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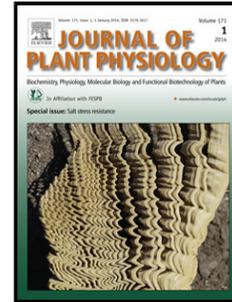
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**The proportion of nitrate in leaf nitrogen, but not changes in root growth, are associated with decreased grain protein in wheat under elevated [CO<sub>2</sub>]**

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## ABSTRACT

The atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]) is increasing and predicted to reach ~550 ppm by 2050. Increasing [CO<sub>2</sub>] typically stimulates crop growth and yield, but decreases concentrations of nutrients, such as nitrogen ([N]), and therefore protein, in plant tissues and grains. Such changes in grain composition are expected to have negative implications for the nutritional and economic value of grains. This study addresses two mechanisms potentially accountable for the phenomenon of elevated [CO<sub>2</sub>]-induced decreases in [N]: N uptake per unit length of roots as well as inhibition of the assimilation of nitrate (NO<sub>3</sub><sup>-</sup>) into protein are investigated and related to grain protein. We analysed two wheat cultivars from a similar genetic background but contrasting in agronomic features (*Triticum aestivum* L. cv. Scout and Yitpi). Plants were field-grown within the Australian Grains Free Air CO<sub>2</sub> Enrichment (AGFACE) facility under two atmospheric [CO<sub>2</sub>] (ambient, ~400 ppm, and elevated, ~550 ppm) and two water treatments (rain-fed and well-watered). Aboveground dry weight (ADW) and root length (RL, captured by a mini-rhizotron root growth monitoring system), as well as [N] and NO<sub>3</sub><sup>-</sup> concentrations ([NO<sub>3</sub><sup>-</sup>]) were monitored throughout the growing season and related to grain protein at harvest. RL generally increased under e[CO<sub>2</sub>] and varied between water supply and cultivars. The ratio of total aboveground N (TN) taken up per RL was affected by CO<sub>2</sub> treatment only later in the season and there was no significant correlation between TN/RL and grain protein concentration across

cultivars and [CO<sub>2</sub>] treatments. In contrast, a greater percentage of N remained as unassimilated [NO<sub>3</sub><sup>-</sup>] in the tissue of e[CO<sub>2</sub>] grown crops (expressed as the ratio of NO<sub>3</sub><sup>-</sup> to total N) and this was significantly correlated with decreased grain protein. These findings suggest that e[CO<sub>2</sub>] directly affects the nitrate assimilation capacity of wheat with direct negative implications for grain quality.

**Keywords:** Free Air CO<sub>2</sub> Enrichment (FACE); Nitrogen; Nitrogen Uptake; Nitrogen Utilisation Efficiency; Root uptake; *Triticum aestivum* L.

## Introduction

Since the industrial revolution, atmospheric [CO<sub>2</sub>] has progressively increased to a current concentration of ~400 ppm, and is predicted to reach ~550 ppm by 2050 (Hartmann, 2013). This >35% increase in [CO<sub>2</sub>] will significantly affect all plant systems (Ainsworth and Long, 2005; Kimball, 1983; Poorter and Navas, 2003) but for wheat, more specifically, it was reported that aboveground biomass production and yield will typically increase by 17 to 20% while concentrations of nutrients such as N will decrease by 9 to 15% in plant tissues and grains (Ainsworth and Long, 2005; Jablonski et al., 2002; Taub et al., 2008; Ziska et al., 2004). A large part of N in plant tissues is present in protein, so that protein concentrations are often directly related to total N concentrations, a relationship that is particularly strong in cereal grains (Mosse, 1990). A drop in grain protein may have significant consequences for the public health especially of poorer countries where C3 grains contribute large proportions of protein intake (Myers et al., 2014).

Several hypotheses were proposed to explain decreases in [N] under increasing [CO<sub>2</sub>]: The most straightforward hypothesis proposes that increased biomass growth is not matched by a similar increase in N uptake (Taub and Wang, 2008). This effect is termed “growth dilution” and results in increased yield with concomitantly occurring decreased concentrations of N and protein (Pleijel and Uddling, 2012). Limitations to N uptake, and therefore a failure of keeping pace with N supply to match growth enhancement, can be caused either by a progressive limitation of the amount of N available to plant roots or by a changed nutrient uptake capacity of roots (Pleijel and Uddling, 2012). While root biomass is often increased under e[CO<sub>2</sub>] with roots becoming more numerous, longer, thicker and faster growing (Madhu and Hatfield, 2013; VanVuuren et al., 1997), root specific activity can be down-regulated, possibly related to less efficient root architectures such as a marked shift towards surface rooting (Fitter et al., 1996).

Another potential explanation for a failure of N uptake under e[CO<sub>2</sub>] can be the effect of CO<sub>2</sub> enrichment on N metabolism in the shoot (Pleijel and Uddling, 2012). For example, Bloom et al. (2014, 2012) proposed that e[CO<sub>2</sub>] directly inhibits the N metabolism of plants, particularly the assimilation of NO<sub>3</sub><sup>-</sup> into proteins. In C3 plants, photorespiration supplies much of the energy required for NO<sub>3</sub><sup>-</sup> assimilation but because photorespiration is generally inhibited under e[CO<sub>2</sub>], NO<sub>3</sub><sup>-</sup> assimilation is reduced. In a recent field study, wheat leaves grown under elevated [CO<sub>2</sub>] had a significantly greater percentage of N remaining as unassimilated NO<sub>3</sub><sup>-</sup> in the leaf providing the first direct support for this hypothesis on field grown crops (Bloom et al.,

2014). However, how  $\text{NO}_3^-$  accumulation might correlate with decreases in grain N was not addressed in that study.

