

Viewpoints

Net photosynthetic CO₂ assimilation: more than just CO₂ and O₂ reduction cycles

Summary

Net photosynthetic assimilation in C₃ plants is mostly viewed as a simple balance between CO₂ fixation by Rubisco-catalyzed carboxylation and CO₂ production by photorespiration (and to a lower extent, by day respiration) that can be easily manipulated during gas exchange experiments using the CO₂ : O₂ ratio of the environment. However, it now becomes clear that it is not so simple, because the photosynthetic response to gaseous conditions involves 'ancillary' metabolisms, even in the short-term. That is, carbon and nitrogen utilization by pathways other than the Calvin cycle and the photorespiratory cycle, as well as rapid signaling events, can influence the observed rate of net photosynthesis. The potential impact of such ancillary metabolisms is assessed as well as how it must be taken into account to avoid misinterpretation of photosynthetic CO₂ response curves or low O₂ effects in C₃ leaves.

Introduction

Photosynthesis of C₃ plants is the net result of concurrent processes in which CO₂ is fixed (carboxylation) and released (photorespiration, day respiration). Gas exchange measurements are essential for the characterization of leaf photosynthetic properties, including stomatal conductance, carboxylation rate, or water use efficiency. The cornerstone of photosynthesis is the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), that can fix either CO₂ (carboxylation) or O₂ (oxygenation). Oxygenation is the starting point of photorespiration, in which CO₂ is liberated in mitochondrial glycine-to-serine conversion by the glycine decarboxylase-serine hydroxymethyl transferase (GDC-SHMT) complex. Rubisco's bi-functionality is visible in photosynthetic Rubisco-limited models of net assimilation (A) based on Michaelis kinetics as follows (Farquhar *et al.*, 1980; Von Caemmerer & Farquhar, 1981; Von Caemmerer, 2013):

$$A = v_c \times \left(1 - \frac{\Gamma^*}{c_c}\right) - R_d \quad \text{Eqn 1}$$

where v_c is ribulose 1,5-bisphosphate (RuBP) carboxylation rate, Γ^* the compensation point in the absence of day respiration, c_c the

CO₂ mole fraction at carboxylation sites, and R_d day respiration (nonphotorespiratory CO₂ evolution). Here, Γ^* reflects the intrinsic O₂ : CO₂ partitioning of Rubisco fixation and subsequent photorespiratory CO₂ evolution. Hence, Γ^* can vary slightly if the stoichiometry of photorespiratory CO₂ release with respect to O₂ fixation changes from the theoretical value of one half (Cousins *et al.*, 2011; Keech *et al.*, 2012; Abadie *et al.*, 2016a). Since Rubisco is not the sole limiting factor under a range of CO₂ : O₂ conditions, the more general expression of assimilation is given by:

$$A = w \times \left(1 - \frac{\Gamma^*}{c_c}\right) - R_d \quad \text{Eqn 2}$$

where w stands for the minimum of Rubisco carboxylation, RuBP regeneration and triose phosphates utilization. It can be seen in Eqn 2 that if there are alternative sinks (that may or may not be directly associated with CO₂ fixation or release), A would be impacted because electron or triose phosphates consumption affect w . In addition, Eqns 1 and 2 neglect the contribution of non-Rubisco carboxylation events (e.g. anaplerotic CO₂ fixation), overlooks the contribution of processes other than (photo) respiration in CO₂ release, and assumes that R_d is constant. Still, they are extremely efficient in modeling photosynthesis in the vast majority of cases, because alternative CO₂ influx or efflux processes (or alternative electron consumptions) are associated with rather modest fluxes. That is, measurements are often performed using unstressed plants under conditions where photosynthesis is sufficiently high, e.g. at ambient CO₂ and saturating light. However, it becomes problematic when photosynthesis is examined at extreme CO₂ mole fractions, typically near or below the compensation point (where A has low values), because usually minor processes can become proportionally large. It can also be of importance when very fine measurements (such as internal conductance) are performed because even small numerical errors have pervading consequences (Harley *et al.*, 1992; Hanson *et al.*, 2016). Furthermore, day respiration (R_d) encompasses all sorts of CO₂-producing events, from the tricarboxylic acid pathway (TCAP) to the oxidative pentose phosphate pathway. The assumption that R_d remains constant is currently believed to be incorrect, considering direct measurements or metabolic studies that all suggest R_d varies with light intensity, CO₂ and O₂ (Tcherkez *et al.*, 2017).

Despite these limitations, CO₂ response curves of net photosynthesis have been used worldwide for > 30 years to compute maximal carboxylation capacity or maximal electron fluxes and also estimate internal conductance and day respiration. As a result, the seminal paper describing mathematical relationships in gas exchange (Von Caemmerer & Farquhar, 1981) has been cited > 4400 times. Recent studies have further discussed how photosynthetic models and CO₂ response curves might be exploited

using Eqn 2 to infer information on photorespiratory nitrogen metabolism (Busch *et al.*, 2018) (as has been done before for phosphate utilization (Harley & Sharkey, 1991)), or suggested alternative mechanisms for CO₂ efflux (Cousins *et al.*, 2011; Bloom & Lancaster, 2018). More generally, as we get more precision in photosynthetic measurements (including online isotopic analyses), it is tempting to infer as many parameters as possible (e.g. internal conductance).

