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REVIEW PAPER

Inorganic nitrogen form: a major player in wheat and Arabidopsis responses to elevated CO_2

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Abstract

Critical for predicting the future of primary productivity is a better understanding of plant responses to rising atmospheric carbon dioxide (CO_2) concentration. This review considers recent results on the role of the inorganic nitrogen (N) forms nitrate (NO_3^-) and ammonium (NH_4^+) in determining the responses of wheat and Arabidopsis to elevated atmospheric CO_2 concentration. Here, we identify four key issues: (i) the possibility that different plant species respond similarly to elevated CO_2 if one accounts for the N form that they are using; (ii) the major influence that plant–soil N interactions have on plant responses to elevated CO_2; (iii) the observation that elevated CO_2 may favor the uptake of one N form over others; and (iv) the finding that plants receiving NH_4^+ nutrition respond more positively to elevated CO_2 than those receiving NO_3^- nutrition because elevated CO_2 inhibits the assimilation of NO_3^- in shoots of C_3 plants. We conclude that the form and amount of N available to plants from the rhizosphere and plant preferences for the different N forms are essential for predicting plant responses to elevated CO_2.

Keywords: Ammonium, biomass, CO_2 acclimation, growth, nitrate, plant–soil interactions, protein yield, yield.

Introduction

The concentration of CO_2 in the atmosphere has risen from 322 ppm in 1969 to >400 ppm today and is likely to double by the end of the century (IPCC, 2013). Human well-being will depend strongly on plant responses to such CO_2 changes. Unfortunately, these responses are diverse and not well understood (Leakey *et al.*, 2009; Bloom, 2010; Terashima *et al.*, 2014; Wang *et al.*, 2013).

A key issue in plant responses to elevated CO_2 is the coordination between carbon (C) and nitrogen (N) metabolism to meet plant demands for growth (Stitt and Krapp, 1999). Indeed, accounting for internal plant nitrogen status is central to computer models that simulate crop productivity under elevated CO_2 (Yin, 2013). N is an integral constituent of proteins, nucleic acids, chlorophyll, co-enzymes, phytohormones, and secondary metabolites, and thus is the mineral element that organisms require in greatest amounts (Epstein and Bloom, 2005; Bloom, 2015*b*). Plants acquire N from their environment in several forms such as NO_3^- and NH_4^+ that are highly distinct, both chemically and biologically. Plant preferences for each N form, along with the availability of each form from the soil, are now receiving more attention (Jackson *et al.*, 2008; Bloom *et al.*, 2012; Boudsocq *et al.*, 2012; Britto and Kronzucker, 2013).

The following reviews the literature on the interactions between N form and atmospheric CO_2 concentration to examine the importance of N form in plant responses to elevated

CO_2. The working hypothesis is that N form is responsible for many of the differences in plant responses to elevated CO_2 because of the variation in NO_3^- and NH_4^+ availability from soils (Burger and Jackson, 2004) and the distinct effects that NO_3^- and NH_4^+ assimilation have on plant C metabolism (Bloom, 1988). We focus on wheat and Arabidopsis, model species for monocots and dicots, respectively.

Wheat and Arabidopsis as plant models in CO_2 studies

Of major concern is the influence of rising atmospheric CO_2 on crop yields and food quality. Wheat plays a unique role in human affairs: it covers more of the earth's surface than any other food crop and has the third highest yields (FAO, 2013). It provides ~20% of the carbohydrate as well as 20% of the protein in the human diet, ranking first among crops in developing countries as a protein source (FAO, 2013).

Wheat, when exposed to elevated CO_2, exhibited decreased stomatal conductance and increased water-use efficiency (Del Pozo *et al.*, 2007; Qiao *et al.*, 2010) (Fig. 1B, C), especially in NH_4^+-fed plants during the earliest phase of vegetative growth (Fig. 2C, D). Plants at elevated CO_2 senesced earlier and experienced accelerated grain development (Sild *et al.*, 1999). In most cases, the biomass of both shoots and roots increased (Rubio-Asensio *et al.*, 2015) (Fig. 3A, C, F).

Wheat grain yield under elevated CO_2 varies greatly with growth conditions (Amthor, 2001; Wang *et al.*, 2013). Under elevated CO_2 ranging from 450 µmol mol^{-1} to 800 µmol mol^{-1}, grain yields increased an average of 10% in 57 experiments (Pleijel and Uddling, 2012), 24% in 59 experiments (Wang *et al.*, 2013), and 31% in 156 experiments (Amthor, 2001). Grain yield of plants receiving NH_4^+ showed a greater enhancement by elevated CO_2 than those receiving NO_3^- (Carlisle *et al.*, 2012). Shoot protein concentration (Wang *et al.*, 2013), grain protein concentration (Taub *et al.*, 2008), and grain protein yield (Pleijel and Uddling, 2012) decreased an average of ~10%.

Wheat, when exposed to elevated CO_2, exhibited enhanced photosynthesis (Figs 1A, 2A, B). This stimulation abated, however, after days to weeks of exposure, a phenomena known as CO_2 acclimation (Sicher and Bunce, 1997). CO_2 acclimation was associated with an increase in carbohydrate concentration and a decrease in (i) total N concentration (Weigel and Manderscheid, 2012); (ii) organic N concentration in NO_3^--fed plants (Bloom *et al.*, 2010) (Fig. 3E), (iii) proteins (Ziska *et al.*, 2004; Wieser *et al.*, 2008; Fernando *et al.*, 2012*a*, *b*; Högy *et al.*, 2013; Jauregui *et al.*, 2015*b*); and (iv) Rubisco activity and content (Zhang *et al.*, 2009). Several meta-studies (Amthor, 2001; Taub *et al.*, 2008; Pleijel and Uddling, 2012; Wang *et al.*, 2013; Feng *et al.*, 2015) confirm that the influence of elevated CO_2 on wheat yield and protein varies greatly, indicating the presence of strong interactions between CO_2 and other factors (Fernando *et al.*, 2014). In particular, soil N availability seems to be a major driver of wheat responses to elevated CO_2 (Hocking and Meyer, 1991; Stitt and Krapp, 1999; Wieser *et al.*, 2008; Erbs *et al.*, 2010), although soil N availability alone is not sufficient to explain these responses (Stitt and Krapp, 1999; Weigel and Manderscheid, 2012; Feng *et al.*, 2015).