N uptake and assimilation of  $\text{NO}_3^-$  into amino acids and then protein is greatest during the vegetative growth stages but diminishes during the reproductive phase. Grain [N] is therefore largely dependent on N remobilised from senescing vegetative plant parts, the remobilisation process recycling previously taken up and assimilated N, to fill the seeds (Yang and Zhang, 2006). Especially in water limited environments, where wheat matures under terminal drought, the proportion of grain N derived from remobilisation processes can be as high as 95% (Palta and Fillery, 1995).

In the current study, two mechanisms potentially accountable for the phenomenon of  $e[\text{CO}_2]$ -induced decreases in grain [N] are addressed: N uptake per unit length of roots as well as inhibition of the assimilation of  $\text{NO}_3^-$  into protein are investigated and related to grain protein. Two wheat (*Triticum aestivum* L.) cultivars from a similar genetic background, cv. Yitpi and cv. Scout, were tested. In contrast to cv. Yitpi, cv. Scout is characterised as a cultivar with very good early vigour. Vigorous wheat cultivars have been shown to have greater root biomass accumulation as well as greater early N uptake (Liao et al., 2004).

The current study was conducted within the Australian Grains Free Air  $\text{CO}_2$  Enrichment (AGFACE) facility, a low-rainfall, semi-arid cropping system in the south-eastern Australian grains belt, a good representative for similar agro-ecosystems that account for about 15% of the global wheat growing area (Fischer et al., 2014). Crops in AGFACE grow either under a  $\text{CO}_2$  concentration predicted for 2050 (~550 ppm,  $e[\text{CO}_2]$ ) or ambient  $[\text{CO}_2]$  (~400 ppm,  $[\text{aCO}_2]$ ). Growth (aboveground biomass accumulation and root length) as well as N dynamics (N and nitrate) were monitored continuously throughout the season. We tested the following hypotheses: (i) Root growth is stimulated by elevated  $\text{CO}_2$ , and is greater in the vigorous cv. Scout compared to cv. Yitpi. Differences in root growth between  $\text{CO}_2$  treatments and cultivars are associated with differences in plant N and grain protein status. (ii) Wheat leaves grown under  $e[\text{CO}_2]$  have a significantly greater percentage of N remaining as unassimilated  $\text{NO}_3^-$  in the leaf and this is associated with differences in grain protein status across  $\text{CO}_2$ - treatments and cultivars.

## Materials and Methods

### Plant material

*Triticum aestivum* L. cv. 'Scout' and cv. 'Yitpi' were chosen for their similar genetic background but otherwise contrasting agronomic features. Cultivar 'Scout' has the pedigree Sunstate/QH71-6/Yitpi and was developed by the LongReach Plant Breeders technical team. Scout was characterised as a cultivar with very good early vigour (Pacificseeds, 2009; Seednet, 2005).

### Experimental site description

The Australian Grains Free Air  $\text{CO}_2$  Enrichment (AGFACE) facility is located on a 7.5 ha site 7 km west of Horsham, Victoria, Australia (36°45'07"S, 142°06'52"E, 127 m above sea level). The major soil type is a Vertosol (Isbell, 2002), which has

approximately 52% clay (from 37 to 66% to a depth of 1.8 m) and 21% sand across all depths. The region has a semi-arid environment (average annual rainfall 445 mm) with dry warm summers, but cool wet winters. More details are given in Fitzgerald et al. (2016) and Mollah et al. (2009). A summary of the climatic conditions during the growing season of this study is given in Table 1.

The experimental design was a split-split-plot within a randomized complete block design with four replications for the whole plots ([CO<sub>2</sub>] treatment). Four Free Air CO<sub>2</sub> Enrichment (FACE) plots (12 m in diameter) with e[CO<sub>2</sub>] (~550 ppm) and four control plots (also 12 m in diameter) with ambient [CO<sub>2</sub>] (a[CO<sub>2</sub>] ~400 ppm) were each split for water treatment and cultivar as follows: In each [CO<sub>2</sub>] plot, each cultivar, cv. Scout and cv Yitpi, was grown in two sub-plots, one under rain-fed conditions and one under well-watered (rain-fed plus additional water supply) conditions. Each sub-plot had a length of 4 m, was 1.4 m wide and consisted of 6 rows at 0.244 m spacing. Wheat was sown on 29 May 2014. At sowing, granular fertiliser (8.8% P, 11% S) was incorporated into the soil at rates of 7–9 kg P ha<sup>-1</sup> and 8–11 kg S ha<sup>-1</sup>. According to local practice no N fertiliser was applied as the initial soil nitrate levels were high (approximately 250 kg NO<sub>3</sub><sup>-</sup> ha<sup>-1</sup>). Following sowing, holes were drilled into the soil at an angle of 45° and to a depth of 75 cm with a vehicle-mounted hydraulic core-sampler. Holes were oriented parallel to rows between rows three and four in each of the four sub-plots per [CO<sub>2</sub>] plot. Acrylic clear mini-rhizotron tubes (1056 cm in length by 7 cm in internal diameter) were inserted into the drilled holes. CO<sub>2</sub> injection started on 4 June 2014, before emergence and was continued until crops reached physiological maturity. Harvest occurred on 20 Nov 2014 for the rain-fed sub-plots and 2 Dec 2014 for the well-watered sub-plots. The CO<sub>2</sub> release tubes of the FACE system were adjusted to the increasing canopy height and positioned approximately 150 mm above the canopy at any developmental stage. Engineering description for the CO<sub>2</sub> injection system and its performance are given in Mollah et al. (2009).

### **Water treatment**

In order to achieve uniform and high germination rates, 34 mm water was added to both, rain-fed and well-watered sub-plots, before sowing. It was originally planned that rain-fed sub-plots would receive only rainfall throughout the growing season but because in 2014 the total amount of growing season rainfall (GSR) (108 mm) was well below the long-term average (274 mm), additional irrigation had to be applied repeatedly during the season to keep crops alive. Therefore, rain-fed sub-plots received a total of 119 mm of additional water in four applications (18, 41, 20 and 40 mm on 4 Sep, 24 Sep, 16 Oct and 23 Oct, respectively). In contrast, well-watered sub-plots received an additional 300 mm of water in twenty applications from 21 July to 7 November 2014 at approximately weekly intervals (Table 1).