However, to interpret photosynthetic response curves appropriately, metabolic imperatives of nonphotosynthetic and nonphotorespiratory pathways (that is, metabolisms other than simply photosynthesis and photorespiration) which both depend on CO₂ (or O₂) mole fraction in the short-term and influence leaf net CO₂ exchange, have to be taken into account. In fact, even though their associated fluxes are generally small, there might be particular situations where they have a significant impact on the photosynthesis rate and/or parameters derived therefrom.

In this Viewpoint, we intend to examine such nonphotosynthetic and nonphotorespiratory metabolic processes and their dependence on CO₂ (or O₂), using recent results that have provided molecular or metabolic insights on short-term response to CO₂ and O₂, with associated experimental flux measurements in C₃ plants (summarized in Fig. 1). Here, we will not discuss photochemistry (and associated redox control) or day respiration themselves, which have been shown to be influenced by CO₂ : O₂ as explained elsewhere (Heber *et al.*, 1992; Laisk *et al.*, 1997; Tcherkez *et al.*, 2017). As will become apparent later, quantitative data on metabolic fluxes associated with nonphotorespiratory and nonphotosynthetic processes in illuminated leaves are relatively

scarce and the present article should be viewed as a call for more research in this area.

Alternative carboxylation activity

In C₃ plants, Rubisco is the CO₂-fixing enzyme of photosynthesis (as reflected in photosynthetic models, see earlier). However, there are other carbon-fixing enzymes, in different metabolic pathways: phosphoenolpyruvate carboxylase (PEPC), carbamoyl phosphate synthase (CPS), acetyl-CoA carboxylase, etc. Amongst these, PEPC is certainly the most significant considering both its protein abundance and carbon flux (Chollet *et al.*, 1996). However, it has been recently suggested that the reverse reaction catalyzed by chloroplastic NADP-dependent malic enzyme (CO₂ + pyruvate + NADPH → malate + NADP) is of importance (Bloom & Lancaster, 2018). This is extremely unlikely, considering the very high *K_m* (Michaelis constant) of the enzyme (in mM) compared to the very low concentration of stromal pyruvate (in μM) (Wheeler *et al.*, 2008; Krueger *et al.*, 2011), the unfavorable NADPH : NADP ratio in the light (≈ 1 in the steady-state) (Takahama *et al.*, 1981), and the low carbon-13 (¹³C) enrichment in C-atom position of malate other than C-1 and C-4 during ¹³CO₂ labeling (Tcherkez *et al.*, 2009). In fact, if the chloroplastic malic enzyme synthesized malate, it would consume chloroplastic pyruvate. Therefore, malate molecules would inherit the ¹³C-pattern of chloroplastic pyruvate in C-1 to C-3 atom positions, while it would inherit the ¹³C signature of dissolved CO₂ in C-4. Since chloroplastic pyruvate is highly ¹³C-labeled (Abadie *et al.*, 2018), there would be little difference in the positional %¹³C between