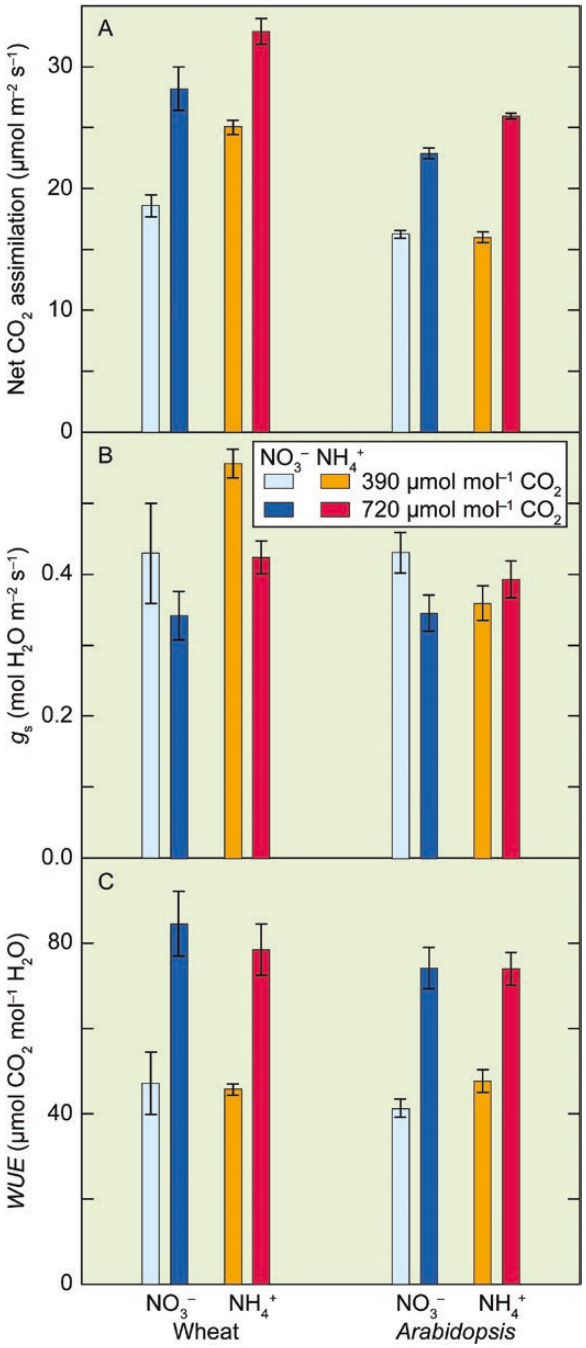

Fig. 1. Net CO_2 assimilation, stomatal conductance (g_s), and water-use efficiency (WUE) of wheat and Arabidopsis plants as affected by the N form (NO_3^- versus NH_4^+) and CO_2 level (390 versus 720 µmol mol^{-1}). Measurements were assessed via a portable photosynthesis system (Licor-6400-40 with fluorimeter). Plant age was 14 d after sowing in wheat and 45 d after sowing in Arabidopsis. Light level during measurements was 1000 µmol photons m^{-2} s^{-1} in both species. Leaf temperature was maintained at 25 °C in wheat and 22 °C in Arabidopsis, and the vapor pressure deficit was maintained at 1.8 kPa in wheat and 1.5 kPa in Arabidopsis. Shown are the means (± SE) for five replicate plants per treatment. These data are unpublished (AJB, University of California, Davis, CA, USA). Details about the wheat growth conditions are provided in Cousins and Bloom (2004), whereas details about the Arabidopsis growth conditions are provided in Bloom *et al.* (2012). (This figure is available in colour at *JXB* online.)

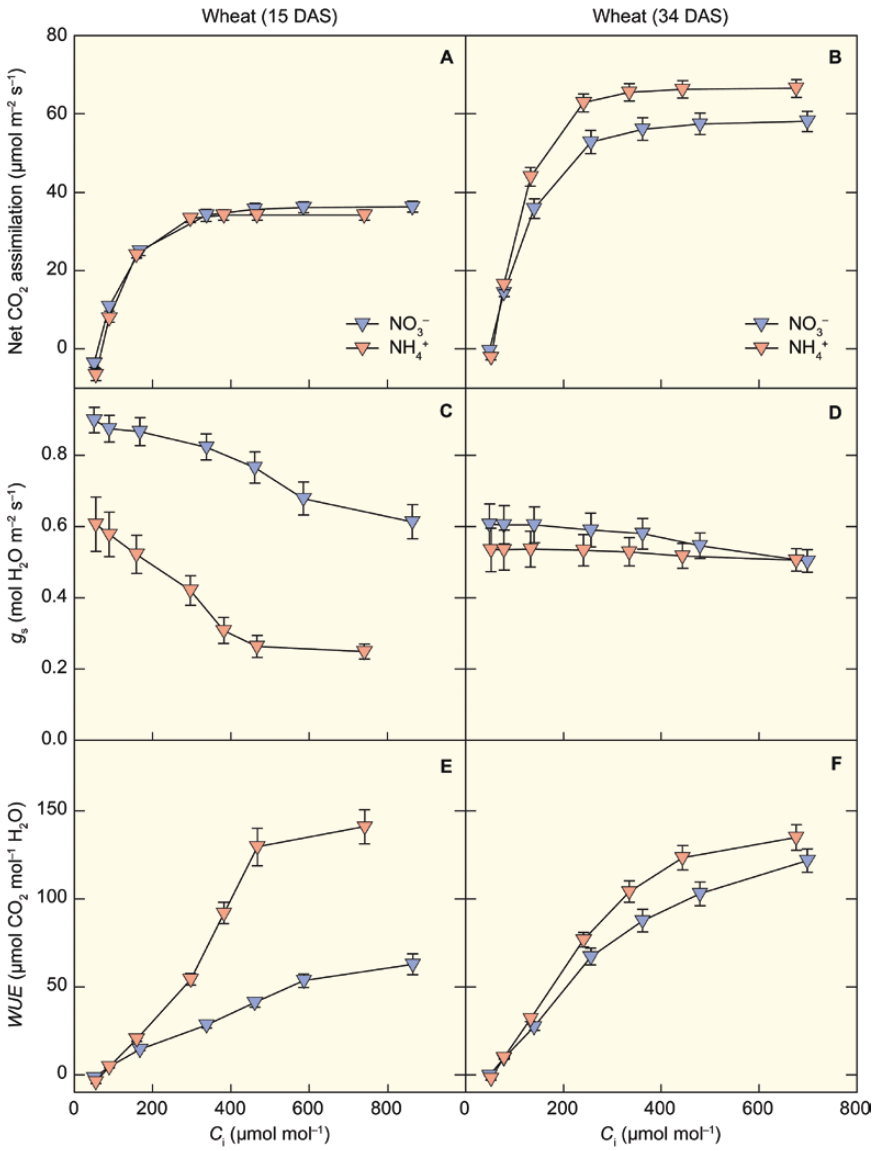

Fig. 2. Net CO_2 assimilation, stomatal conductance (g_s), and water-use efficiency (WUE) of wheat plants 15 days after sowing (DAS) (left panels) and 34 DAS (right panels) as a function of internal CO_2 concentration (C_i) and N form (NO_3^- versus NH_4^+). Net CO_2 assimilation was calculated using the A/C_i curve fitting utility described in Sharkey *et al.* (2007). Measurements were assessed with a portable photosynthesis system (Licor-6400-40) at a light level of 1500 μmol photons m^{-2} s^{-1}. Each NO_3^- or NH_4^+ response curve includes measurements of plant growth at ambient and elevated CO_2 atmospheric concentration. Measurement: leaves were first adapted to 400 μmol CO_2 mol^{-1}, and then stepwise down to 50 μmol CO_2 mol^{-1}, after which the CO_2 concentration surrounding the leaf was increased as follows: 50, 100, 200, 400, 550, 700, and 900 μmol CO_2 mol^{-1}. Shown are the means ±SE for 10–15 plants per treatment. Details about the plant growth conditions are provided in Bloom *et al.* (2012). (This figure is available in colour at *JXB* online.)

Arabidopsis thaliana has unique advantages as a model species for examining both molecular mechanisms involved in the responses to elevated CO_2 (Engineer *et al.*, 2014; Jauregui *et al.*, 2015*a*, 2016) and plant evolution to an elevated CO_2 world (Ward and Kelly, 2004; Li *et al.*, 2014). The vegetative growth phase of Arabidopsis is short and can be accelerated by increasing the light photoperiod and light intensity. Large numbers of plants can be grown in a short time and in a small space under well-controlled environmental conditions. For this species, there are numerous genotypes and molecular tools available that allow precise dissection of its responses to various factors (Easlon *et al.*, 2015).

Elevated CO_2 stimulated photosynthesis in Arabidopsis (Markelz *et al.*, 2014; Easlon *et al.*, 2015) (Fig. 1A). This was associated with an increase in the total non-structural carbohydrates and starch (Cheng *et al.*, 1998; Teng *et al.*, 2006; Markelz *et al.*, 2014). In most cases, elevated CO_2 decreased the total N concentration and organic N concentration (Sun *et al.*, 2002; Teng *et al.*, 2006; Bloom *et al.*, 2010; Takatani *et al.*, 2014) (Fig. 3M), Rubisco activity and content (Cheng *et al.*, 1998), and stomatal density and aperture (Teng *et al.*, 2006). Decreased stomatal density and aperture resulted in decreased stomatal conductance and enhanced water-use efficiency (Fig. 1B, C), especially under stress conditions (Leymarie *et al.*, 1999). At elevated CO_2, Arabidopsis plants had higher biomass (Rubio-Asensio *et al.*, 2015) (Fig. 3I, K, N), reproductive biomass, and accelerated development (Ward and Strain, 1997; Tocquin *et al.*, 2006; Li *et al.*, 2014).