### **Aboveground dry weight and grain yield**

Aboveground dry weight (ADW) measurements were taken at (1) 54 days after sowing (DAS) corresponding to start of tillering (Decimal Code (DC) 21 according to (Zadoks et al., 1974)), (2) 96 DAS; flag leaf just visible (DC 37), (3) 124 DAS; mid-booting (DC 45), (4) 138 DAS; mid-anthesis with half of the spikes with anthers (DC 65), (5) 152 DAS; mid milk-development (DC 75), and (6) 180 DAS; physiological maturity (DC 90). At each sampling date, three plants were hand-harvested from each of the four sub-

plots (two cultivars, two water treatments) within each [CO<sub>2</sub>] plot. Samples were dried for 72 h at 70°C before dry mass (g plant<sup>-1</sup>) was determined. At DC 90, grain yield (g plant<sup>-1</sup>) was determined as well.

### Root length

Root growth dynamics were measured with a mini-rhizotron root growth monitoring system (CI-600™, CID Bio-Science Inc., Camas, WA, USA) (Taylor et al., 1990). This system consists of a cylindrical scanner and acrylic clear tubes allowing for non-destructive and repeated monitoring of root growth of the same sample (Dannoura et al., 2008; Kirkham et al., 1998). Images of the root system were acquired by inserting the scanner inside the tubes. The scanner then rotated clockwise, so that the top of the tube was pictured in the centre of the image analysed with the CI-600 Digital Root Scanner software (CID Bio-Science Inc., Camas, WA, USA). Each image was 21.6 cm by 19.6 cm. The tubes allowed for four depths, ~0-16, 17-32, 33-48, and 48-64 cm. Root growth was recorded at nine times during the season: 54 DAS corresponding to start of tillering (DC 21), 68 DAS; first node visible (DC 31), 83 DAS; stem elongation (DC 34), 96 DAS; flag leaf (FL) just visible (DC 37), 109 DAS; FL developing (DC 39), 124 DAS; mid-booting (DC 45), 138 DAS; mid-anthesis with half of the spikes with anthers (DC 65), 152 DAS; mid milk-development (DC 75) and 166 DAS; dough development (DC 85). The images were analysed for captured root length per image using the software RootSnap!™ version 1.2.8.23 (CID Bio-Science Inc., Camas, WA, USA). Root length values are reported as the total root length (RL) per rhizotron access tube (sum of the four images per tube).

### N and nitrate

N and nitrate were analysed on plant material hand-harvested for ADW and grain yield determination outlined above. The plant material used for N and nitrate analyses consisted earlier in the season of green leaves only and with progressing development the proportion of senescing leaves steadily increased. All analyses were made on oven dried material (70°C) ground to a fine powder with a Retsch ball mill (Haan, Germany). Biomass and grain N were analysed by combustion with an elemental analyser (CNS, LECO TruMac MI, USA). NO<sub>3</sub><sup>-</sup> present in leaves was analysed by HPLC according to Maas et al. (1986). The HPLC system consisted of a Knauer HPLC pump model 100 and a Knauer differential refractometer model 98.00 (Knauer, Berlin, Germany) with an Agilent IonoSpher 5A anion exchange column (250 x 4.6 mm; Agilent Technologies, Amstelveen, The Netherlands). The mobile phase contained 25 mM potassium biphthalate (pH 4.3). About 50 mg oven-dried leaf material (weighed to .01 mg) was extracted with 5 mL of deionised water in a shaking water bath at 40°C for 2-3 hours. Extracts were filtered and centrifuged twice (30,000 and 40,000 xg) before injection.

### NUp, NUtE and grain protein

Nitrogen Uptake (NUp) was defined as the total N taken up by the plant (g N plant<sup>-1</sup>) at maturity. Nitrogen Utilisation Efficiency (NUtE) was defined as the grain yield (g plant<sup>-1</sup>) divided by the total amount of N (g plant<sup>-1</sup>) taken up at maturity (Hawkesford, 2014). Grain protein (%) was calculated as following:

Protein (%) = Grain N (%) x 5.7 (Mosse, 1990)

As a surrogate for N uptake capacity per unit of root length, we also calculated the ratio of the total aboveground N content divided by total captured RL at 54 DAS; start of tillering (DC 21), (2) 96 DAS; flag leaf just visible (DC 37), (3) 124 DAS; mid-booting (DC 45), (4) 138 DAS; mid-anthesis (DC 65), (5) 152 DAS; mid milk-development (DC 75), and (6) 180 DAS; physiological maturity (DC 90).

## Statistical analyses

Statistical analyses were performed in R (R Core Team 2015). The aov function was used with an error term defining the split-split-plot design with repeated measurements, with whole plots (rings, CO<sub>2</sub> as the whole plot factor) split for irrigation, and each irrigation sub-plot split again for cultivars. Homogeneity of variances was tested using Levene's tests (function LeveneTest from R package DescTools, (Signorell, 2016)), and where variances were significantly different, data were ln-transformed before running ANOVAs. Residual distribution of the fitted models was checked visually using the plot(lm) function. The correlations between total captured RL and nitrate concentration in leaves vs grain protein were analysed at 152 DAS corresponding for milk-development stage (DC 75). All graphs were created with the Minitab software 17<sup>th</sup> Edition. Statistical effects were regarded significant at p≤0.05. Marginally significant p values (p≤0.1) were considered for discussion purpose.