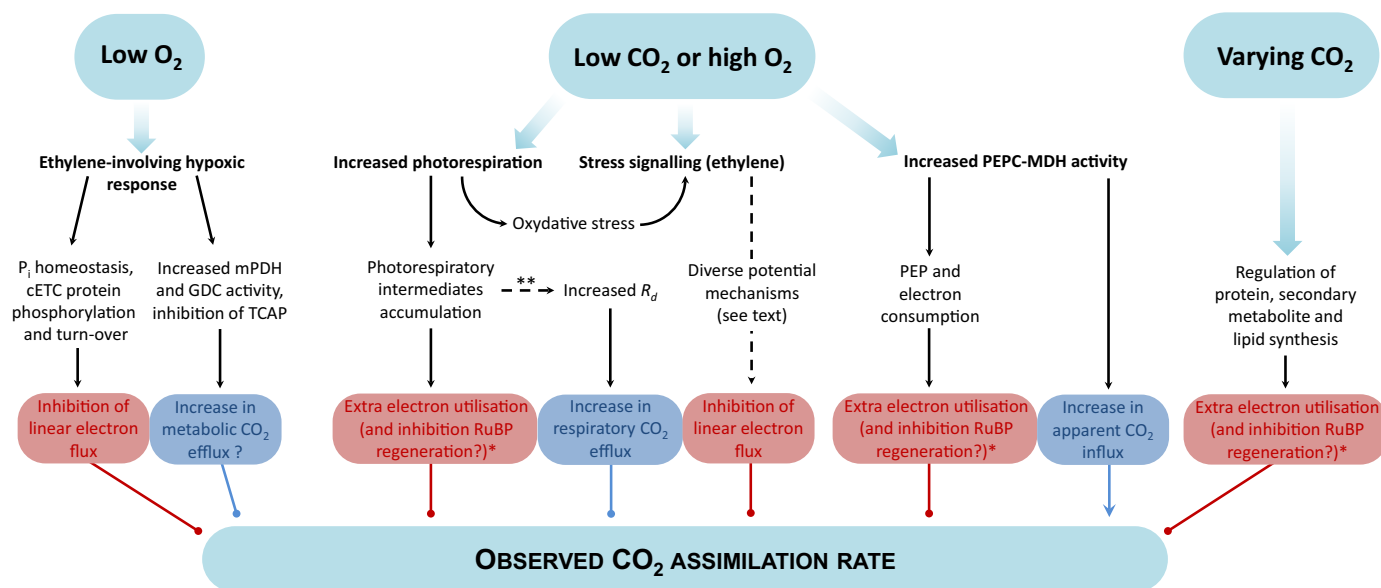


Fig. 1 Summary of metabolic pathways affected by CO₂ : O₂ in the short-term and possibly affecting observed CO₂ assimilation rate. For simplicity, effects on photosynthesis are shown as falling into two categories: effect on chloroplastic electron transfer chain and/or ribulose 1,5-bisphosphate (RuBP) regeneration (red) and on CO₂ production or consumption (blue). See the text for more details on numerical flux values. The asterisk (*) stands for possible variation in the net effect due to possible decrease on redox pressure (e.g. under high light) and re-utilization of chloroplastic phosphate to facilitate RuBP regeneration (Harley & Sharkey, 1991). The double asterisks (**) represent the link between the nitrogen demand by photorespiration and the stimulation of day respiration, further discussed elsewhere (Tcherkez *et al.*, 2017). cETC, chloroplastic electron transport chain; GDC, glutamate decarboxylase; mPDH, mitochondrial pyruvate dehydrogenase; PEP, phosphoenolpyruvate; P_i, free phosphate; R_d, day respiration; TCAP, tricarboxylic acid pathway.

C-atom positions in malate synthesized *de novo*. Experimental evidence is to the contrary (Abadie & Tcherkez, 2019).

PEPC fixes bicarbonate onto phosphoenolpyruvate and forms oxaloacetate in the cytosol, which can be then reduced to malate (by malate dehydrogenases, MDHs) in various cellular compartments. Isotopic labeling has demonstrated that this sequence of two reactions does occur in the illuminated leaf (Tcherkez *et al.*, 2009; Abadie *et al.*, 2017b). PEPC-derived oxaloacetate is also essential to sustain aspartate production, as demonstrated by both intramolecular natural ^{13}C abundance in leaf aspartate (Melzer & O'Leary, 1987) and isotopic labeling (Abadie *et al.*, 2017b). Nevertheless, it is generally assumed that PEPC activity is of little importance for steady-state C_3 photosynthesis (Von Caemmerer, 2013) and it is commonly not included in photosynthetic models while it is recognized to contribute to lowering the observed $^{12}\text{C}/^{13}\text{C}$ isotope fractionation during photosynthesis (Raven & Farquhar, 1990).

PEPC-MDH activity is extremely relevant for photosynthesis measurement since it leads to CO_2 fixation (via the rapid equilibrium between CO_2 and bicarbonate) thus potentially, can increase apparent photosynthesis rates. It also consumes two electrons per CO_2 to reduce oxaloacetate to malate, thereby impacting slightly the quantum yield of photosynthesis. In addition, PEPC-MDH activity is directly linked to (1) photorespiration via the utilization of reductive power generated by the GDC-SHMT complex and (2) TCAP replenishment to sustain glutamate and glutamine metabolism which is stimulated by photorespiration. In fact, mutants affected in MDH show an alteration in photorespiratory metabolism (Cousins *et al.*, 2008; Tomaz *et al.*, 2010) and mutants affected in glutamine-2-oxoglutarate amino transferase do exhibit an increase in PEPC activity that correlates to leaf glutamine content (Ferrario-Mery *et al.*, 2002). Quite critically, PEPC activity does vary with CO_2 and O_2 mole fraction. First, the enzyme appears to be slightly less phosphorylated at high $\text{CO}_2 : \text{O}_2$ ratio, suggesting a decline in activity. Second, phosphoenolpyruvate provision by pyruvate Pi dikinase (PPDK) varies, due to a significant loss in PPDK phosphorylation and increase in activity at low CO_2 (Abadie *et al.*, 2016b). Third, direct *in vivo* PEPC-MDH activity measurements by NMR have shown that while being rather small at high photosynthesis ($0.3 \mu\text{mol m}^{-2} \text{s}^{-1}$), PEPC activity increases to $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ or more at low CO_2 ($140 \mu\text{mol mol}^{-1}$) or high oxygen (100% O_2), representing up to 40% of observed net assimilation under high O_2 mole fraction (Abadie & Tcherkez, 2019). Taken as a whole, anaplerotic CO_2 fixation is not a negligible actor in CO_2 response curves, and rather counterintuitively, it decreases as CO_2 increases.

Electron and carbon utilization by anabolism

It is well-known that nitrogen (or sulfur) assimilation, amino acid and protein synthesis, lipid production and secondary metabolism represent significant carbon and electron sinks, thereby potentially inhibiting sucrose synthesis or RuBP regeneration and thus CO_2 assimilation. The extra electron consumption causes an 'offset' between total electron flux (for example measured by fluorescence