The influence of elevated CO_2 on growth and biomass production and partitioning in Arabidopsis varies greatly,

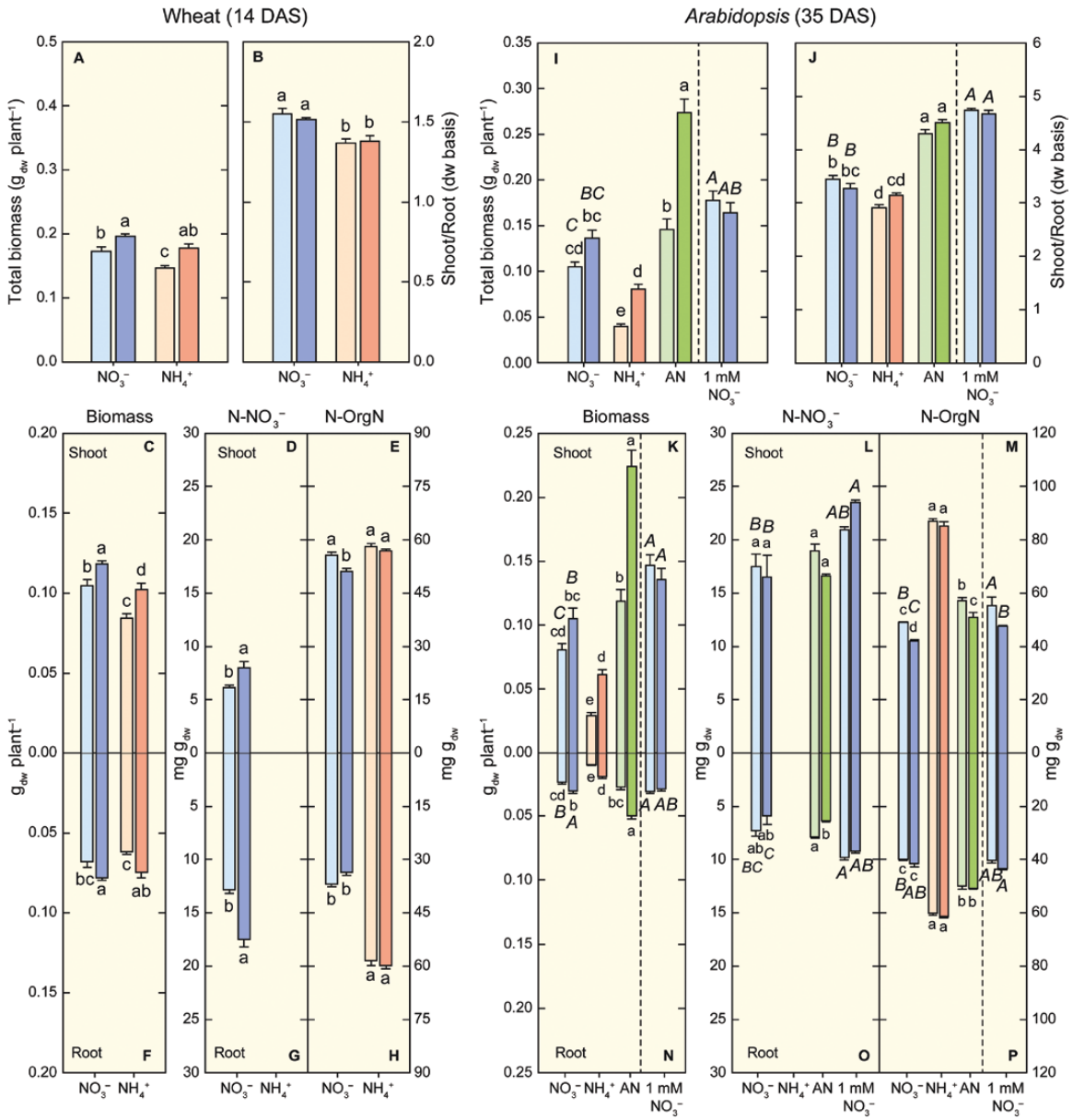

Fig. 3. Total biomass production (g_{dw}) and partitioning (shoot to root ratio) (upper panels) and biomass production and nitrogen balance of the shoot and root (lower panels) for wheat 14 days after sowing (DAS) and Arabidopsis 35 DAS as affected by source of nitrogen (NO_3^- versus NH_4^+) and atmospheric CO_2 concentration (410 µmol mol^{-1}, light blue and light orange, or 720 µmol mol^{-1}, dark blue and dark orange). Data of plant growth with 0.2 mM of NO_3^- or 0.2 mM of NH_4^+ are from (Rubio-Asensio *et al.*, 2015). Additionally, unpublished data are presented for Arabidopsis for which the plants were grown with 0.1 mM NO_3^- plus 0.1 mM NH_4^+ (NH_4NO_3 or AN, left side of dotted line) or 1 mM NO_3^- alone (right side of dotted line). Each bar represents the mean ±SE of 5–12 plants. The effect of CO_2×N source or CO_2×N dose and its interactions were tested for significance by two-way ANOVA with SPSS, and results are presented in Table 1. Means of treatments N source×CO_2 that are significantly different are labeled with different lower case letters; means of treatments N dose×CO_2 that are significantly different are labeled with different upper case letters.

depending on the N source and its availability to the plants (Fig. 3I, K, N) (Bloom *et al.*, 2012; Niu *et al.*, 2013; Takatani *et al.*, 2014), and the age of the experimental material. Young plants showed the greatest stimulation at elevated CO_2 (Takatani *et al.*, 2014; Easlon *et al.*, 2015) and older plants showed less stimulation (Bloom *et al.*, 2010, 2012). The shoot/root ratio was sensitive to N source and availability, but not CO_2 treatment (Fig. 3J).

Wheat and Arabidopsis show major differences in their resource allocation patterns. For example, wheat had about half the shoot:root ratio of Arabidopsis (Fig. 3B, J). The NO_3^- concentration in wheat roots was about double that in shoots, whereas the NO_3^- concentration in Arabidopsis shoots was about double that in roots (Fig. 3D, G, L, O). These species also show distinct responses to N source: (i) both species grew larger under NO_3^- as a sole N source than under NH_4^+, but they differed in tolerance to NH_4^+, whereby wheat was less sensitive than Arabidopsis in the early phase of growth (Fig. 3A, I); (ii) both species had a higher organic N concentration when receiving NH_4^+ rather than NO_3^-, but the increase in organic N concentration in NH_4^+ plants was much less pronounced for wheat than for Arabidopsis (Fig. 3E, H, M, P); and (iii) wheat receiving NH_4^+ had higher rates of CO_2 assimilation than those receiving NO_3^-, whereas Arabidopsis

Table 1. *Two-way ANOVA of the data in Fig. 3 that examined the effects of N source and CO_2 level or N dose and CO_2 level*

Experiment	Factor	Total biomass	S/R	Shoot			Root		
				Biomass	NO_3^-	Org-N	Biomass	NO_3^-	Org-N
Wheat	N source (S)	***	***	***	–	***	ns	–	***
	CO_2	***	ns	**	–	ns	***	–	*
	S×CO_2	ns	ns	ns	–	*	ns	–	*
Arabidopsis	N source (S)	***	***	***	ns	***	***	ns	***
	CO_2	***	ns	***	ns	***	***	*	ns
	S×CO_2	***	**	***	ns	*	***	ns	ns
Arabidopsis	N dose (D)	***	***	***	***	***	ns	***	ns
	CO_2	ns	ns	ns	ns	***	ns	ns	*
	D×CO_2	*	ns	*	ns	ns	*	ns	ns

The symbols indicate statistical significance; ***P<0.001; **P<0.01; *P<0.05; ns, not significant.

receiving NH_4^+ had higher CO_2 assimilation rates than those receiving NO_3^- only in the plants at elevated CO_2 (Fig. 1A).

Despite these differences, wheat and Arabidopsis generally showed similar responses to elevated CO_2. In both species, elevated CO_2 accelerated net CO_2 assimilation (Fig. 1A) and growth (Fig. 3A, I). Elevated CO_2 inhibited shoot NO_3^- assimilation in both species (Fig. 7). Overall, these results indicate that plant responses to elevated CO_2 were highly influenced by the N form used by the plants and that the responses to elevated CO_2 were similar in the two species if one took into account the N form that they were using.

Plant–soil nitrogen interactions

Nitrogen availability

In wheat, when the availability of N from the soil solution declined to levels insufficient for sustaining maximum growth, foliar N and chlorophyll concentrations decreased, which slowed photosynthetic electron transport (Zhang *et al.*, 2013). In Arabidopsis, 2 d of nitrogen deprivation repressed the expression of most genes involved in photosynthesis, chlorophyll synthesis, and plastid protein synthesis; induced many genes for secondary metabolism; and changed expression of many genes involved in mitochondrial electron transport (Scheible *et al.*, 2004). In most plants, N deficiency caused changes in N metabolism that in turn triggered changes in C metabolism, because photosynthesis, photorespiration, and respiration supply the C skeletons and energy required to assimilate NO_3^- and NH_4^+ into amino acids (Huppe and Turpin, 1994). Plants also adjusted their morphology under N-deficient conditions via partitioning more biomass to the root than the shoot (Poorter and Nagel, 2000; Ikram *et al.*, 2012) (Fig. 4A). Ultimately, if plants cannot sufficiently meet their N requirements for growth, they sacrifice production of total biomass (Delgado *et al.*, 1994; Theobald *et al.*, 1998; Erbs *et al.*, 2010). At the other end of the spectrum, excess N in the rhizosphere can suppress growth (Tocquin *et al.*, 2006), possibly as a consequence of an osmotic effect.