## Results

### Aboveground dry weight and root length

Plants grown under e[CO<sub>2</sub>] had on average 17% greater aboveground dry weight (ADW) than under a[CO<sub>2</sub>] with cv. Yitpi showing a trend towards greater response to increased [CO<sub>2</sub>] (26%) than cv. Scout (8%, CO<sub>2</sub> x cultivar interaction, Fig. 1A). Well-watered plants had 30% greater ADW compared to rain-fed ones and increases in ADW under well-watered conditions were particularly evident later in the season (significant DAS x irrigation interaction, Fig. 1A).

Captured total root length (RL) increased from early tillering (54 DAS) to flag leaf emergence (109 DAS) in both wheat cultivars assessed. After peaking at 109 DAS, RL then plateaued until the last measurement at 166 DAS. The increase in RL was generally greater under well-watered conditions than under rain-fed ones (significant DAS x irrigation interaction), and under elevated than ambient [CO<sub>2</sub>] (CO<sub>2</sub> x DAS interaction, Fig. 1B). In addition, RL was 10% greater in cv. Yitpi when grown under e[CO<sub>2</sub>] and rain-fed conditions (marginal significant CO<sub>2</sub> x irrigation x cultivar x DAS interaction, Fig. 1B).

### N and nitrate

Total N content (mg N plant<sup>-1</sup>) increased in both wheat cultivars from early tillering (54 DAS) to physiological maturity (180 DAS), in a similar pattern to total ADW. This increase was more pronounced under well-watered conditions as evidenced by a significant DAS x irrigation interaction. There was a tendency towards greater N content in cv. Yitpi than cv. Scout, especially under well-watered conditions (Fig. 2A).

N concentrations [N] significantly decreased from early tillering (54 DAS) to physiological maturity (180 DAS), and this decrease was slower for irrigated than rain-fed crops (significant DAS x irrigation interaction). Shoot [N] decreased under e[CO<sub>2</sub>] but only in cv. Yitpi than cv. Scout (significant CO<sub>2</sub> x cultivar interaction, Fig. 2B). Cultivar Yitpi maintained on average greater [N] than Scout, particularly under well-watered conditions (significant cultivar x irrigation effect, Fig. 2B)

The percentage of N remaining as unassimilated NO<sub>3</sub><sup>-</sup> in the leaf (NO<sub>3</sub><sup>-</sup>/total N) significantly increased from tillering to maturity. CO<sub>2</sub> treatment also significantly affected the NO<sub>3</sub><sup>-</sup>/total N ratio with e[CO<sub>2</sub>] grown crops having on average a 16% greater NO<sub>3</sub><sup>-</sup>/total N ratio than a[CO<sub>2</sub>] grown crops (significant CO<sub>2</sub> effect). Rain-fed crops had on average 19% greater NO<sub>3</sub><sup>-</sup>/total N ratio than crops that were well-watered. In addition, cv. Scout had on average 14% greater NO<sub>3</sub><sup>-</sup>/total N ratio than cv. Yitpi, especially under e[CO<sub>2</sub>] and irrigated conditions (significant CO<sub>2</sub> x cultivar x irrigation interaction, Fig. 2C).

The ratio of total N (TN) in mg taken up per cm captured root length (RL, TN/RL) was greater under ambient than e[CO<sub>2</sub>] later in the season (significant CO<sub>2</sub> x DAS interaction, Fig. 2D). The TN/RL ratio was also affected by a marginally significant irrigation x cultivar interaction with cv. Scout showing a greater TN/RL ratio than cv. Yitpi under rain-fed but not well-watered conditions (Fig. 2D).

### **Grain yield, grain protein, N uptake and NUtE**

Grain yield per plant was greater under e[CO<sub>2</sub>] (Table 2). Irrigation treatment significantly enhanced grain yield by 128%. There was no significant difference in grain yield between the two wheat cultivars. Grain protein concentration (%) decreased by 12% in e[CO<sub>2</sub>] grown wheat compared to a[CO<sub>2</sub>]. Grain protein concentration (%) was 15% higher in rain-fed than well-watered treatments but did not differ between the two wheat cultivars (Table 2).

Nitrogen uptake at maturity (NUp) was not affected by e[CO<sub>2</sub>], but was 86% greater in well-watered crops than in rain-fed ones. There was no significant difference in NUp between the two cultivars (Table 2). NUtE was 18% higher in well-watered crops than in rain-fed crops. No significant effects of [CO<sub>2</sub>] and cultivar were observed for NUtE (Table 2).

### **Correlations with grain protein**

There was a significant negative correlation between the percentage of N remaining as unassimilated NO<sub>3</sub><sup>-</sup> in the leaf (NO<sub>3</sub><sup>-</sup>/total N) and grain protein across cultivars and CO<sub>2</sub>-treatments under both, rain-fed and well-watered treatments (Fig. 3A). There was no correlation between total N per captured RL (TN/RL) and grain protein, neither under rain-fed conditions nor under well-watered conditions (Fig. 3B).

## **Discussion**

### **Grain protein**

The 12% loss in grain protein under e[CO<sub>2</sub>] in our study is consistent with reports of other FACE studies (Ainsworth and Long, 2005; Kimball et al., 1993; Leakey et al.,

2009; Ziska et al., 2004,) and previous AGFACE results (e.g. Fernando et al., 2014). Several mechanisms have been proposed to explain protein decreases under e[CO<sub>2</sub>], most prominently limitations to N uptake and inhibition of NO<sub>3</sub><sup>-</sup> assimilation (Bloom et al., 2014; Taub et al., 2008). In this study, we investigated associations of grain protein with root growth and leaf NO<sub>3</sub><sup>-</sup> to assess the potential importance of these mechanisms for our water-limited system.

