucial for carbo-
oxylase efficiency. We have previously shown using proteo-
omics that elevated CO₂ leads to a decline in Rubisco activase
content (Aranjuelo et al., 2015). Here, our analysis focused on
a number of specific proteins, which did not include Rubisco
activase. Photosynthetic products (sugars) were affected by
CO₂ mole fraction, with significantly less sucrose and gener-
ally more fructose ($P=0.06$); as a result, the fructose-to-sucrose
ratio was larger at elevated CO₂ in two countries (Germany
and Italy) (Fig. 2B). In other words, elevated CO₂ was asso-
ciated with a reconfiguration of sugar metabolism, likely
including a larger allocation of carbon to starch and a futile
sucrose synthesis–degradation cycle.

**Effect of CO₂ on grain composition**

We found that elevated CO₂ had a clear effect on grain com-
position, leading to lower contents of N, amino acids, and
microelements (Fig. 3). The general effect of elevated CO₂ on
N content (and proteins) in grains has been documented be-
fore, and has been suggested to be linked to perturbations in
both leaf-to-grain N transport (e.g. glutamine) and amino acid
metabolism in grains (e.g. in the lysine degradation pathway;
see the Introduction and Soba et al., 2019). Here, it is remark-
able that Mg was found to be more abundant in leaves but less
abundant in grains, suggesting that elevated CO₂ compromised
microelement remobilization (the same occurred with Ca but
this was not significant in grains). Nutrient quantitation during
grain filling in different cultivars of spring wheat has suggested
that Mg is remobilized from the lower stem and leaves to grains,
with little effect of cultivar (Tiryakioğlu et al., 2014). Growth
at elevated CO₂ has been found to affect Mg content in wheat
(Sanchez de la Puente et al., 2000; Högy et al., 2013; Aranjuelo
et al., 2015; Beleggia et al., 2018). Mg remobilization involves
chlorophylls and Rubisco degradation in leaves, and Mg cir-
culation via the phloem. This raises the question of whether
phloem transport was affected by elevated CO₂ in the current
study. On the one hand, glutamine and glutamate were found
to be more abundant in grains at elevated CO₂ (Fig. 3), sug-
gestting that phloem N export from leaves was not affected.
On the other hand, leaves contained significantly less K, which
is the cornerstone of phloem ion movement. Therefore, our data
do not provide a firm answer to this question. Nevertheless,
we recognize that the higher glutamine (glutamate) content
in grains could also have originated from a lower efficiency
of glutamine utilization and thus a lower incorporation of N
to anabolism. Grains were specifically depleted in GABA, me-
thonine, tyrosine, alanine, and threonine. Interestingly, these
amino acids belong to distinct pathways: aspartate metabolism
(methionine and threonine), glutamate degradation/GABA shunt
(GABA and alanine), and aromatics (tyrosine). The common branching point is pyruvate, since aspartate can come
from anaplerotic phosphoenolpyruvate (PEP) carboxylase fix-
ation (which forms oxaloacetate), alanine comes from pyruvate,
and the biosynthesis of aromatics requires PEP. Therefore, it
is possible that the decrease in amino acids was caused by a
specific effect of elevated CO₂ on (phosphoenol)pyruvate syn-
thesis via glycolysis. For example, pyruvate Pi dikinase, which
resynthesizes PEP from pyruvate, has been shown to be es-
tential for starch accumulation at the late grain-filling stage
(Prioul et al., 2012).

Importantly, these metabolic effects depended on ‘condi-
tions’, that is, country and species/cultivar (Supplementary Figs
S2, S3). It was not possible here to separate the specific con-
tributions of country and species/cultivar, since not all plant
lines were cultivated in each country. There was nevertheless
a strong effect of location, with the effect of elevated CO₂ on
some micro- and macro-elements being country specific. For
example, the effect of elevated CO₂ on grain Pb content was
largest in Australia, where Pb was the least abundant at the
field location (Supplementary Fig. S1). Similarly, there was a
very limited effect of CO₂ on grain N content (with generally
low values) in Australia (Supplementary Fig. S3), where N fer-
tilization was the lowest (Table 1), while the effect on leaf N
content was strong (Supplementary Fig. S2). There was also a
strong effect of CO₂ on grain free amino acid content in both
Australia and the USA, despite the fact that the sites in the two
countries used different fertilization levels and wheat species.
Therefore, our results show the importance of growth condi-
tions (i.e. location) to delineate the effects of elevated CO₂;
in particular, soil composition (and not only N fertiliza-
tion) seems to be important. Yet, the decrease in threonine and Mg
content in grains was independent of ‘conditions’, that is, it
was not associated with a CO₂ × conditions interaction effect.

**Potential metabolic markers of yield**

We used the dataset to examine possible relationships be-
tween yield and grain metabolic properties, using multi-
ivariate and univariate statistics (Fig. 4). Because the country
of origin (and associated conditions such as climate, fertil-
ization, and sowing density) and species/cultivar (i.e. ‘condi-
tions’) represented a confounding factor, we carried out two
types of analyses: (i) independent of and (ii) accounting for
‘conditions’. In doing so, we assumed that the variable ‘condi-
tions’ can be a driver of yield regardless of other variables (e.g.
contents of metabolites or micro-/macro-elements). Growth
at elevated CO₂ was not a confounding factor, since there
was no significant effect of CO₂ on yield overall (Fig. 4A,
C). Only two significant and strong drivers of yield remained
after the elimination of the ‘conditions’ effect: glutamine and
valine (Fig. 4E, F).