or O_2 evolution), and electron consumption for CO_2 (and O_2) fixation only. Experimentally, it could be desirable to ensure that such an offset is 'real' by comparing fluorescence-derived electron transport rate (ETR) and maximal electron transport computed from photosynthetic response curves (A/c_c curves). We note, however, that such a comparison can be complicated by (1) variations in the coefficient used to convert the yield of PSII photochemistry (ΦPSII) to ETR due to changes in, e.g. pseudocyclic electron flux, and (2) the fact that alternative fluxes are relatively small and possibly within the experimental error of ETR estimates. Regardless of these technical issues, several studies have taken advantage of the offset to estimate the electron flux to nitrate (nitrite) reduction using the assimilatory quotient (Rachmilevitch *et al.*, 2004; Bloom *et al.*, 2010; Eichelmann *et al.*, 2011). They have suggested that nitrogen assimilation is promoted under photorespiratory conditions (this conclusion being also supported by other measurements such as enzyme activities, and natural nitrogen-15 (^{15}N) abundance) (Rachmilevitch *et al.*, 2004; Bloom *et al.*, 2010). However, the other earlier-cited electron-consuming biosynthetic processes should not be overlooked. In fact, in Arabidopsis, cytosolic protein synthesis has been shown to depend on photosynthesis, with much higher polysome abundance at light (compared to dark) (Piques *et al.*, 2009) and a positive effect of CO_2 mole fraction on the phosphorylation of the ribosomal protein RPS6 and the translation initiation factor eIF4B, and a negative effect on the translation initiation inhibitor eIF4A (Boex-Fontvieille *et al.*, 2013). Accordingly, isotopic labeling of peach leaves with $^{14}\text{CO}_2$ along a A/CO_2 curve has shown that protein synthesis increases proportionally with photosynthesis (at about 1% of photosynthesis) as CO_2 mole fraction increases (Escobar-Gutiérrez & Gaudillère, 1997). These results clearly indicate that protein production is not constant and when expressed in absolute units ($\mu\text{mol m}^{-2} \text{s}^{-1}$), increases with photosynthesis.

Chloroplastic fatty acid synthesis also strictly occurs in the light, and requires NADPH as well as acetyl-CoA formed from pyruvate by chloroplastic pyruvate dehydrogenase (pPDH) (Bao *et al.*, 2000). Contrary to its mitochondrial counterpart (mPDH), pPDH is not down-regulated by phosphorylation in the light, allowing efficient conversion of pyruvate to acetyl-CoA in the chloroplast (Tovar-Méndez *et al.*, 2003). It is generally believed that acetyl-CoA carboxylase, which represents the committed step of fatty acid synthesis, is activated by redox power to match NADPH availability in the chloroplast (Rawsthorne, 2002). That said, increasing CO_2 mole fraction has little effect on the flux to fatty acid synthesis (Williams & Harwood, 1997) and in fact, ^{14}C -labelling has suggested that lipids represent about 1% of net fixed carbon regardless of CO_2 mole fraction (Escobar-Gutiérrez & Gaudillère, 1997). Fatty acid synthesis with ^{13}C -labelling has also shown that the flux represents about $0.1 \mu\text{mol carbon m}^{-2} \text{s}^{-1}$ in Arabidopsis in a slightly CO_2 -enriched atmosphere ($\approx 450 \mu\text{mol mol}^{-1} \text{CO}_2$), i.e. $\approx 1\%$ of photosynthesis (Bao *et al.*, 2000). However, the scarcity of quantitative measurements of lipid and protein synthesis should be noted, and the way the associated flux in electron, carbon or nitrogen equivalents varies with photosynthetic conditions is not so well-known. In addition, there is currently little quantitative knowledge on reserve remobilization

that may contribute to electron and ATP utilization but would remain invisible upon labeling.

Secondary compounds can also represent a small, but significant part of carbon utilization. At the leaf level, the synthesis of isoprenoids has been shown to be generally $< 0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ and to depend on CO_2 and O_2 mole fraction (Rasulov *et al.*, 2009). In general, isoprene emission decreases at high CO_2 or low O_2 and this has been related to pools of intermediates and phosphate, reductant supply as well as phosphoenolpyruvate utilization (Monson & Fall, 1989; Loreto & Schnitzler, 2010; Rasulov *et al.*, 2016). Interestingly, isoprene emission has also been shown to be modulated by nitrogen nutrition, with lower values at high nitrogen due to the competition for electrons and phosphoenolpyruvate (Rosenstiel *et al.*, 2004). The carbon flux to phenylpropanoids has been suggested to be large because they can ultimately represent up to 30% of plant biomass (Maeda & Dudareva, 2012). But at the leaf level, chlorogenate synthesis has been measured precisely and found to be small ($< 1\%$ of photosynthesis) in sunflower and remarkably, has a bell-shaped pattern (i.e. decreased at low photosynthesis, and in 0.03% O_2 , maximal under 'standard' $\text{CO}_2:\text{O}_2$ conditions) (Abadie *et al.*, 2018). Such compounds are of interest because their synthesis starts in the chloroplast and requires NADPH, phosphoenolpyruvate and erythrose-4-phosphate. The latter is directly abstracted from the Calvin cycle and therefore depends on RuBP regeneration metabolism.

Photosynthesis and stress signaling

Changing the $\text{CO}_2:\text{O}_2$ environment, in particular low CO_2 or high O_2 , represents stressful conditions for photosynthesizing leaves. There is now substantial evidence that photorespiration is a source of reactive oxygen species (ROS, such as peroxisomal H_2O_2) that have to be detoxified (Noctor & Foyer, 1998; Foyer & Noctor, 2005) and trigger a rapid gene expression response (Eisenhut *et al.*, 2017). In addition, stress-related signaling events involving ethylene are believed to be of considerable importance.

Ethylene has long been known for its potential (and controversial) stimulation of stomatal closure, with considerable species-specific variations (Kays & Pallas, 1980; Pallas & Kays, 1982; Tanaka *et al.*, 2005; Iqbal *et al.*, 2011). Conversely, ethylene can antagonize abscisic acid (ABA)-induced stomatal closure under water deficit (Tanaka *et al.*, 2005), and in the long-term, might stimulate plant growth (Pierik *et al.*, 2006). Ethylene stimulates antioxidants (flavonols) synthesis in guard cells and thus counteracts ROS accumulation thereby down-regulating the ABA response in Arabidopsis (Watkins *et al.*, 2014) and tomato (Watkins *et al.*, 2017). In fact, this effect was not observed in Arabidopsis *ethylene-overproducing1* (*ein1*) and tomato *Neverripe* (*Nr*) mutants affected in ethylene signal transduction (Watkins *et al.*, 2014, 2017); accordingly, mutants affected in flavonol synthesis have a higher ROS content in guard cells and are more sensitive to ABA.