Total aboveground N per root length (as a surrogate for specific N uptake) was affected by cultivar and irrigation, also by [CO<sub>2</sub>] treatment (Fig. 2D), but despite this effect, and a significant response of root length to e[CO<sub>2</sub>] (Fig. 1B), the relationship with grain protein concentration across cultivars and [CO<sub>2</sub>] treatments was weak and not significant (Fig. 3B). From these data, we suggest that root growth stimulation or any changes in specific root uptake activity under e[CO<sub>2</sub>] were insufficient to alleviate the negative effect of e[CO<sub>2</sub>] on grain protein. We also suggest that root N uptake is not closely associated with the observed depression in grain protein under e[CO<sub>2</sub>].

In contrast, NO<sub>3</sub><sup>-</sup> as a proportion to total N in leaves was significantly greater under e[CO<sub>2</sub>] (Fig. 2C), which is an independent corroboration of previous reports (Bloom et al., 2012; Bloom et al., 2010; Bloom et al., 2002; Lekshmy et al., 2013), including one from an earlier FACE experiment on wheat (Bloom et al., 2014). Also in accordance to Bloom et al (2014), the CO<sub>2</sub> effect on total N concentration was less clear and only significant for one cultivar (Fig. 2B). These results support the findings that e[CO<sub>2</sub>] inhibits NO<sub>3</sub><sup>-</sup> photo-assimilation in C<sub>3</sub> plants (Bloom et al., 2010; Bloom et al., 2002). Under conditions of slower NO<sub>3</sub><sup>-</sup> assimilation, but sufficient NO<sub>3</sub><sup>-</sup> supply, the proportion of NO<sub>3</sub><sup>-</sup> in total N may increase in leaves, either by accumulation of NO<sub>3</sub><sup>-</sup> or by a decrease in total N, or both. In our study, the proportion of unassimilated NO<sub>3</sub><sup>-</sup> was not only greater under e[CO<sub>2</sub>] but also greater under rain-fed as compared to irrigated conditions. This is in line with reports of decreased activity of two major enzymes of NO<sub>3</sub><sup>-</sup> assimilation pathway, NO<sub>3</sub><sup>-</sup> reductase (NR) and glutamine synthetase (GS), under drought (Nagar et al., 2015).

There was a close inverse association between leaf NO<sub>3</sub><sup>-</sup> as a proportion to total N and grain protein across cultivars and CO<sub>2</sub>-treatments for each water treatment. Grain protein concentration is generally strongly correlated with organic N forms in the flag leaf such as the free amino acids (e.g. Barneix, 2007), and also associated with N supply and total leaf N concentrations (e.g. Erbs et al 2001). Under low N supply the proportion of free NO<sub>3</sub><sup>-</sup> in total N is low and free nitrate may even be below detection limits (Bloom et al. 2010, 2014) In contrast, our results indicate that it is slower NO<sub>3</sub><sup>-</sup> assimilation under e[CO<sub>2</sub>] that limits organic N in the leaves and therefore reduces the N pool available for filling grains. In addition, environmental conditions considerably influence the filling of grains and water availability, as such, plays a particularly important role (Jenner et al., 1991). Continuing favourable water supply conditions for photosynthesis during grain filling can prolong carbohydrate delivery to grains and thereby increase yield but depress grain protein, which is consistent with greater grain yield and lower grain protein concentrations in well-watered compared to rain-fed crops in our study (Table 2). Within each water treatment, the significant correlations between leaf NO<sub>3</sub><sup>-</sup>/N and grain protein indicate a co-dependence of both parameters (Fig. 3A). Using increased NR activity as a proxy for reduced NO<sub>3</sub><sup>-</sup> accumulation, Croy and Hageman (1970) reported a positive correlation between leaf NO<sub>3</sub><sup>-</sup> reductase activity and grain protein in wheat. A similar dependence was found for tritordeum

(*Hordeum chilense x Triticum turgidum*) by Barro et al. (1994). In agreement with the findings of the current study, Barro et al. (1994) also reported that grain protein accumulation appeared to be more related to nitrate reduction than to nitrate uptake. Those cited studies were conducted under  $a[\text{CO}_2]$  only. To our knowledge, the current study is the first to show such co-dependence of leaf  $\text{NO}_3^-/\text{N}$  and grain protein under  $e[\text{CO}_2]$ .

### Aboveground growth and N dynamics

Overall,  $e[\text{CO}_2]$  stimulated grain yield in both wheat cultivars and water treatments, which is consistent with results reported in other FACE (e.g. Ainsworth and Long, 2005; Kimball et al., 1995) and AGFACE studies (Fitzgerald et al., 2016; Tausz-Posch et al., 2015; Tausz-Posch et al., 2012). Aboveground dry weight (ADW) per plant was significantly increased by  $e[\text{CO}_2]$ , and this stimulation tended to be greater for cv. Yitpi than cv. Scout. Confirming strong limitations by water availability in this environment, well-watered plants yielded higher and grew significantly better than rain-fed crops, especially in later stages of crop development when rain-fed crops ran out of water and experienced terminal drought (Palta and Fillery, 1995) (Fig. 1A).

Total N content per plant followed closely the trend observed for ADW accumulation; but there was no significant effect of  $\text{CO}_2$ . Although N content is closely associated with biomass accumulation, and  $e[\text{CO}_2]$  commonly increases the N content of wheat crops in line with biomass increases (Kimball et al., 2002), the magnitude of this effect is dependent on the magnitude of the biomass stimulation itself (Lam et al., 2012), and the effect may be offset by decreases in biomass N concentration [N]. In this present data-set,  $e[\text{CO}_2]$  decreased [N] in ADW on average by 14%, albeit significant only for cv. Yitpi, which had generally a greater [N] than cv. Scout. Such a decrease is in close agreement with previous studies reporting  $e[\text{CO}_2]$ -induced decreases in tissue [N] of 9 – 15% for  $\text{C}_3$  crops (Jablonski et al., 2002, Taub et al., 2008, Ziska et al., 2004).