In summary, N availability *per se* or consistency of N availability interacts strongly with conditions that affect C metabolism such as atmospheric CO_2 concentration (Stitt and Krapp, 1999) (Fig. 5), especially during rapid vegetative growth (Bloom, 1988). Nitrogen additions to elevated CO_2 systems enhance plant growth responses (Reich *et al.*, 2006), retard CO_2 acclimation (Stitt and Krapp, 1999; Ainsworth and Rogers, 2007), and increase protein concentration (Taub and Wang, 2008). Hence, the importance of accounting for the role of N availability in plant responses to elevated CO_2.

The form of N in the soil

Plants obtain N from the soil solution mainly in the forms of NO_3^- and NH_4^+ (Epstein and Bloom, 2005). The proportion of these two forms that a plant absorbs depends on both its preference between forms and the relative availability of these forms in the rhizosphere (Britto and Kronzucker, 2013). The preferences of plants between NO_3^- and NH_4^+ depends on a wide and dynamic range of environmental and physiological factors (Britto and Kronzucker, 2013), but a plant generally performs better under the N form that is dominant in the soil of its natural habitat (Gigon and Rorison, 1972). For instance, species native to acid and reducing soils, such as those found in mature forests or arctic tundra, tend to prefer NH_4^+, whereas those native to alkali or aerobic soils tend to prefer NO_3^- (Maathuis, 2009). The availability of NO_3^- and NH_4^+ depend on the soil–microbial interactions (nitrification rates) in the vicinity of the root, as well as the fertilization rates in agricultural soils (Britto and Kronzucker, 2002; Burger and Jackson, 2004). The distribution and concentration of NO_3^- and NH_4^+ in soils show great spatial and temporal heterogeneity (Jackson and Caldwell, 1993), but in temperate agricultural soils, the predominant form of N available to plants is NO_3^- (Andrews, 1986).

Plants depend on NO_3^- as an N source even in locations where soil NO_3^- concentrations tend to be relatively low. For instance, many flood-tolerant plants such as paddy rice, which grow in wetland soils subject to NO_3^- leaching and denitrification (microbial conversion of NO_3^- to N_2), develop aerenchyma that supply the rhizosphere with oxygen, which promotes nitrification on root surfaces. The NO_3^- thus generated is immediately absorbed by the root (Koch *et al.*, 1991; Kronzucker *et al.*, 2000; Rubinigg *et al.*, 2002; Li *et al.*, 2008).

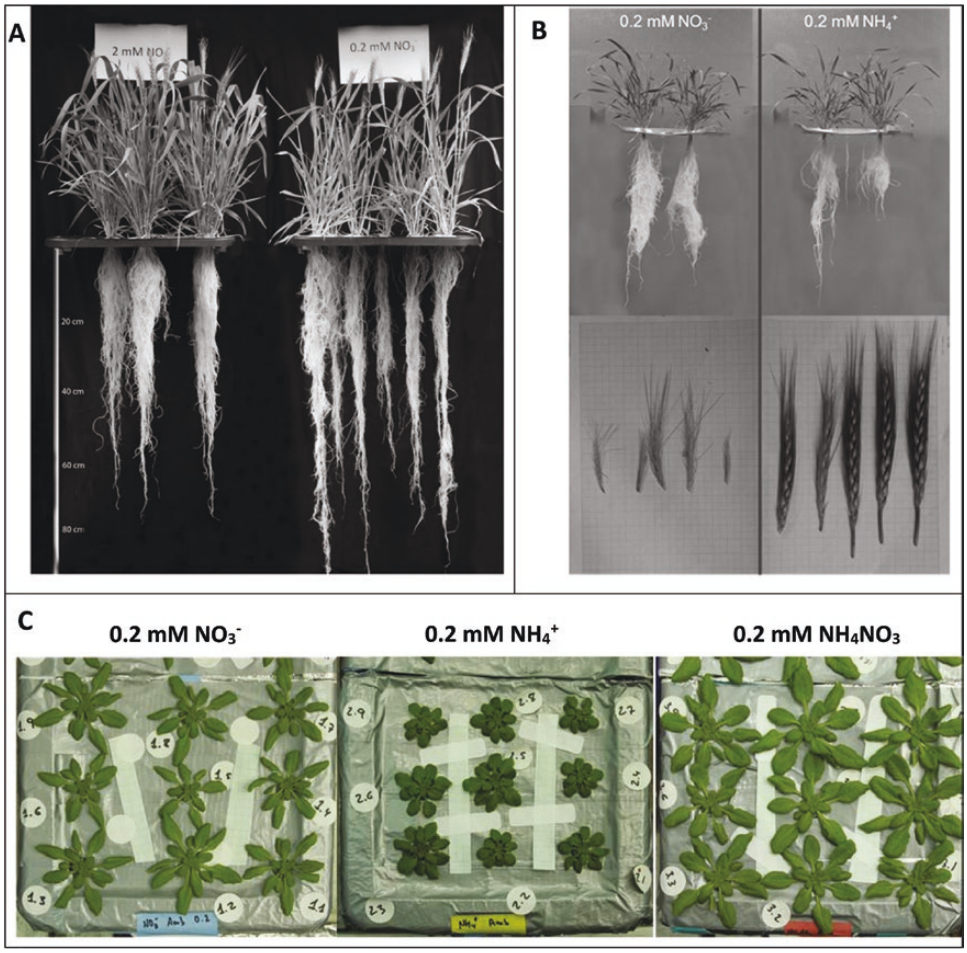

Fig. 4. Effect of dose and form of N fertilization on growth and development of wheat and Arabidopsis (cv. Columbia). (A) Sixty-day-old wheat plants (cv. Yecora rojo) affected by the NO_3^- dose (from left to right): 2 mM NO_3^- and 0.2 mM NO_3^- (Rubio-Asensio *et al.*, 2014). Total fresh biomass at this stage was 173 ± 6 and 61.5 ± 7 for plants grown in 2 mM and 0.2 mM NO_3^-, respectively. Shoot to root ratio on a fresh weight basis at this stage was 1.91 ± 0.033 and 1.19 ± 0.27 for plants grown in 2 mM and 0.2 mM NO_3^-, respectively. (B) Forty-day-old wheat plants (cv. Veery 10) and its ears after growing for 36 d in nutrient solution with 0.2 mM NO_3^- or 0.2 mM NH_4^+ as the sole N source. Total fresh biomass at this stage was 77.13 ± 4.56 and 58.39 ± 3.13 for plants grown in NO_3^- and NH_4^+, respectively. The shoot to root ratio on a fresh weight basis was 0.74 ± 0.022 and 1.15 ± 0.04 for plants grown in NO_3^- and NH_4^+, respectively (unpublished data of AJB and co-workers, UC Davis, USA). (C) Thirty-five-day-old Arabidopsis plants affected by N form (NO_3^-, NH_4^+, or a mix of NO_3^- and NH_4^+) (see Fig. 2 for more details). Nutrient solution composition and growth conditions for wheat and Arabidopsis are described in Bloom *et al.* (2010). Photos: José Salvador Rubio-Asensio. (This figure is available in colour at *JXB* online.)

Also, forest soils in which NH_4^+ is the major N source have high rates of gross nitrification that indicate a small but ecologically important NO_3^- pool (Stark and Hart, 1997). Finally, plants that can conduct symbiotic N fixation are more prevalent in soils deficient in N, but these plants cease N fixation whenever NO_3^- becomes available in the rhizosphere (Cabeza *et al.*, 2014).

