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# Will rising atmospheric $CO_2$ concentration inhibit nitrate assimilation in shoots but enhance it in roots of $C_3$ plants?

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Bloom et al. (2019) proposed that rising atmospheric $CO_2$ concentrations "inhibit malate production in chloroplasts and thus impede assimilation of nitrate into protein of $C_3$ plants, a phenomenon that will strongly influence primary productivity and food security under the environmental conditions anticipated during the next few decades". Previously we argued that the weight of evidence in the literature indicated that elevated atmospheric [$CO_2$] does not inhibit $NO_3^-$ assimilation in $C_3$ plants (Andrews et al. 2019). New data for common bean (*Phaseolus vulgaris*) and wheat (*Triticum aestivum*) were presented that supported this view and indicated that the effects of elevated atmospheric [$CO_2$] on nitrogen (N) assimilation and growth of $C_3$ vascular plants were similar regardless of the form of N assimilated. Bloom et al. (2019) strongly criticised the arguments presented in Andrews et al. (2019). Here we respond to these criticisms and again conclude that the available data indicate that elevated atmospheric [$CO_2$] does not inhibit $NO_3^-$ assimilation of $C_3$ plants. Measurement of the partitioning of $NO_3^-$ assimilation between root and shoot of $C_3$ species under different $NO_3^-$

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supply, at ambient and elevated $CO_2$ would determine if their $NO_3^-$ assimilation is inhibited in shoots but enhanced in roots at elevated atmospheric $CO_2$.

**Introduction**

Nitrate ($NO_3^-$) is likely to be the main form of nitrogen (N) available to, and taken up and assimilated by, most vascular plants in disturbed/ cultivated (well aerated) soils (Andrews et al. 2013; Cameron et al. 2013). Most $C_3$ vascular plants have the ability to assimilate $NO_3^-$ in their root and shoot with the relative importance of the two parts of the plant dependent on genotype and environmental conditions, in particular, $NO_3^-$ availability (Andrews 1986). For many species, the proportion of total plant $NO_3^-$ assimilation in the shoot increases with increased $NO_3^-$ supply. Within the plant, $NO_3^-$ is reduced to $NO_2^-$ by the enzyme nitrate reductase (NR) and then this $NO_2^-$ is reduced to $NH_4^+$ by nitrite reductase (NiR). In turn, the $NH_4^+$ is assimilated into the amino acids glutamine and glutamate via the glutamine synthetase (GS)/ glutamate synthase (GOGAT) pathway (Andrews et al. 2004; Lea and Miflin, 2011; Xu et al. 2012).

Nitrate reductase is located in the cytosol of root and shoot cells and, for most species tested, uses NADH as the reductant for the conversion of $NO_3^-$ to $NO_2^-$ (Xu et al. 2012). The NiR enzyme ($NO_2^- > NH_4^+$) is located in the plastids of roots and other non-photosynthetic tissue, and the chloroplasts of photosynthetic tissue, and uses ferredoxin (Fd) as a reductant (Hanke and Mulo 2013). Within the plastids/ chloroplasts, GS (GS2) catalyses the ATP-dependent conversion of this $NH_4^+$ and glutamate to glutamine, while GOGAT catalyses the NADH- or Fd- dependent conversion of glutamine and 2-oxoglutarate to form two molecules of glutamate. The NADH-dependent GOGAT is located predominantly in non-photosynthesising cells where reductant for $NO_3^-$ reduction and glutamate synthesis is initially supplied by the oxidative pentose phosphate pathway (Bowsher et al. 2007). The Fd-dependent GOGAT activity is much greater than NADH-GOGAT in shoot/ leaves, where the ATP and reductant for NiR, GS2 and Fd-GOGAT can be derived directly from photosystems I and II in illuminated chloroplasts (Lea and Miflin 2011).

Over several papers, Bloom and co-workers argued that $C_3$ plants respond more positively to elevated [$CO_2$] with $NH_4^+$ (assimilated primarily in roots) than with $NO_3^-$ as N source because elevated $CO_2$ inhibits their photoreduction of $NO_3^-$ and hence reduces total

plant N assimilation and growth (Bloom 2015a,b; Rubio-Asensio and Bloom 2017). They argued that under ambient $CO_2$, photorespiration stimulates the export of malate from chloroplasts to the cytoplasm and this malate in the cytoplasm generates NADH that drives the reduction of $NO_3^-$ to $NO_2^-$. Under elevated $CO_2$, however, photorespiration is inhibited which causes a decrease in transport of malate from the chloroplast to the cytoplasm and consequently decreased generation of NADH and associated $NO_3^-$ assimilation in the cytoplasm. Previously we argued that the weight of evidence in the literature indicates that elevated atmospheric [$CO_2$] does not inhibit $NO_3^-$ assimilation in $C_3$ plants (Andrews et al. 2019). Also, new data for common bean (*Phaseolus vulgaris*) and wheat (*Triticum aestivum*) were presented that supported this view and suggested that the effects of elevated atmospheric [$CO_2$] on N assimilation and growth of $C_3$ vascular plants will be similar regardless of the form of N assimilated. Bloom et al. (2019) responded to Andrews et al. (2019) and three other papers (Dier et al. 2018; Abadie and Tcherkez 2019; Tcherkez and Limami 2019) that "purport to present counterevidence" to their proposal "that rising atmospheric $CO_2$ concentrations inhibit malate production in chloroplasts and thus impede assimilation of $NO_3^-$ into protein in shoots of $C_3$ plants". Here we focus on their response to Andrews et al. (2019).