### Root growth and N dynamics

Elevated  $[\text{CO}_2]$  increased root length in wheat, albeit inconsistently across cultivars and growing conditions, which is consistent with trends generally observed under  $e[\text{CO}_2]$  (Madhu and Hatfield, 2013). Effects of  $e[\text{CO}_2]$  on root characteristics such as total root dry weight and/or root length may reflect indirect consequences of increased total crop growth and adaptation mechanisms to increased rate of carbon and nutrient cycling in an agro-ecosystem (Pacholski et al., 2015), and greater root growth might be necessary to sustain the increase in aboveground biomass under  $e[\text{CO}_2]$  (Chaudhuri et al., 1990).

The response of root growth to  $e[\text{CO}_2]$  can depend on cultivar (Benlloch-Gonzalez et al., 2014a) and/or environmental conditions (Benlloch-Gonzalez et al., 2014b; Pritchard et al., 2006). In the current study, cv. Yitpi had ~20% greater root length than early vigour cv. Scout. Early vigour in cereals has been previously linked to a deeper and bigger root system in field-grown crops (Liao et al., 2004; Richards and Lukacs, 2002) but this could not be confirmed for our study, at least not for the root depths investigated. Similarly, (Benlloch-Gonzalez et al., 2014a), reported of a suppression of root growth responses of vigorous wheat lines under  $e[\text{CO}_2]$ .

Both, root length and root uptake capacity per unit length (a measure of root function), can determine genetic differences in N uptake in wheat (Melino et al., 2015; Palta and Watt, 2009). Using N-uptake per measured root length as a surrogate for root N uptake capacity, we found some compensation for the differences in root length between cultivars with cv. Scout showing greater N uptake per measured RL than cv. Yitpi.

## Conclusions

In partial support of our first hypothesis, our data confirm a stimulation of root growth by  $e[\text{CO}_2]$ , but this stimulation is not uniform, but dependent on cultivar and growing conditions. In contrast to this hypothesis, we found little evidence that differences in root length are associated with differences in plant or grain N status. In support of our second hypothesis, and recent results from a FACE experiment under irrigated conditions (Bloom et al., 2014), we found that the proportion of  $\text{NO}_3^-$  to total N in leaves was greater in  $e[\text{CO}_2]$ -grown wheat under the semi-arid conditions of the current study. In addition, the increased proportion of  $\text{NO}_3^-$  in total leaf N was not only associated with decreased N concentrations in vegetative plant parts, but also with grain protein depression across cultivars and  $\text{CO}_2$ -treatments. To our knowledge, this is the first study to show the strong co-dependence of leaf  $\text{NO}_3^-/\text{N}$  and grain protein under  $e[\text{CO}_2]$ , potentially helping to unravel the mechanisms leading to decreased grain N under  $e[\text{CO}_2]$ .

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Figure legends:

Fig. 1. Total aboveground dry weight (total ADW, A) and total captured root length (total captured RL, B) of wheat cultivars “Yitpi” (○ and ●) and “Scout” (△ and ▲) grown under ambient [CO<sub>2</sub>] (a[CO<sub>2</sub>]) of 400 μmol mol<sup>-1</sup>; △ and ○ with dashed lines) and elevated [CO<sub>2</sub>] (e[CO<sub>2</sub>]) of ~550 μmol mol<sup>-1</sup>; ▲ and ● with continuous lines) in rain-fed (left panels) and well-watered (right panels) sub-plots. Symbols are means ± SE of four replicate plots. (DAS: days after sowing, Irri: irrigation treatment, CV: cultivar) ANOVA effects with P<0.100 are listed except for DAS, which was significant at P<0.001 in all cases.

Fig. 2. Total N content (A), and N concentration (B) in aboveground biomass as well as nitrate as a percentage of total N in leaves (C) and total N in aboveground biomass per captured root length (D) of wheat cultivars “Yitpi” (○ and ●) and “Scout” (△ and ▲) grown under ambient [CO<sub>2</sub>] (a[CO<sub>2</sub>]) of ~400 μmol mol<sup>-1</sup>; △ and ○ with broken lines) and elevated [CO<sub>2</sub>] (e[CO<sub>2</sub>]) of ~550 μmol mol<sup>-1</sup>; ▲ and ● with continuous lines) in rain-fed (left panels) and well-watered (right panels) sub-plots. Symbols are means ± SE of four replicate plots. (DAS: days after sowing, Irri: irrigation treatment, CV: cultivar) ANOVA effects with P<0.100 are listed except for DAS, which was significant at P<0.001 in all cases.

Fig. 3. Correlation between nitrate as a percentage of total N in leaves and grain protein (A), as well as total N per captured root length (TN/RL) and grain protein (B) of wheat cultivars “Yitpi” and “Scout” grown under ambient [CO<sub>2</sub>] (a[CO<sub>2</sub>]) of ~400 μmol mol<sup>-1</sup>) and elevated [CO<sub>2</sub>] (e[CO<sub>2</sub>]) of ~550 μmol mol<sup>-1</sup>) in rain-fed (○ symbols with broken lines) and well-watered (● symbols with continuous lines) plants at milk-development stage (152 DAS).