Plant acquisition of the two N forms, NO_3^- and NH_4^+, has disparate effects on plant C metabolism (Haynes and Goh, 1978; Stitt *et al.*, 2002; Patterson *et al.*, 2010; Hachiya and Noguchi, 2011). Assimilation of NO_3^- consumes several times the energy than that of NH_4^+ (Bloom *et al.*, 1989, 1992): reduction of NO_3^- to NH_4^+ consumes the equivalent of ~10 ATP, whereas the conversion of NH_4^+ to glutamate consumes the equivalent of ~2 ATP. Specificially, photo-assimilation of NO_3^- to NH_4^+ oxidizes NADPH or NADH and six reduced ferrodoxins, and so plants dependent on NO_3^- as an N source must efficiently partition reductant generated during the light reactions of photosynthesis to cover the extra demands of NO_3^- assimilation (Patterson *et al.*, 2010). To do this, plants receiving NO_3^- increase their electron transport rate (ETR) and generally have lower rates of CO_2 assimilation (Huppe and Turpin, 1994). Wheat and Arabidopsis were consistent with this trend, having lower CO_2 assimilation rates under NO_3^- nutrition than NH_4^+ nutrition, especially at elevated CO_2 concentration (Figs 1A, 2B). In contrast, NH_4^+ becomes toxic to plants if it accumulates to high levels within their cells (Li *et al.*, 2014). Plants receiving excess NH_4^+ address this problem by increasing respiration to provide both energy for NH_4^+ efflux across the plasma membrane of root cells (Britto *et al.*, 2001) and C skeletons needed for NH_4^+ assimilation (Bloom *et al.*, 1992; Cramer and Lewis, 1993).

These differences in C metabolism when plants use NO_3^- or NH_4^+ as a sole N source often result in large differences in plant growth and development (Fig. 4B, C). Both wheat and Arabidopsis receiving NH_4^+ nutrition accumulated less biomass than those receiving NO_3^- nutrition (Fig. 3A, I). These differences were dramatic in Arabidopsis, which could

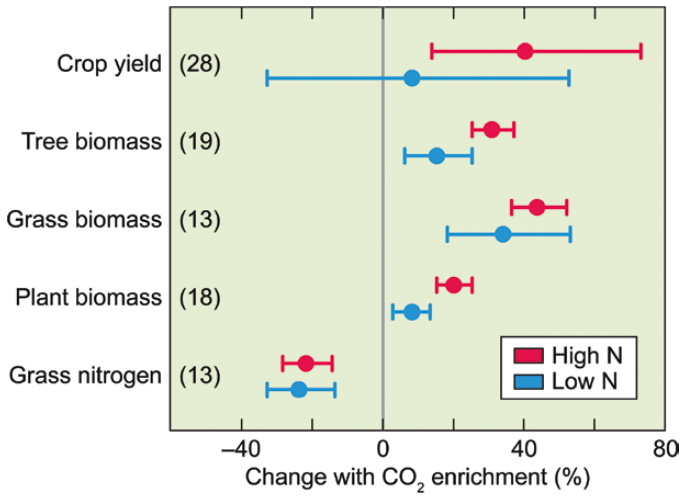

Fig. 5. Influence of nitrogen fertilization on plant responses to atmospheric CO_2 levels. Shown are the changes in crop yield, above-ground biomass, and grass total nitrogen concentrations from plants grown at ambient CO_2 concentration (~366 ppm) compared with those grown at elevated CO_2 concentration (~567 ppm). 'High N' (red) and 'Low N' (blue) designate, respectively, plants receiving greater and lesser amounts of nitrogen fertilizer. In parentheses are the numbers of experiments used to estimate the response of each parameter. The response of each parameter was based on a different meta-analysis, and so the response of tree biomass to CO_2 enrichment included more and different experiments from that of plant biomass. Circles and error bars designate means and confidence intervals, respectively, for crops, trees, C_3 grasses, and all plant species. (After Curtis and Wang, 1998; Wand *et al.*, 1999; Ainsworth and Long, 2005; de Graaff *et al.*, 2006.)

be considered an NH_4^+-sensitive species (Fig. 4C). The higher shoot-to-root ratio that is observed after a long exposures to NH_4^+ nutrition often derives from the detrimental effect of NH_4^+ on root growth (Bauer and Berntson, 2001; Guo *et al.*, 2007; Carlisle *et al.*, 2012) (Fig. 3B for 14-day-old plants versus Fig. 4B for 40-day-old plants). Plants usually grow fastest and produce more biomass when they receive both NO_3^- and NH_4^+ simultaneously (Bloom *et al.*, 1993; Gloser *et al.*, 2002; Boudsocq *et al.*, 2012). In Arabidopsis (Figs 3I, 4C) or in wheat (Gentry *et al.*, 1989), the mix of both NO_3^- and NH_4^+ in a nutrient solution produced more biomass than only one N source in a nutrient solution. Unfortunately, the physiological mechanisms responsible for this response to NO_3^- and NH_4^+ mixtures are still not well understood (Hachiya *et al.*, 2012; Britto and Kronzucker, 2013).

In summary, the transport and assimilation of NO_3^- and NH_4^+ are processes that require precise co-ordination with C metabolism, and the large influence of NO_3^- and NH_4^+ on C metabolism can obscure the effects of other factors, such as atmospheric CO_2 level and N availability, when plants have access to both forms of N.

The direct effect of atmospheric CO_2 and the indirect effect of nitrogen in the soil on plant performance

Plant performance depends on complex and balanced interactions among C gain from the atmosphere, N gain from the soil, and plant water status (Fig. 6A). The relationships among these processes depend strongly on atmospheric CO_2 concentration, whereby CO_2 concentration itself alters net CO_2 assimilation, stomatal density and aperture, and photorespiration (Fig. 6B). (i) Carbon fixation is generally CO_2 limited under current CO_2 atmospheres in plants conducting C_3 carbon metabolism. Elevated CO_2 atmospheres increase internal CO_2 concentration (C_i), and so accelerate net CO_2 assimilation, which in turn generates more carbohydrates (Geiger *et al.*, 1998). (ii) Lower stomatal density and aperture are common responses of plants to elevated CO_2 atmospheres (Zeiger, 1983; Woodward, 1987), which are responsible for decreased leaf transpiration rates (Long *et al.*, 2004; Leakey *et al.*, 2009). These decrease plant water use and thereby increase soil moisture availability unless elevated CO_2 dramatically enhances canopy size (Leakey *et al.*, 2009). (iii) Rubisco catalyzes either the carboxylation or oxygenation of the substrate RuBP, initiating respectively either the C_3 carbon fixation or photorespiratory pathways. The relative rates of the carboxylation and oxygenation reactions depend on the relative concentrations of CO_2 and O_2 at the active site of the enzyme (Ogren, 1984). Elevated CO_2 atmospheres favor C_3 carbon fixation, and thus less reductant from the photosynthetic electron transport is diverted into photorespiration for re-assimilating NH_4^+ and re-fixing CO_2 (Leegood *et al.*, 1995; Wingler *et al.*, 2000). Slower photorespiration, however, entails decreased export of malate from the chloroplast to the cytosol, and therefore interferes with maintaining a favorable cytosolic NADH/NAD ratio (Backhausen *et al.*, 1994; Igamberdiev *et al.*, 2001) that empowers NO_3^- reduction (Bloom, 2015*a*).

The indirect effects of the amount and form of N that plants are absorbing from the soil could be as important to plant performance as the direct effects of elevated CO_2 (Fig. 6C). When soil N concentrations are very low or very high, plant performance (e.g. growth rate, development, biomass production, and partitioning) should derive, respectively, from N deficiencies or toxicities and will be less responsive to CO_2 concentrations. Indeed, studies that measured the availability of rhizosphere N found that the phenomenon of CO_2 acclimation was more pronounced under N deficiencies, both in wheat (Brooks *et al.*, 2000; Sinclair *et al.*, 2000; Wall *et al.*, 2000; Kimball *et al.*, 2001; Del Pozo *et al.*, 2007; Erbs *et al.*, 2010) and in Arabidopsis (Sun *et al.*, 2002; Tocquin *et al.*, 2006). When N availability from the rhizosphere does not limit plant growth, N form may strongly influence plant responses to elevated CO_2 (Bloom *et al.*, 2012). Under these conditions, the balance between NO_3^- and NH_4^+ assimilation and interactions with atmospheric CO_2 concentration should influence plant performance.