**Response to Bloom et al. (2019)**

Bloom et al. (2019) listed several points in our paper (Andrews et al. 2019) where in their view we made false claims in relation to the literature or misinterpreted it. For example, the first point raised was that our statement "the weight of evidence in the literature indicates that elevated atmospheric [$CO_2$] does not inhibit $NO_3^-$ assimilation and growth of $C_3$ vascular plants" consists of four studies that exposed plants to a specific nitrogen form. This is a misinterpretation of our arguments. The weight of evidence in the literature that we referred to included considerable data sets from free air carbon dioxide (FACE) trials carried out under conditions (cultivated/ aerated soils) in which $NO_3^-$ was likely to have been the main form of N available to plants (Andrews et al. 2013, 2019). These studies indicated that a wide range of $C_3$ species, including wheat, show increased growth under $CO_2$ enrichment, especially if they receive high applications of N (a selected eight references were given for this point that included meta-analyses and reviews). Continuing, Bloom et al.

(2019) stated that the four studies we highlighted actually support their conclusions: this is not the case. These studies indicated that for wheat (Hocking and Meyer 1991), tobacco (*Nicotiana tabacum*; Geiger et al. 1999; Matt et al. 2001) and cucumber (*Cucumis sativus*; Dong et al., 2017) supplied $NO_3^-$ as the sole N source under controlled environment or glasshouse conditions, greatest growth and reduced N accumulation across treatments occurred under elevated $CO_2$ with high $NO_3^-$ supply. Considering the first study (Hocking and Meyer 1991), Bloom et al. (2019) argued that wheat with $NO_3^-$ as a sole N source accumulated less reduced N per DW in its shoots under elevated than ambient $CO_2$ affirming that elevated $CO_2$ inhibited total $NO_3^-$ assimilation. This is a major point of difference in our views. The results of Hocking and Meyer (1991) for wheat are similar to those presented for wheat and common bean under $NO_3^-$ nutrition in Andrews et al. (2019). Specifically, elevated $CO_2$ substantially increased growth of common bean and wheat under $NO_3^-$ nutrition. Also, for both species, at limiting and optimal $NO_3^-$ supply, total plant reduced N was greater at elevated than ambient $CO_2$ indicating that greater $NO_3^-$ assimilation had occurred at elevated $CO_2$. Nevertheless, the proportional increase in total plant N content was not as great as that for DW and thus tissue N content per unit DW was consequently lower with elevated $CO_2$. The results of Hocking and Meyer (1991) must be interpreted carefully as elevated $CO_2$ was only supplied during the day and it could be argued that plants may have increased the proportion of $NO_3^-$ assimilation carried out at night to mitigate $CO_2$ inhibition of shoot $NO_3^-$ assimilation during the light period. However, elevated $CO_2$ was maintained over the 24 h in the three other studies highlighted (Geiger et al. 1999; Matt et al. 2001; Dong et al. 2017).

The increase in total plant DW relative to total plant N and the resultant decrease in tissue N per unit DW at elevated $CO_2$ has been termed 'N dilution' and has been linked to increased accumulation of non-structural carbohydrates and plant secondary compounds (Taub and Wang 2008). In some cases, elevated $CO_2$ can increase photosynthesis in the short term, but if photosynthate utilisation is inadequate, a source sink imbalance can arise, leading to end-product (carbohydrate) accumulation and subsequent down-regulation of photosynthesis linked to lower ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) concentration and activity (Ainsworth and Rogers 2007; Zheng et al. 2019; Beechey-Gradwell et al. 2020). In their Summary, Bloom et al. (2019) stated that "hundreds of papers" support their proposal that rising atmospheric $CO_2$ concentrations inhibit malate

production in chloroplasts and thus impede assimilation of $NO_3^-$ into protein in shoots of $C_3$ plants. We strongly disagree with this statement, but many papers do report a decrease in tissue N/ protein content $g^{-1}$ DW with elevated $CO_2$. We emphasise that the general effects of elevated [$CO_2$] on growth and N assimilation of wheat with $NO_3^-$ or $NH_4^+$ as N source, and common bean under $NO_3^-$, $NH_4^+$, urea and $N_2$ fixation nutrition were similar regardless of N form supplied. In all cases, total plant DW and total plant reduced N were greater at elevated [$CO_2$] but tissue N $g^{-1}$ DW was lower. These results led to our suggestion that the effects of elevated atmospheric $CO_2$ concentration on N assimilation and growth of $C_3$ plants will be similar regardless of the form of N assimilated including $NO_3^-$.