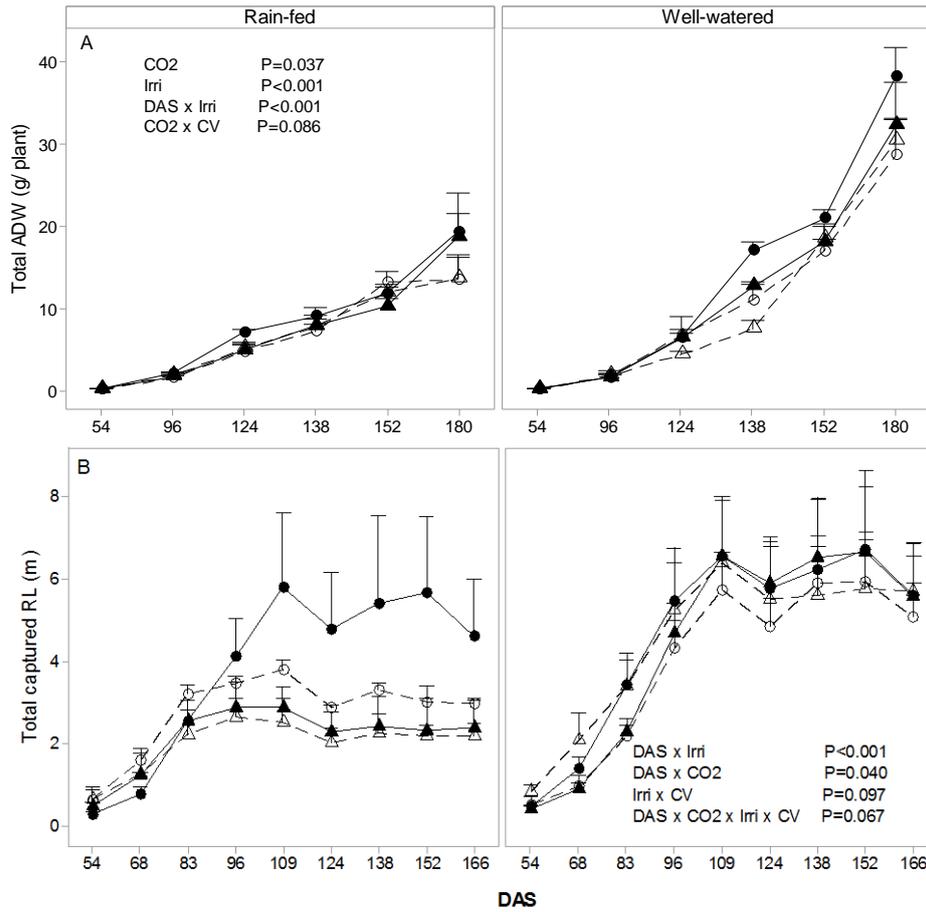


Figure 1

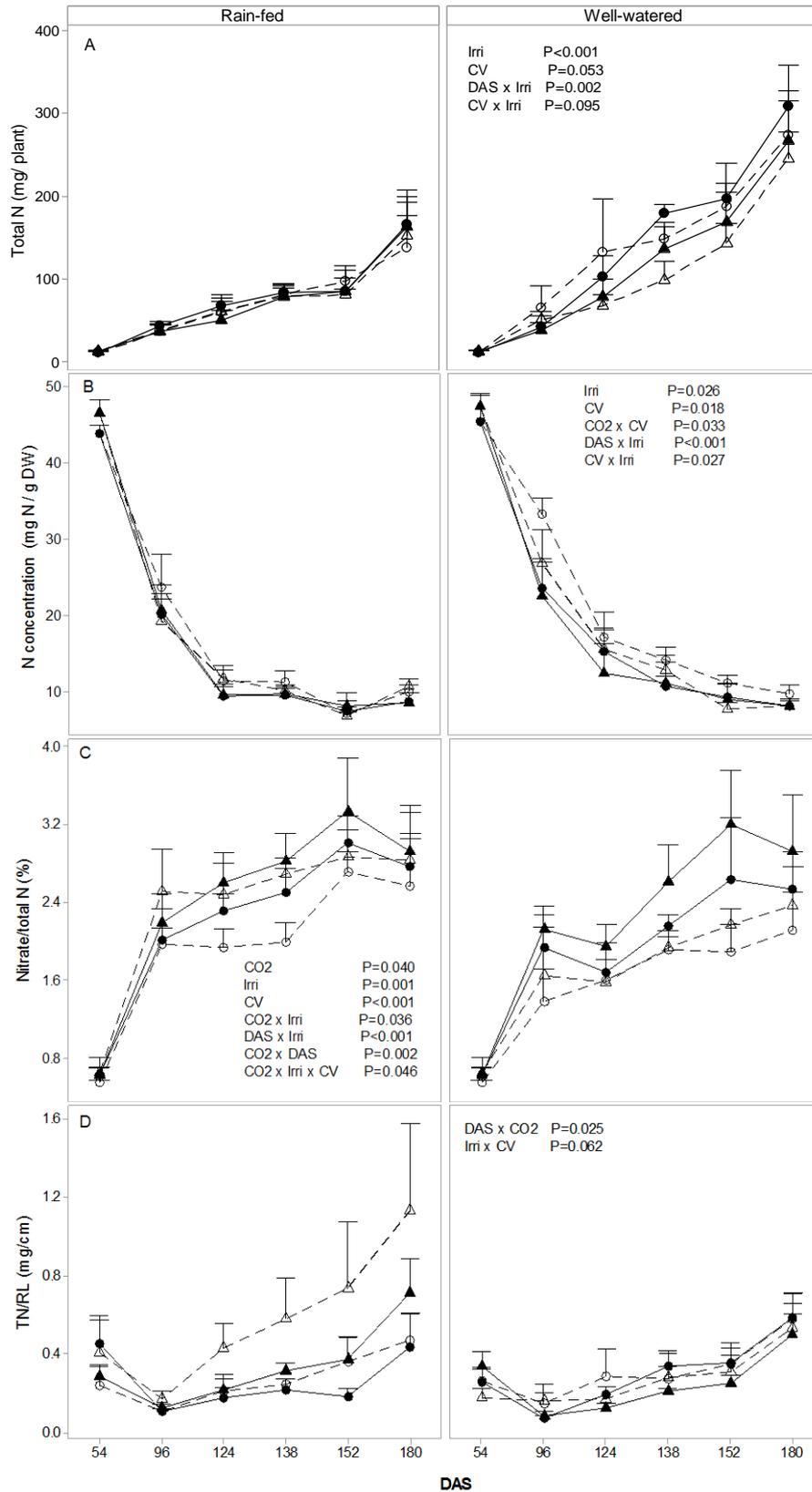


Figure 2

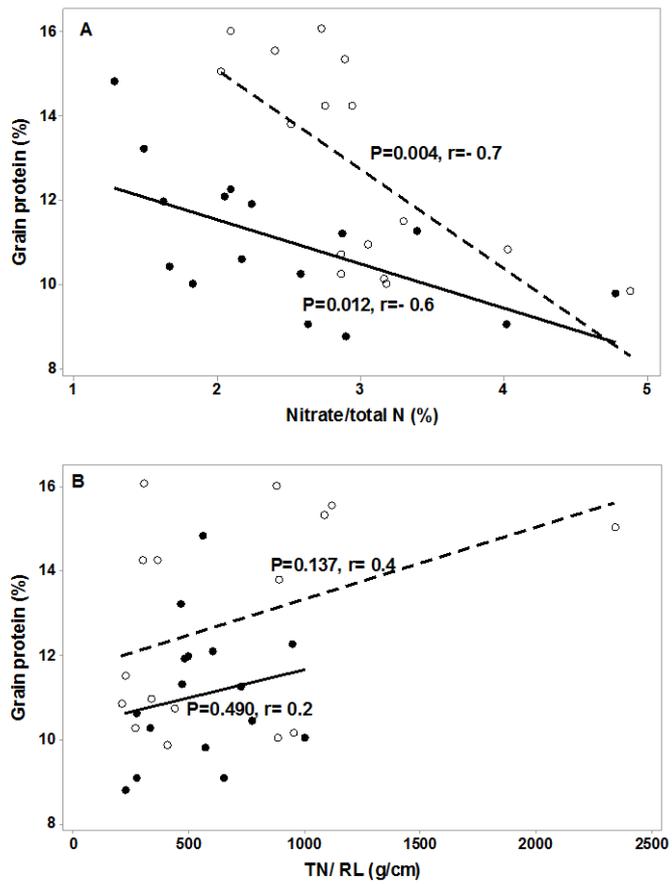


Figure 3

Table 1. Minimum, maximum and average temperatures (°C), growing season rainfall (GSR, mm) and irrigation (water added in mm) at the AGFACE facility in Horsham, Australia, in 2014. Pre-sowing irrigation for all sub-plots occurred on 23 April 2014 to achieve optimal germination. All rainfall and irrigation amounts are absolute values (mm) within each month. Temperature values (°C) are means of daily averages for each month.

						2014												
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Irrigation (mm)	GSR (mm)	Σ GSR plus irrigation (mm)			
Min Temp (°C)	13	12	10	9	7	5	3	1	4	6	8	11						
Max Temp (°C)	32	31	27	22	19	14	13	15	20	24	27	28						
Avg Temp (°C)	22	22	18	15	13	10	8	8	12	15	17	19						
Rainfall (mm)	17	1	15	42	25	35	34	11	11	7	17	6						
Rain-fed (mm)				34					59	60			119	108	261			
Well-watered (mm)				34			27	67	90	99	17		300	115	449			

Table 2. Grain yield (g plant<sup>-1</sup>), grain protein concentration (%), N Uptake (NUp, g N plant<sup>-1</sup> at maturity) and N Utilisation Efficiency (NUtE, grain yield per plant N taken up at maturity) of two wheat cultivars (Scout and