Responses to elevated CO_2 depend on nitrogen form

Acquisition of different N forms

Elevated CO_2 influences root absorption of soil NO_3^- and NH_4^+ in several ways. (i) The anion NO_3^- does not bind

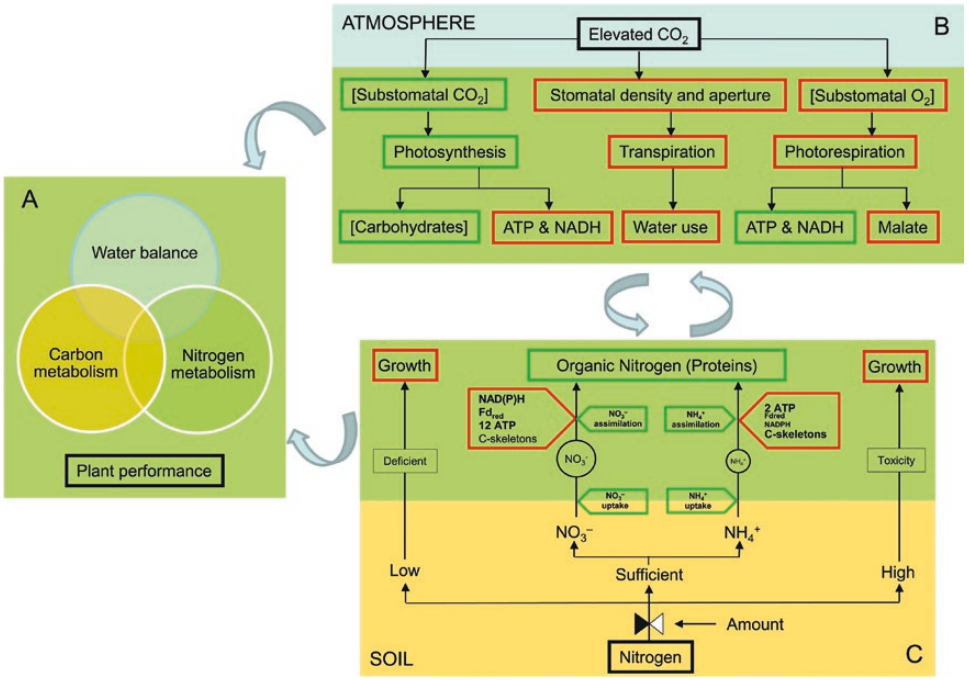

Fig. 6. A plant's performance at all stages of development is defined by fine-tuning the relationships among C metabolism, N metabolism, and water status (A). Atmospheric CO_2 concentration (B) and nitrogen form and concentration in the soil (C) strongly influence these relationships. Understanding plant performance as influenced by CO_2 level and N in soil has a further level of complexity because the plant response to elevated CO_2 (B) would change the N form preferences (NO_3^- versus NH_4^+) and also the rates of N absorption. Similarly, the N form and dose that plants take up from the soil (C) with its different influences on plant energetics and metabolism would change the rates of CO_2 assimilation (B). Shown in red or green processes or products that decrease or increase, respectively, its rate or concentration. Additional explanation of this figure appears in the text.

to the cation exchange complex of soils, whereas the cation NH_4^+ binds strongly; therefore, NO_3^- is usually more mobile through soils than NH_4^+. Elevated CO_2 decreases leaf transpiration, mass flow of water from the soil to the roots, and movement of NO_3^- through the soil to root surfaces (McGrath and Lobell, 2013; Jauregui *et al.*, 2015*b*;). Thus elevated CO_2 may alter rhizosphere availability of NO_3^- more than that of NH_4^+. (ii) Higher soil moisture levels that may result from less transpiration at elevated CO_2 may decrease soil nitrification rates because of hypoxic conditions, and therefore may decrease the soil NO_3^- concentration (Niklaus *et al.*, 2001; Carrillo *et al.*, 2012). In water-limited environments, however, higher soil moisture levels at elevated CO_2 may increase nitrification and thereby increase soil NO_3^- concentration (Schleppi *et al.*, 2012). (iii) Elevated CO_2 tends to impede root NO_3^- uptake (Cheng *et al.*, 2012) (Fig. 7). In six annual grassland species, elevated CO_2 did not affect NH_4^+ uptake whereas the NO_3^- uptake rate decreased (Jackson and Caldwell, 1996). (iv) In soils, NH_4^+ moves ~10 times more slowly than NO_3^-, and depletion zones for immobile cations such as NH_4^+ typically form around the surface of roots (Tinker and Nye, 2000). Elevated CO_2 promotes root growth (Fig. 3F, N) (Niu *et al.*, 2013; Arndal *et al.*, 2014; Hachiya *et al.*, 2014) and total root length (Benlloch-Gonzalez *et al.*, 2014), particularly if N is not limiting (de Graaff *et al.*, 2006). Enhanced root growth and root density at elevated CO_2 thus is more crucial for plant capture of relatively immobile cations such as NH_4^+ than mobile anions such as NO_3^- (Silberbush and Barber, 1983).

These findings support that elevated CO_2 may differentially influence plant NO_3^- and NH_4^+ absorption.

Nitrogen assimilation

Another major factor that influences plant responses to elevated CO_2 is the disparate energy requirements for NO_3^- and NH_4^+ assimilation into amino acids. To examine this factor, experiments must control atmospheric CO_2 concentration and rhizosphere N concentration and form. Unfortunately, the balance between NO_3^- and NH_4^+ available from the rhizosphere may not reflect the actual dependence of a plant on NO_3^- and NH_4^+ assimilation because of rapid microbial transformations between NO_3^- and NH_4^+ in most soils (Burger and Jackson 2004) as well as because of the differences in root absorption of the two forms. Moreover, the extent to which NO_3^- assimilation is coupled to respiratory versus photosynthetic electron transport is difficult to assess. Consequently, relatively few laboratories have examined this topic.

In 1998 we tested the hypothesis that elevated CO_2 concentration increases absorption and assimilation of NO_3^- in wheat (Smart *et al.*, 1998). This hypothesis was based on two ideas: (i) higher carbon gain at elevated CO_2 would provide the additional energy necessary to increase NO_3^- absorption and assimilation; and (ii) higher concentrations of nonstructural carbon metabolites (glucose and sucrose) would increase the activity of NO_3^- assimilation enzymes (Kaiser and Brendle-Behnisch, 1991) and therefore would increase NO_3^- assimilation. We found, however, that wheat at elevated CO_2 failed to increase biomass production, NO_3^- absorption,

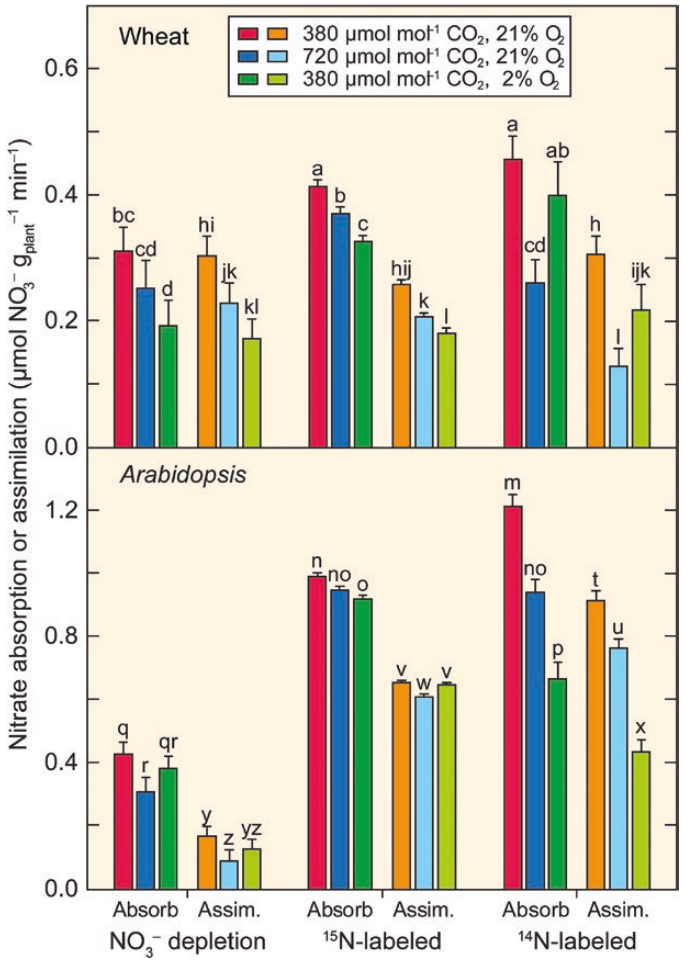

Fig. 7. Three methods for assessing nitrate absorption (Absorb) and assimilation (Assim.) in wheat and Arabidopsis plants where the shoots were exposed to atmospheres containing 380 µmol mol^{-1} CO_2 and 21% O_2, 720 µmol mol^{-1} CO_2 and 21% O_2, or 380 µmol mol^{-1} CO_2 and 2% O_2. Shown are means and SEs in µmol NO_3^- g^{-1} plant min^{-1}. Within a species and for absorption separately from assimilation, bars labeled with different letters differ significantly (P<0.05, n=6–18). (Data from Bloom *et al.*, 2010.) (This figure is available in colour at *JXB* online.)

and NO_3^- assimilation. Instead, organic N concentration declined, and NO_3^- accumulated in shoots at elevated CO_2.