Unlike stomatal aperture, the photosynthetic machinery is negatively impacted by ethylene. Using photosynthetic CO_2 response curve after a brief pre-exposure to ethylene, a decline in maximal chloroplastic electron transport rate has been found

(Taylor & Gunderson, 1986; Wullschlegel *et al.*, 1992). Other studies have further shown a decrease in Rubisco activity itself (Tholen *et al.*, 2008) or carbon export from leaves (Woodrow *et al.*, 1988). Phosphoproteomics analyses have shown that when $\text{CO}_2:\text{O}_2$ conditions vary, there are rapid changes in a number of signaling pathways (Abadie *et al.*, 2016b) and amongst them, ethylene response is the most represented. This includes ACC synthase and the central transducer EIN2, with a close correlation with the $\text{CO}_2:\text{O}_2$ ratio, suggesting that both ethylene synthesis and perception are enhanced at low photosynthesis (Supporting Information Table S1). Although the specific effect of gaseous conditions on ethylene synthesis is less documented than in fruits (where it is well-known that CO_2 inhibits ethylene synthesis), available evidence indicates that in leaves, minimal carbon assimilation is required for carbon provision to ethylene synthesis (Grodzinski *et al.*, 1982; Guy & Kende, 1984) but increased CO_2 mole fraction (but not super-high CO_2) inhibits ethylene synthesis (Guo *et al.*, 2014). ROS-generating conditions (high photorespiration, low $\text{CO}_2:\text{O}_2$ ratio) interact with ethylene signaling, as suggested by (1) the increased expression at low CO_2 of 2-cysteine peroxiredoxin A (2CP A) within a few hours and (2) the fact that 2CP B contains a strong ethylene response element in its promoter (Heiber *et al.*, 2007). In addition, in *Chlamydomonas* the retrograde ROS-signaling protein TER23 (ethylene-dependent EGY1 in Arabidopsis) participates in the fast phosphorylation response and thus on cyclic vs linear electron flux in the chloroplast under stressful conditions (Schönberg *et al.*, 2017). Accordingly, in nonsenescent leaves, ethylene has been shown to cause changes in antioxidant contents (Hodges & Forney, 2000) and a considerable increase in biosynthesis of phenolic compounds (Tomás-Barberán *et al.*, 1997; Liu *et al.*, 2016), thereby consuming sugar phosphates and reductive power in the chloroplast. Ethylene also perturbs polyamine synthesis and S-adenosylmethionine metabolism (Aziz *et al.*, 1997) and a beneficial effect on nitrogen and sulfur assimilation has been proposed (Iqbal *et al.*, 2012). Taken as a whole, ethylene-mediated stress signaling under unfavorable photosynthetic gaseous conditions (low $\text{CO}_2:\text{O}_2$) has direct or indirect effects on photosynthetic electron transfer chain and this should in principle alter the quantum yield of photosynthesis in the short-term.

Specific effects of low oxygen

It is common practice to use low O_2 mole fraction (typically 2% O_2 or less) in gas exchange experiments to eliminate oxygenation and thus infer the contribution of photorespiration to net CO_2 exchange. However, in addition to variable consequences on stomatal conductance, low O_2 is associated with strong metabolic effects that may change carboxylation activity.

First, low O_2 causes a well-known alteration of (sugar) phosphate homeostasis in the chloroplast (and thus ATP generation) that can lead to a decrease in photochemical quenching and photosynthesis rate (Sharkey & Vassey, 1989). In addition, hypoxia has been demonstrated to reconfigure the STN7-dependent phosphorylation of proteins of the chloroplastic electron transfer chain (Bergner *et al.*, 2015; Shapiguzov *et al.*, 2016), typically LHC proteins that

are responsible for the photosynthetic state shift (state I/state II). This directly impacts on the balance between the cyclic electron flux around photosystem I and the linear electron flux in the short term, and thus the quantum yield of photosynthesis.

Second, despite the production of oxygen by photosynthesis, the low- O_2 atmosphere triggers a hypoxic response even in the short-term (here, 'short-term' means a time frame of 1 to 2 h, that is, the typical time required to carry out a photosynthetic response curve). From a metabolic perspective, hypoxia is associated with a reorganization of glycolysis, TCAP and nitrogen assimilation, with considerable accumulation of biomarkers metabolites such as the nitrogenous compounds alanine, β -alanine, glutamate or γ -aminobutyrate (Limami *et al.*, 2014). Such changes are associated with an increase in nitrogen demand (and thus nitrate assimilation using electrons of the chloroplastic electron chain) as shown by the decrease in phosphorylation of nitrate reductase under hypoxia (Lothier *et al.*, 2018). Hypoxia is also associated with nitric oxide production from nitrite produced by nitrate reductase (for a review, see Planchet *et al.*, 2017). Also, there is a potential change in CO_2 evolution: a substantial decrease in mPDH phosphorylation (Lothier *et al.*, 2018) and thus an increase in pyruvate decarboxylation activity, alanine synthesis (that abstracts pyruvate from catabolism) and γ -aminobutyrate synthesis by glutamate decarboxylation (Abadie *et al.*, 2017a). It has been suggested that under 0.03% O_2 , the extra nitrogen demand represents about $0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$, i.e. about $2 \mu\text{mol electrons m}^{-2} \text{s}^{-1}$ (Abadie *et al.*, 2018). However, the overall effect of hypoxia on day respiratory CO_2 efflux is not very well-known (for a specific discussion, see Tcherkez *et al.*, 2012).