We highlight one other area of our work criticised by Bloom et al. (2019). Bloom et al. (2019) stated that we used shoot nitrate reductase activity (NRA) and shoot organic N concentration as proxy measures for shoot $NO_3^-$ assimilation *in planta* but NRA seldom limits $NO_3^-$ assimilation, and organic N in shoots is derived not only from shoot $NO_3^-$ assimilation but also from import of amino acids generated by $NO_3^-$ assimilation in roots. The main point made in Andrews et al. (2019) relating to the experiments carried out was that for wheat and common bean under low and high $NO_3^-$ supply, total plant reduced N was greater at elevated than ambient $CO_2$. For both species, the shoot is likely to have been the main site of $NO_3^-$ assimilation at ambient $CO_2$ (Andrews et al. 1992, 2013). However, we acknowledge that we did not measure the contribution of the root to reduced N in the shoot of either species at ambient or elevated $CO_2$. Generally, NR is a substrate ($NO_3^-$) induced enzyme and tissue NRA often correlates with tissue $NO_3^-$ assimilation although we concede it is unlikely to give an accurate measure of its $NO_3^-$ assimilation *in situ* (Andrews et al. 2013; Bloom et al. 2019). In Andrews et al. (2019), leaf NRA for wheat increased with increased $NO_3^-$ supply and the associated increased total plant reduced N at ambient and elevated $CO_2$. Also, the *in vivo* NRA assay used relies on endogenous NADH to reduce $NO_3^-$ to $NO_2^-$ (NADH is not included in the assay buffer), and thus similar values for lamina NRA at ambient and elevated $CO_2$ indicate that NADH was not limiting $NO_3^-$ reduction under elevated relative to ambient $CO_2$. Bloom et al. (2019) stated that exposure to elevated $CO_2$ atmosphere stimulates root assimilation. Again, we did not determine if this was the case for wheat or common bean in our study. However, in our view, if elevated $CO_2$ did inhibit shoot $NO_3^-$ assimilation, it seems highly unlikely that for common bean or wheat, a shift from shoot to root $NO_3^-$ assimilation could be great enough to give increased $NO_3^-$

assimilation per plant with elevated than ambient $CO_2$ at optimum $NO_3^-$ supply but this needs testing.

The two most common methods used to quantify the partitioning of $NO_3^-$ assimilation between root and shoot are measurement of the relative proportions of total plant NRA in the two plant parts and xylem sap analysis for $NO_3^-$ and reduced N. The proportion of xylem sap N as $NO_3^-$ -N is taken as the proportion of total plant $NO_3^-$ assimilation carried out in the shoot. Data from both sets of measurements must be interpreted carefully. For example, as outlined above, tissue NRA is unlikely to give an accurate measure of $NO_3^-$ assimilation in situ. The main weakness of xylem sap analysis for $NO_3^-$ and reduced N is that it does not indicate the proportion of xylem sap N that is cycling (as organic N) between root and shoot. This can be substantial in some cases. Modelling approaches have been developed to counter possible inaccuracies in determining the partitioning of $NO_3^-$ assimilation between root and shoot from NRA distribution in the plant and xylem sap analysis. Generally, models involve the quantitative measurement of $NO_3^-$ uptake, its movement, storage and assimilation in the different parts of the plant and cycling of reduced N in the phloem and xylem (Jeschke and Pate 1991). We agree with Bloom et al (2019) that for some species, root assimilation increases in importance under elevated $CO_2$ but data are few and this effect is inconsistent (Table 1). For example, focussing on the studies quoted by Bloom et al. (2019), a quantitative modelling approach indicated that the proportion of total plant $NO_3^-$ assimilation in the shoot decreased from 80% at ambient $CO_2$ to 57% at elevated $CO_2$ for *Nicotiana tabacum* supplied 5 mol $m^{-3}$ applied $NO_3^-$ (Kruse et al. 2002). However, for poplar (*Populus tremula* x *P. alba*), a quantitative modelling approach indicated that elevated $CO_2$ shifted the partitioning of $NO_3^-$ assimilation towards the root at low $NO_3^-$ supply but not at high $NO_3^-$ supply (Kruse et al. 2003). The NRA distribution data of Jauregui at al. (2016) indicated that for Arabidopsis supplied 0.8 mol $m^{-3}$ $NO_3^-$, almost all $NO_3^-$ was assimilated in the shoot at ambient and elevated $CO_2$ (Table 1).