Yitpi) under ambient and elevated [CO<sub>2</sub>] and two different water supply treatments (rain-fed and well-watered). Data are means  $\pm$  SE of means (n=4). P-values of ANOVA effects with CO<sub>2</sub>, irrigation (Irrig), cultivar (CV) are reported. None of the interactions were significant. ns P  $\geq$  0.1.

	CO <sub>2</sub> level	cv. Scout		cv. Yitpi		P-values		
		Rain-fed	Well-watered	Rain-fed	Well-watered	CO <sub>2</sub>	Irrig	CV
Grain yield (g plant <sup>-1</sup> )	a[CO <sub>2</sub> ]	3.3 $\pm$ 0.6	8.3 $\pm$ 0.7	3 $\pm$ 0.7	7.8 $\pm$ 1.7	0.067	<0.001	ns
	e[CO <sub>2</sub> ]	4.8 $\pm$ 0.71	8.5 $\pm$ 1.2	4.5 $\pm$ 1.1	9.1 $\pm$ 1.1			
Grain protein concentration (%)	a[CO <sub>2</sub> ]	13.8 $\pm$ 1.03	10.4 $\pm$ 0.6	13.9 $\pm$ 1.06	12.3 $\pm$ 0.9	0.067	0.028	ns
	e[CO <sub>2</sub> ]	11.5 $\pm$ 1.3	10.4 $\pm$ 0.7	12 $\pm$ 1.4	11 $\pm$ 0.9			
NUp (g N plant <sup>-1</sup> )	a[CO <sub>2</sub> ]	0.15 $\pm$ 0.04	0.25 $\pm$ 0.03	0.14 $\pm$ 0.04	0.27 $\pm$ 0.04	ns	0.001	ns
	e[CO <sub>2</sub> ]	0.16 $\pm$ 0.04	0.27 $\pm$ 0.06	0.17 $\pm$ 0.04	0.31 $\pm$ 0.05			
NUtE (g yield g <sup>-1</sup> N taken up)	a[CO <sub>2</sub> ]	23 $\pm$ 3.6	34.4 $\pm$ 2.6	22.3 $\pm$ 3.2	28.6 $\pm$ 4.5	ns	0.046	ns
	e[CO <sub>2</sub> ]	31.9 $\pm$ 3.9	34.2 $\pm$ 3.9	29 $\pm$ 4.4	30.7 $\pm$ 3			