This result highlights the well-known role of glutamine
in grain protein synthesis: glutamate is a major amino acid
in wheat stem phloem (Hayashi and Chino, 1986) used as a
primary source of N for conversion to glutamine and, fur-
thermore, glutamine is the most represented residue in accu-
mulated proteins. It has been shown that glutamine accounts
for ~40% of free amino acids not only in phloem sap from the
spikelet peduncle but also in endosperm cavity sap, showing its
crucial role as a N source during grain development (Fisher
and Macnicol, 1986). In addition, glutamine has the highest
supply rate from the vascular bundle to the developing endosperm, at ~0.5 µmol grain⁻¹ d⁻¹ (Ugalde and Jenner, 1990). Valine was negatively related to yield (Fig. 4E), suggesting that increased valine degradation rather than valine synthesis is beneficial to grain production. Proteomics analyses have shown that acetolactate synthase, which catalyses the first step of valine and leucine biosynthesis, is most abundant at the beginning of grain development (pre-filling stage) and then declines up to maturity (Tasleem-Tahir et al., 2012), while methylmalonate semialdehyde dehydrogenase, which is involved in branched-chain amino acid degradation, appears at the last stage of grain filling (Vensel et al., 2005) and its content increases with ABA (Zhang et al., 2012). Accordingly, metabolic profiling has shown that branched-chain amino acids (valine, leucine and isoleucine) tend to increase during grain filling and then sharply decline (Shewry et al., 2012). Valine metabolism can have several roles. First, valine degradation generates acetyl-CoA (via methylmalonate semialdehyde), which can in turn be used for respiration or lipid synthesis. Second, valine can be converted to leucine via 2-oxoisovalerate. Labelling with 14C-valine in developing wheat spikes has shown up to 30% conversion to leucine (Kolderup, 1978), and during grain development, the valine content correlates with that of leucine (Martín Del Molino et al., 1988). Interestingly, a quantitative trait locus analysis has shown that 2-methylmalate, an intermediate of leucine synthesis, correlates positively with the number of tillers and thermal time to heading, and negatively with yield (Hill et al., 2015).

Conclusions

Taken as a whole, our results show that growth at elevated CO₂ in FACE experiments had different effects on leaf and grain metabolism, but no significant effect on yield. Elevated CO₂ appeared to be detrimental to photosynthesis and leaf proteins (e.g. Rubisco large subunit) and to alter grain composition, in particular %N, amino acids (e.g. threonine and alanine), and minerals (Mg). The yield was related to metabolic features of grains but this relationship was not influenced by elevated CO₂.

The lack of a positive effect of CO₂ fertilization can be explained not only by the down-regulation of carbon fixation (i.e. of several photosynthetic parameters) but also by an effect on grain metabolism itself. In particular, elevated CO₂ appeared to be detrimental to N metabolism, with a decrease in several amino acids and, accordingly, a positive effect of elevated CO₂ on yield was observed only in the USA, where N fertilization was the highest.

Elevated CO₂ also had an effect on Mg redistribution between source leaves and grains. The detrimental effect of CO₂ on the content of microelements has been found elsewhere for Zn (Myers et al., 2014). A meta-analysis has recently highlighted the decrease in the content of many elements (including macroelements such as S) in crops cultivated at elevated CO₂ (Loladze, 2014). Interestingly, recent experiments in which Mg availability was varied have shown that low Mg causes a significant decline in grain starch content and yield (Ceylan et al., 2016). Here, despite significantly lower Mg content in grains at elevated CO₂, starch content and yield were not significantly affected, suggesting that the decrease in Mg associated with elevated CO₂ was too small (<10%) to affect yield. In addition, the relationship between Mg content in grains and yield is not driven by genotype (Oury et al., 2006). We nevertheless recognize that further work is required to determine the reason for the decreased Mg content in grains found here. For example, isotopic labelling with 25Mg (or 26Mg) would be helpful to examine the role played by phloem Mg transport during remobilization from leaves at elevated CO₂.

The overall effect on N, K, and Mg nutrition found here raises the question of whether specific fertilization management strategies could compensate for the lack of effect of elevated CO₂ observed at most of the FACE sites. Previous studies compared ammonium-based and nitrate-based fertilizers and found that with nitrate, plants tended to show higher photosynthetic acclimation (Bowler and Press, 1996; Geiger et al., 1999; Cruz et al., 2014), which in turn limits the response of production or yield to elevated CO₂. In addition, high nitrate availability tends to exaggerate photosynthetic acclimation, due to down-regulation of the expression of photosynthetic genes (Vicente et al., 2017). Therefore, in an effort to find optimal fertilization strategies adapted to local field conditions, both the quantity and the quality of N fertilizer seems to be important. The fact that nutrients other than N are also affected by elevated CO₂ further indicates that the nutrient balance itself is also of importance. A specific multifactorial experiment (nutrient compositions × CO₂) would be necessary to determine the best solutions to improve yield responsiveness to CO₂. In addition, the selection of varieties with a high harvest index, which is associated with optimal allocation and efficient nutrient remobilization capacity, might be desirable to obtain better grain yield and quality at elevated CO₂.

Finally, we found that wheat grain yield was related to glutamine and valine grain content, regardless of country and cultivar, showing the importance of amino acid metabolism for grain maturation. However, while the role of glutamine is clear (as a N source and utilization for storage proteins, which are glutamine rich), understanding the metabolic role(s) of valine requires further work, such as tracing with 13C-valine and isotope-assisted metabolomics. This will be addressed in a future study.

Supplementary data

The following supplementary data are available at JXB online.

Fig. S1. Elemental composition of soil (top layer) at the different sites used in this study.

Fig. S2. Leaf features significant for a CO₂ × conditions (country, cultivar, and time) effect.

Fig. S3. Grain features significant for a CO₂ × conditions (country, cultivar, and time) effect.

Fig. S4. Correlation plot showing the best variables obtained by subset sampling in linear models of yield.
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