Hypoxia may also lead to changes in the expression of genes encoding proteins that play key roles in photosynthesis (summarized in Table S2). A comparison of the transcriptome of various tissues of Arabidopsis photosynthetic tissues subjected to oxygen (and CO_2) deprivation showed that there is a rapid (within 2 h) change in the translation of mRNAs encoding photosystem II proteins CP22 (involved in nonphotochemical quenching) and OHP2 (involved in PSII assembly) as well as Rubisco chaperonins, suggesting an increase in turn-over of the photosynthetic machinery (Mustroph *et al.*, 2009). In the longer term, it is believed there is an increase in the expression of genes encoding chlorophyll binding proteins (LHCs), as suggested by the comparison of transcriptome profiles under normoxia or hypoxia (for 2 h), in wild-type and mutants affected in the N-end-rule pathway and exhibiting a constitutive response to hypoxia (including the typical group VII ethylene response factors) (Gibbs *et al.*, 2011).

Taken as a whole, hypoxia directly affects photosynthesis via rapid changes in metabolism and synthesis of the photosynthetic machinery. Consequently, gas exchange experiments carried out at low oxygen are not simply representative of photorespiration-free conditions.

The problem of photorespiration and nitrogen demand

Photorespiration does not only exist in a metabolic cycle whereby O_2 fixation (oxygenation) leads to glycine synthesis, subsequent CO_2 release and recycling of serine to 3-phosphoglycerate (PGA).

In fact, photorespiration has been shown to involve alternative pathways (such as formate oxidation) (Peterhansel *et al.*, 2012; Obata *et al.*, 2016), and at high oxygenation rates, to be associated with glycine and serine accumulation (Abadie *et al.*, 2016a). Also, metabolic comparisons using gas chromatography-mass spectrometry (GC-MS) profiles (Carroll *et al.*, 2015) has shown that high photorespiration leads to a metabolic phenotype similar to some photorespiratory mutants (Abadie *et al.*, 2016a). It is worth noting that despite the amino acid build-up, photorespiration *per se* does not represent a nitrogen assimilation pathway since it strictly depends on the glutamine synthetase/glutamine-2-oxoglutarate amino transferase cycle (Wingler *et al.*, 2000). Photorespiratory recycling (or lack thereof) of glycerate, glycine and serine has been controversial for a long time (Harley & Sharkey, 1991; Dirks *et al.*, 2012; Tcherkez, 2013; Busch *et al.*, 2018). The abstraction of serine and glycine molecules from the photorespiratory cycle might be detrimental for photosynthesis because it leads to an extra-demand in electrons (for nitrogen assimilation in glyoxylate amination to glycine) and PGA, potentially impeding RuBP regeneration. Using a modeling exercise that neglects (changes in) internal conductance (g_m), glycine and serine build-up has been suggested to be of considerable importance, with about 30% of serine not recycled to PGA, thus shaping the CO_2 response curve of photosynthesis (Busch *et al.*, 2018) due to serine accumulation rate of up to $0.9 \mu\text{mol m}^{-2} \text{s}^{-1}$ and nitrogen assimilation rates up to $1.2 \mu\text{mol m}^{-2} \text{s}^{-1}$. The nonquantitativity of glycine-to-serine conversion has been demonstrated experimentally (Abadie *et al.*, 2016a), but metabolite accumulation represents only a few percent of oxygenation rate. In effect, using both metabolite kinetics and isotopomer distribution, it has been shown that glycine and serine accumulation rates taken together never exceed $0.09 \mu\text{mol m}^{-2} \text{s}^{-1}$ regardless of the $CO_2 : O_2$ ratio in sunflower, and often represent $< 0.05 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Abadie *et al.*, 2016a, 2018). Therefore, glycine and serine nonrecycling increase the diversion of electrons to assimilate nitrogen, and maybe contribute to shaping CO_2 assimilation curves, but the impact is numerically very small.

Furthermore, leaf nitrogen assimilation (from nitrate) is unlikely to be as high as $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ as suggested by modeling (Busch *et al.*, 2018). It should be recognized that measuring instantaneous leaf nitrogen assimilation is, perhaps surprisingly, more complicated than one would think. The assimilatory quotient method, which leads to high values, can be compromised by alternative electron utilization (e.g. sulfur assimilation, PEPC-MDH activity, anabolism), as outlined earlier. Elemental analysis of plant organic matter generally shows that nitrogen represents 2–5% of total organic matter (while carbon is about 40%), and therefore suggests that the nitrogen assimilation flux falls within 1/20th and 1/8th of net CO_2 assimilation. However, such an estimate is flawed simply because (1) roots can assimilate nitrogen in many species, (2) leaf-assimilated nitrogen is redistributed to other organs (and a small amount is lost as gaseous NH_3), and (3) a considerable amount of carbon is lost by respiration. Using isotopic labeling (e.g. using ^{15}N) is also challenging because of the isotopic dilution by endogenous nitrates, the utilization of 'new' nitrogen atoms to synthesize less easily extractable compounds (like proteins) and nitrogen redistribution to other organs. However, available