In a similar experiment on tobacco, relative growth rate increased with elevated CO_2 only at the seedling stage, and decreased afterwards (Geiger *et al.*, 1998). Elevated CO_2 increased nitrate reductase activity, but the levels of unassimilated NO_3^- in plant tissues were also higher under elevated CO_2. These results suggested that elevated CO_2 impairs plant performance through a direct effect on NO_3^- metabolism. This finding has profound implications for plant responses to elevated CO_2 because most plants rely strongly on NO_3^- as N source.

In shoots, NO_3^- assimilation can be coupled to photosynthetic electron transport either directly (Bloom *et al.*, 1989) or indirectly via shuttling malate between the chloroplast and cytosol (Scheibe *et al.*, 2005). Therefore, competition for photogenerated reductant (NADPH or reduced ferrodoxin) between NO_3^- photo-assimilation and CO_2 assimilation from the light reactions of photosynthesis might decrease NO_3^- photo-assimilation at elevated CO_2 (Bloom *et al.*, 2002, 2010). Measurements of net CO_2 and net O_2 fluxes from wheat and Arabidopsis canopies showed that net CO_2 fluxes were relatively insensitive to N form while net O_2 fluxes were not (Bloom *et al.*, 2002; Rachmilevitch *et al.*, 2004). These studies showed that at light levels ([photosynthetic flux densities (PFDs)] near full sunlight, net O_2 evolution increased with rising internal CO_2 concentration (C_i) in plants receiving NH_4^+ but not NO_3^-. Thus, conditions that limited reductant production such as low light intensity or conditions that increased the competition for reductant such as elevated internal CO_2 decreased NO_3^- photo-assimilation. Presumably NO_2^- reduction proceeds only when the availability of reduced ferrodoxin exceeds that needed for the reduction of NADP to NADPH for C_3 carbon fixation. Giving priority to carbon fixation seems an appropriate strategy in that plants can store moderate levels of NO_3^- with little difficulty until reductant becomes available, but cannot directly store significant amounts of CO_2.

Another important finding was that conditions that decrease photorespiration in C_3 plants, namely elevated CO_2 or low O_2, also decrease NO_3^- assimilation (Searles and Bloom, 2003; Rachmilevitch *et al.*, 2004; Bloom *et al.*, 2010) (Fig. 7). In wheat, Arabidopsis, and seven other C_3 species, photorespiration played a crucial role in NO_3^- assimilation when assessed via methods such as gas exchange, nitrate depletion, and labeling with ^{15}N or ^{14}N. Photorespiration stimulates the export of malate from chloroplasts (Scheibe, 2004) and increases the availability of the reduced form of NADH in the cytoplasm that powers NO_3^- reduction to NO_2^- (Quesada *et al.*, 2000). In contrast, maize conducts C_4 carbon fixation that generates ample amounts of malic acid and NADH in the cytoplasm of mesophyll cells, and elevated CO_2 has little effect on NO_3^- assimilation in maize (Cousins and Bloom, 2003).

Shoot versus root NO_3^- assimilation

The balance between shoot and root NO_3^- assimilation varies within and among species (Andrews, 1986; Epstein and Bloom, 2005; Nunes-Nesi *et al.*, 2010; Andrews *et al.*, 2013). When plants are exposed to ambient CO_2 and receive sufficient rhizosphere NO_3^- to support full growth, shoots usually account for the majority of plant NO_3^- assimilation (Bloom *et al.*, 1992; Cen and Layzell, 2003). Under such conditions, the energy costs of NO_3^- assimilation are borne by photorespiration rather than by mitochondrial respiration, and thereby do not divert energy from other plant activities such as growth (Cousins and Bloom, 2004). CO_2 inhibition of both photorespiration and growth under NO_3^- nutrition in a wide variety of C_3 plants indicates that photorespiratory NO_3^- assimilation is a major contributor to the performance of a whole plant (Bloom *et al.*, 2012).

Mechanisms other than photorespiration power NO_3^- assimilation because mature C_3 plants under NO_3^- nutrition continue to grow at elevated CO_2. One such mechanism is the coupling of shoot NO_3^- assimilation to mitochondrial respiration (Cousins and Bloom, 2004). Another is root

NO_3^- assimilation. Root NO_3^- assimilation is entirely dependent on mitochondrial respiration and may even accelerate at elevated CO_2 because carbohydrate translocation to roots increases (Kruse *et al.*, 2002, 2003; Jauregui *et al.*, 2016).

Growth

Because elevated CO_2 inhibits NO_3^- assimilation in the shoots of C_3 plants, elevated CO_2 should stunt the growth of plants that rely on NO_3^- as the sole N source. The first evidence of such a growth response came from a study on wheat, in which after several weeks of growth under NO_3^- or NH_4^+ as the sole N source and at ambient or elevated CO_2, seedlings that received NH_4^+ accumulated more biomass in response to elevated CO_2 than those that received NO_3^- (Bloom *et al.*, 2002). Similar results were obtained for wheat (Carlisle *et al.*, 2012) and Arabidopsis grown to maturity at subambient, ambient, and elevated atmospheric CO_2 concentrations (310, 410, and 720 µmol mol^{-1}, respectively) (Fig. 8). In a separate study, several species conducting C_3 carbon fixation were more responsive to CO_2 enrichment when they received NH_4^+ as a sole N source than when they received NO_3^- (Bloom *et al.*, 2012) (Fig. 9).

Only a few other studies have examined the interaction between CO_2 level and N form in terms of whole plant growth or biomass production. A doubling of CO_2 level increased the biomass of *Betula alleghaniensis* by 79% under NH_4^+ nutrition and 61% under NO_3^- (Bauer and Berntson, 2001) and the biomass of *Nicotiana tabacum* by 280% under NH_4NO_3 and 240% under NO_3^- (Matt *et al.*, 2001). Neither CO_2 nor (CO_2×N form) influenced biomass accumulation in *Pinus strobes* (Bauer and Berntson, 2001) or *Lolium perenne* (Gloser *et al.*, 2002). Growth of poplar (*Populus tremula*×*P. alba*) did not respond to CO_2 when supplied with relatively large amounts of NO_3^- (Kruse *et al.*, 2003). During 5 months, the shoot growth of young coffee trees was consistently higher under CO_2 enrichment, with plants receiving NH_4^+ showing greater leaf area and total above-ground biomass than those receiving NO_3^- (Silva *et al.*, 2015). Indeed, only a few other studies on plant responses to elevated CO_2 even discuss N form. In a freshwater marsh, an NH_4^+-dominated ecosystem, plants showed little CO_2 acclimation even after a decade of CO_2 enrichment (Rasse *et al.*, 2005), and increased productivity at elevated CO_2 was associated with increased plant N uptake rather than with increased N-use efficiency (Finzi *et al.*, 2007). This field experiment supports that stimulation of photosynthesis under elevated CO_2 in an NH_4^+-dominated ecosystem is not a transient phenomenon (Ellsworth *et al.*, 2004, 2012).

Paddy rice and temperate forest trees, as mentioned in the section 'The form of N in the soil', also grow in NH_4^+-dominated soils, but may depend on NO_3^- for a significant portion of their N. Similarly, legumes may at times depend on NO_3^- as an N source. Elevated CO_2 may inhibit assimilation of this NO_3^-. Accordingly, under CO_2 enrichment, (i) rice yield increases by <12% while rice grain protein concentration decreases by >7% (Long, 2012; Myers *et al.*, 2014); (ii) tree leaf biomass does not change while tree leaf protein concentrations decrease by 14% (Feng *et al.*, 2015); and (iii) legume net annual primary productivity does not change, while legume protein concentration decreases slightly (Myers *et al.*, 2014; Feng *et al.*, 2015).