Bloom et al. (2019) presented new data on $^{15}N$ isotope discrimination in wheat and Arabidopsis that they claim show shoot $NO_3^-$ assimilation decreased and root assimilation increased under elevated $CO_2$, indicating that elevated $CO_2$ inhibited shoot $NO_3^-$ assimilation while it enhanced root $NO_3^-$ assimilation. Their approach is limited for several reasons. For example, there are no data on the N isotope ratio of the source $NO_3^-$, so absolute values for

discrimination cannot be calculated and in the absence of dry matter data for root and shoot, a mass balance of the isotopes is not possible. The measurements of isotope ratios in the roots and in the shoots include both organic and inorganic N. The extent of discrimination in organic N in the roots depends not only on the root NRA but also on the $NO_3^-$ efflux as a fraction of $NO_3^-$ influx. The shoot delta $^{15}N$ gives no independent evidence of shoot $NO_3^-$ assimilation into organic matter. Also, a shift in the partitioning of $NO_3^-$ assimilation from shoot to root under elevated $CO_2$ supply does not confirm inhibition of $NO_3^-$ assimilation in photosynthetic tissue. For example, there is evidence for barley (*Hordeum vulgare*) that reduced levels of reductant (NADH) limit $NO_3^-$ assimilation in roots at high $NO_3^-$ supply (Andrews et al. 1992). Greater root $NO_3^-$ assimilation under elevated $CO_2$ could be due to greater transport of photosynthate to the root which is utilised in the production of reductant and increased root biomass (Hocking and Meyer 1991; Andrews et al. 2006).

**Conclusions and a way forward**

In our view, as argued above, the weight of evidence in the literature indicates that elevated atmospheric [$CO_2$] does not inhibit $NO_3^-$ assimilation in $C_3$ plants (Andrews et al. 2019). If, as proposed by Bloom et al. (2019), inhibition of photorespiration causes a decrease in transport of malate from the chloroplast to the shoot cytoplasm and consequently decreased generation of NADH in the cytoplasm this does not impact on $NO_3^-$ assimilation at plant level. Indeed, there are reports for several $C_3$ species that $NO_3^-$ assimilation per plant was greater at elevated $CO_2$. Nevertheless, total plant DW also increased with elevated $CO_2$, and the proportional increase in total plant N content was not as great as that for DW thus tissue N content per unit DW was lower with elevated $CO_2$. Detailed studies of the partitioning of $NO_3^-$ assimilation between root and shoot of a range of $C_3$ species under different $NO_3^-$ supply, at ambient and elevated $CO_2$ would be a next step to determine if rising atmospheric $CO_2$ concentration inhibits $NO_3^-$ assimilation in shoots but enhances it in roots of this group of plants. Arabidopsis should be included in the study to relate to previous work and also to determine how elevated $CO_2$ affects expression of genes involved in $NO_3^-$ assimilation and associated processes.

**Author contributions**

MA drafted the manuscript with input from all authors and all authors agreed on the final version of the manuscript.

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**Table 1.** Collated values for the effect of elevated atmospheric [$CO_2$] on the proportion of nitrate ($NO_3^-$) assimilation carried out in the shoot of vascular plant species as indicated by the distribution of nitrate reductase activity (NRA) between the two plant parts, xylem sap analysis (XSA) for $NO_3^-$ and reduced N or a quantitative model (M).

| Species | % $NO_3^-$ assimilation in shoot ($NO_3^-$ supply) | | Method used | Comments | References |
|---|---|---|---|---|---|
| | (≤1 mol $m^{-3}$) | (>1.0 mol $m^{-3}$) | | | |
| *Arabidopsis thaliana* | 96 (0.8)<br>97 (0.8) | | NRA | Unaffected by elevated [$CO_2$] | Jauregui et al. (2016) |
| *Betula alleghaniensis* | | 49 (1.6)<br>76 (1.6) | NRA | Decreased at elevated [$CO_2$] | Bauer and Berntson (2001) |
| *Nicotiana tabacum* | | 75 (5)<br>78 (5) | XSA | Unaffected by elevated [$CO_2$] | Kruse et al. (2002) |
| | | 57 (5)<br>80 (5) | M | Decreased at elevated [$CO_2$] | |
| | 88 (1)<br>89 (1) | 89 (2)<br>89 (2) | NRA | Unaffected by elevated [$CO_2$] | Matt et al. (2001) |
| *Pinus stroba* | | 3 (1.6)<br>10 (1.6) | NRA | Unaffected by elevated [$CO_2$] | Bauer and Berntson (2001) |
| *Populus tremula* × *P. alba* | 4 (0.2)<br>30 (0.2) | 44 (2)<br>47 (2) | XSA/M | Decreased at elevated [$CO_2$] at low but not high $NO^-$ supply | Kruse et al. (2003) |