evidence suggests that nitrogen assimilation is much lower than $1 \mu\text{mol m}^{-2} \text{s}^{-1}$, and more likely about $0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$. In fact, with ^{13}C labeling, the flux to *de novo* glutamate synthesis has been found to be $< 0.01 \mu\text{mol glutamate m}^{-2} \text{s}^{-1}$ regardless of $\text{CO}_2 : \text{O}_2$, meaning a total $\leq 0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$, accounting for carbon remobilization for total glutamate synthesis (Abadie *et al.*, 2017b, 2018). Using ^{15}N -labeling, total glutamate synthesis accounting for ^{15}N isotopic dilution has also been found to be about $0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Gauthier *et al.*, 2010). In these experiments, this value of $0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ was probably representative because short-term labeling was used so it is unlikely a very high proportion of the label was allocated to proteins or nucleotides and glutamate was the major product of nitrogen assimilation. Still, if we assume that proteins represent about 1% of net assimilated carbon, this would mean an extra nitrogen demand of about $0.07 \mu\text{mol m}^{-2} \text{s}^{-1}$ (assuming net photosynthesis at $20 \mu\text{mol m}^{-2} \text{s}^{-1}$). A recent estimation of fluxes in Arabidopsis leaves based on mass-balance (flux balance analysis, FBA) has also shown that in mature leaves, nitrogen assimilation from nitrate represents about 0.2% of gross photosynthesis (Shaw & Cheung, 2018), that is, about $0.04 \mu\text{mol m}^{-2} \text{s}^{-1}$. Taken as a whole, instantaneous leaf nitrogen assimilation is an important actor of photosynthetic leaf metabolism but absolute flux-values are important to keep in mind. Nitrogen assimilation is equal to, or $< 0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ in most cases, meaning an electron consumption of about $2 \mu\text{mol m}^{-2} \text{s}^{-1}$. This value probably changes to higher values when particular circumstances increase the nitrogen demand considerably despite low photorespiration, such as hypoxia (see above). Also, it would be interesting to disentangle the metabolism associated with nitrogen assimilation in genetically modified plants where photorespiration has been bypassed and plant biomass increases (Kebeish *et al.*, 2007; South *et al.*, 2019).

Under what conditions are these metabolic pathways of particular importance?

We have discussed the potential importance of metabolisms other than photosynthesis and photorespiration ('ancillary' metabolism such as PEPC fixation, nitrogen assimilation, etc.) when CO_2 and O_2 conditions vary and thus can have an impact on observed net CO_2 assimilation. It is presently difficult to provide precise guidelines to account for such 'ancillary' metabolisms in observed CO_2 exchange rate, simply because the numerical values of fluxes are not known with sufficient certainty. That said, considering their rather low flux values, the impact is numerically small under standard conditions ($400 \mu\text{mol mol}^{-1} \text{CO}_2$, 21% O_2) or more generally, when photosynthesis (and electron utilization to CO_2 fixation itself) is sufficiently high (Table 1). In other words, their potential effect is more likely visible when photosynthesis is low, at low CO_2 or high O_2 because their relative impact on the CO_2 flux might be large. For example, when low CO_2 conditions are used to carry out Laik curves so as to determine the compensation point in the absence of day respiration (Γ^*) and day respiration (R_d), the change in assimilation caused by PEPC fixation (which itself varies with CO_2 mole fraction) could cause an error in estimated Γ^* (Abadie & Tcherkez, 2019).

The alternative utilization of electrons in the chloroplast (for nitrogen and sulfur assimilation, malate synthesis, or secondary metabolism) is also relatively small, but can have subtle consequences on photosynthetic parameters. Typically, most calculations of internal conductance based on fluorescence assume that total electron flux (J_t) is partitioned between oxygenation (J_o) and carboxylation (J_c) and thus $J_t = J_o + J_c$. If the alternative consumption of electrons is not negligible, the relationship becomes $J_t - J_a = J_o + J_c$ where J_a is the consumption of electrons to alternative pathways. For instance, based on the total computed in Table 1, when J_t (ETR) is larger than $50 \mu\text{mol m}^{-2} \text{s}^{-1}$, potential errors in partitioning and conductance calculations must be small. By contrast, under ambient oxygen (21%), when net CO_2 assimilation is $< 8 \mu\text{mol m}^{-2} \text{s}^{-1}$ and thus J_t likely $< 50 \mu\text{mol m}^{-2} \text{s}^{-1}$, potential errors are large. In particular, internal conductance calculations are quite sensitive to potential errors in electron flux, and thus can be affected by J_a . In fact, an error of $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ in electron flux (that is, of about 1%) can lead to an offset of at least $0.01 \text{ mol m}^{-2} \text{s}^{-1}$ in internal conductance (for a detailed sensitivity analysis, see Harley *et al.*, 1992). Unfortunately, as it is apparent in this Viewpoint, the way J_a varies with O_2 and CO_2 is not known with precision. We also note that day respiration (not discussed here) can also impact on internal conductance (further discussed in Harley *et al.*, 1992 and Tcherkez *et al.*, 2017).