Nitrogen remobilization

Nitrogen remobilization within plants is a key factor for their N-use efficiency (Masclaux-Daubresse *et al.*, 2010). As much as 95% of wheat grain N comes from remobilization of N stored in roots and shoots acquired before anthesis (Sheibani and Ghadiri, 2012). The most common response to elevated CO_2 is a decrease in grain protein concentration (Taub and Wang, 2008). This decrease has been associated with increased yield (Fernando *et al.*, 2014; Hawkesford, 2014), suggesting involvement of biomass dilution whereby a certain amount of grain protein is diluted by the additional grain biomass accumulation under elevated CO_2

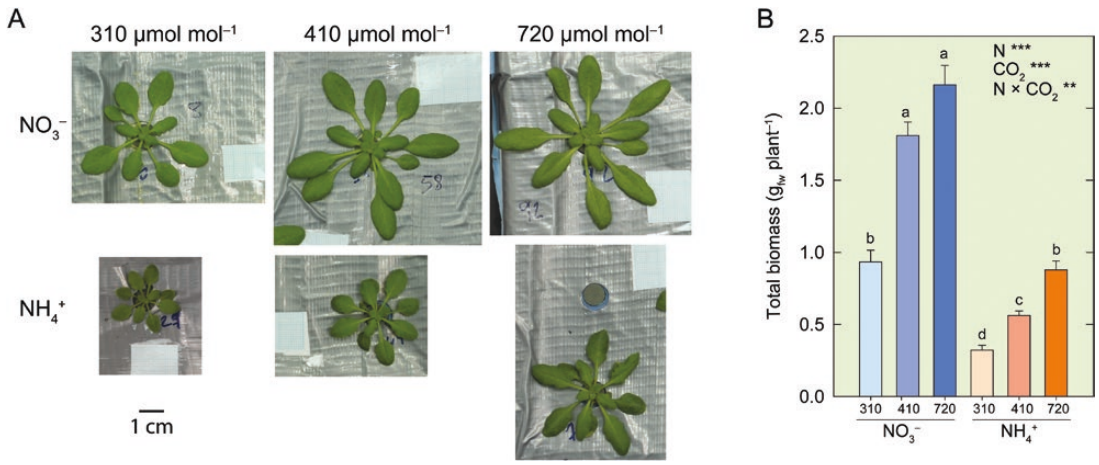

Fig. 8. (A) Growth of 32-day-old Arabidopsis plants at subambient CO_2 (310 µmol mol^{-1}), ambient CO_2 (410 µmol mol^{-1}), or elevated CO_2 (720 µmol mol^{-1}) and supplied with 0.2 mM NO_3^- or 0.2 mM of NH_4^+ as sole N source. (B) Means and SE of the total biomass of the Arabidopsis plants shown in (A). Results of ANOVA for the factors N, CO_2, and the interaction N×CO_2 are also shown. Unpublished data from AJB, University of California, Davis. (This figure is available in colour at *JXB* online.)

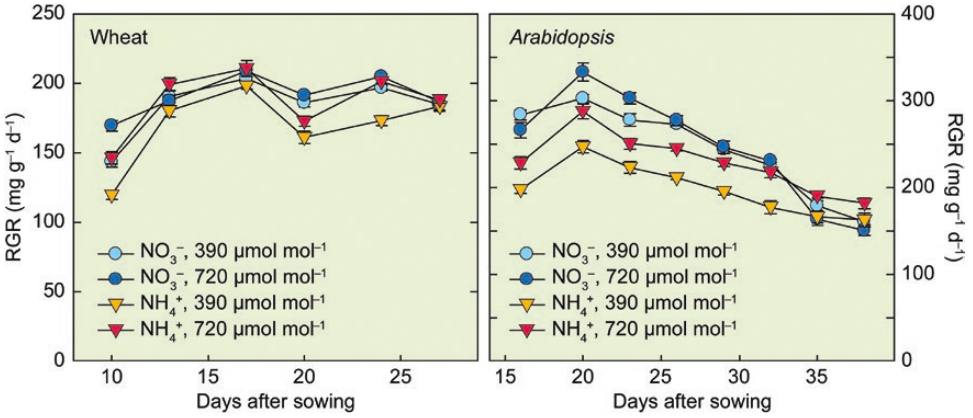

Fig. 9. Relative growth rate (on a FW basis) of wheat and Arabidopsis as affected by the form of nitrogen (NO_3^- versus NH_4^+) and atmospheric CO_2 level (390 versus 720 µmol mol^{-1}). Small error bars are incorporated into the symbols. (Data are from Bloom *et al.*, 2012, but include two additional time points for each species.) (This figure is available in colour at *JXB* online.)

(Taub and Wang, 2008). Several recent studies concluded, however, that growth dilution cannot fully explain the decline in grain protein concentration at elevated CO_2 (Taub *et al.*, 2008; Pleijel and Uddling, 2012; Feng *et al.*, 2015; Pleijel and Högy, 2015).

Slower NO_3^- assimilation per unit of plant biomass at elevated CO_2 would contribute to the decline in crop protein concentration (Bloom *et al.*, 2010; Myers *et al.*, 2014; Feng *et al.*, 2015; Jauregui *et al.*, 2015*b*). Rubisco is a major source of N for remobilization, accounting for ~50% of the total soluble protein content in the leaves of C_3 plants (Sage *et al.*, 1987). Slower NO_3^- assimilation directly decreases the amount of Rubisco in source organs; therefore, the availability of Rubisco in the source organs would limit N translocation for grain filling (Mathieu *et al.*, 2015).

Role of nitrogen form in the CO_2 acclimatation and rates of nitrogen fertilization

Most studies, as introduced above, have found that CO_2 acclimation is more pronounced in plants supplied with low amount of N fertilizer. CO_2 acclimation, however, is not a universal response even in wheat (Wall *et al.*, 2000; Weigel and Manderscheid, 2012). In fact, the interactions among atmospheric CO_2 concentrations, wheat grain yield, and grain protein are highly variable (Amthor, 2001; Wang *et al.*, 2013; Fernando *et al.*, 2014). We contend that the disparate amounts and forms of N that roots encounter in the soil solution explain a large portion of this variability. The majority of studies on plant responses to elevated CO_2 have been conducted in soils where microbial N transformations produce rapid changes in soil N pools and forms (Jackson *et al.*, 2008). Soil N pools and forms, in turn, influence both development of root systems (biomass, length, branching, and area) (Bloom *et al.*, 1993) and N uptake capabilities (Houlton *et al.*, 2007). Experiments in solution culture (hydroponics), which provide better control of the amounts and N forms to plants, suggest that plants dependent on NO_3^-—either because it is the only N form available or because a particular species preferentially uses NO_3^- rather than NH_4^+—will exhibit greater CO_2 acclimation and greater protein concentration decline.

Plants using NH_4^+ as their N source are more responsive to CO_2 in terms of growth, yield, and grain protein. Wheat grain yield increased by 11.4% while N accumulated across the 3 years at elevated CO_2 in plants fertilized with ammonium and urea (Han *et al.*, 2015). Thus, most agricultural systems will benefit from greater reliance on NH_4^+ as atmospheric CO_2 rises. Application of NH_4^+ fertilizers plus inhibitors of soil nitrification (microbial conversion of NH_4^+ to NO_3^-) might avoid the bottleneck of NO_3^- assimilation, but would require sophisticated fertilizer management to prevent NH_4^+ toxicity. To address these issues, a better understanding of plant NH_4^+ and NO_3^- assimilation is critical.

Concluding remarks

This article examines plant adaptations to the elevated atmospheric CO_2 concentrations that are anticipated during the next few decades. In particular, extensive evidence is now available that N form, NO_3^- versus NH_4^+, is a key factor in plant responses to elevated CO_2. Recent results support that: (i) responses to elevated CO_2 in different plant species can be better understood if one accounts for N form; (ii) plant–soil interactions have a major influence on plant responses to elevated CO_2; (iii) the influence of N form on plant performance may exceed that of elevated CO_2, and thus may confound interpretation of studies where plants have access to both N forms; (iv) elevated CO_2 may favor the absorption of one form of N over others; and (v) elevated CO_2 inhibits shoot NO_3^- assimilation in C_3 plants, and so plants receiving NH_4^+ nutrition exhibit greater stimulation from CO_2 enrichment.

The role of NO_3^- and NH_4^+ in plant growth and development under elevated CO_2 will become increasingly important at several levels: (i) food quality will suffer, because NO_3^- is the dominant N form in most agricultural systems, and elevated CO_2 inhibits conversion of NO_3^- into protein; (ii) species distributions will change, because C_3 species that prefer NO_3^- as an N source will be at a competitive disadvantage; and (iii) the capacity to sequester CO_2 in organic compounds

will be compromised in plants reliant on NO_3^- as an N source. Normally, both NO_3^- and NH_4^+ are available from the rhizosphere, but data are sparse on the extent to which plants absorb and assimilate each N form and how this affects their responses to elevated CO_2 atmospheres. Such information will be critical for human well-being.

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