Conclusions and perspectives

In these perspectives, we have emphasized the need to have a more integrative view of leaf metabolism, which goes beyond CO_2 and O_2 reduction cycles themselves. That is, even in the short-term, leaf metabolism reacts not only via the $\text{CO}_2 : \text{O}_2$ partitioning of Rubisco kinetics and activation, but also through other pathways that may have direct or indirect effects on observed CO_2 assimilation rate, summarized in Fig. 1. Unfortunately, quantitative data on these aspects are scarce and instantaneous metabolic flux analysis when CO_2 and O_2 conditions vary is insufficiently documented. It must be recognized that it is technically difficult and expensive to carry out detectable isotopic labeling in metabolites, or to apply omics techniques when gaseous conditions change rapidly and are coupled to precise photosynthesis (and transpiration) measurements. That said, recent studies (cited here) have provided experimental estimates of fluxes in some pathways and new metabolomics and fluxomics technologies now offer outstanding capabilities to use fluxomics and thus get more precision in flux determination, remobilization and nonquantitativity of photosynthetic and photorespiratory reactions.

Also, most available data were obtained in common C_3 plants (such as Arabidopsis), and more data are required in C_3 and C_4 crops as well as C_3 species that exhibit particular nitrogen metabolism (i.e. that can affect relationships between photosynthesis and photorespiration) such as legumes. A better knowledge of concurrent, nonphotosynthetic metabolisms affected by gaseous conditions would be instrumental to define important regulations and possibly, improve models of CO_2 capture by leaves in particular (1) when net assimilation is low and thus the impact of nonphotosynthetic metabolism may be proportionally large, and

Table 1 Summary of nonphotorespiratory and nonphotosynthetic pathways discussed here and linked to net photosynthesis under varying CO₂ : O₂ conditions in illuminated leaves, in the short term.

Process	Gas exchange conditions where it prevails	Key metabolic pathway	Consumes electrons or Calvin cycle intermediates?	Electron flux (μmol m ⁻² s ⁻¹)	Produces or fixes CO ₂ ?	CO ₂ flux (μmol m ⁻² s ⁻¹)
Anaplerotic fixation ^a	Increases at low CO ₂ : O ₂ ratio	PEPC-MDH activity	Yes (reduction of oxaloacetate to malate)	0.6–2.2	Fixes	+0.3 to +1.1
Nitrogen assimilation	Increases at low CO ₂ : O ₂	GS/GOGAT cycle (which is mobilized by recycling of photorespiratory NH ₃)	Yes (ferredoxin-dependent GOGAT activity)	0.1–1	Produces, potentially (<i>de novo</i> 2-oxoglutarate synthesis)	–0.01 to –0.1
Sulfur assimilation	Increases at high O ₂ ?	Asp pathway (Met) and photorespiratory or nonphotorespiratory Ser synthesis (Cys)	Yes (sulfate reduction)	Unknown (probably ≈0.2)	In principle, produces (Asp and folate metabolism)	Unknown
Lipid synthesis ^a	Variations with CO ₂ : O ₂ poorly known	Mostly chloroplastic glycolysis (pyruvate synthesis) and cPDH for fatty acid synthesis	Yes (NADPH and triose phosphates utilization)	≈0.4	Produces (cPDH activity)	≈–0.1
Protein synthesis	Increases as photosynthesis increases?	Cytosolic (and chloroplastic) mRNA translation	Indirectly (ATP and nitrogen assimilates utilization)	1?	No (but coupled to respiratory CO ₂ production via ATP and nitrogen requirement)	
Secondary metabolism ^a	Complicated, poorly-known pattern	Various (mevalonate pathway, methylerythritol pathway, phenylpropanoids, etc.)	Yes (triose phosphates, erythrose-4-phosphate, etc.)	≈0.1	Sometimes (depends on synthesized metabolites)	≈–0.1
Hypoxic metabolism	Low O ₂	Fermentative or alternative electron-consuming processes	Maybe (increased nitrogen assimilation)		Produces, potentially (GABA synthesis, pyruvate conversion to ethanol, ...)	Unknown
Stress response	High O ₂ or low CO ₂ (high photorespiration)	Oxidative stress and photosynthetic downregulation mediated by ethylene	Yes (ROS detoxification)	?	Possibly produces, via changes in polyamine and C ₁ metabolisms	?
Day respiration	Increases at high photorespiration	Glycolytic pyruvate production, TCAP, OPPP	No (unless recycles organic acids from the chloroplast)	Up to 5	Produces	Usually within –0.2 and –1
Total						–1 to +0.7

The CO₂ flux is shown as negative when it is an efflux, and positive when it is an influx. Note the significant number of question marks (?), demonstrating the need in more quantitative analyses. See the text for further details on flux values. ^aElectron consumption and CO₂ exchange for anaplerosis, lipid synthesis and secondary metabolism only account for NADPH utilization and CO₂ fixation/generation from key precursors (pyruvate/phosphoenolpyruvate) and thus does not account for electrons needed to synthesize precursors themselves. The flux indicated for secondary metabolism here only accounts for isoprene + chlorogenate synthesis as an example. Abbreviations: cPDH, chloroplastic pyruvate dehydrogenase; GABA, γ-aminobutyrate; GOGAT, glutamine 2-oxoglutarate aminotransferase; GS, glutamine synthetase; OPPP, oxidative pentose phosphate pathway; PEPC-MDH, phosphoenolpyruvate carboxylase-malate dehydrogenase; ROS, reactive oxygen species; TCAP, tricarboxylic acid pathway.


(2) at low O₂ which causes hypoxic metabolism, and thus specific catabolic pathways and changes in nitrogen assimilation.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 Best phosphopeptides involved in signaling in Arabidopsis rosettes that correlate with gaseous conditions.

Table S2 Hypoxia-responding genes related to photosynthesis in wild-type Arabidopsis.

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