



Short- and long-term responses of leaf day respiration to elevated atmospheric CO₂

Yan Ran Sun, Wei Ting Ma, Yi Ning Xu, Xuming Wang, Lei Li, Guillaume Tcherkez, Xiao Ying Gong

► To cite this version:

Yan Ran Sun, Wei Ting Ma, Yi Ning Xu, Xuming Wang, Lei Li, et al.. Short- and long-term responses of leaf day respiration to elevated atmospheric CO₂. *Plant Physiology*, 2023, 191 (4), pp.2204-2217. 10.1093/plphys/kiac582 . hal-03919109

HAL Id: hal-03919109

<https://hal.inrae.fr/hal-03919109>

Submitted on 2 Jan 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright

Short- and long-term responses of leaf day respiration to elevated atmospheric CO₂

Running title: CO₂ response of leaf respiration in the light

Yan Ran Sun¹, Wei Ting Ma¹, Yi Ning Xu¹, Xuming Wang¹, Lei Li¹, Guillaume Tcherkez^{2,3},
Xiao Ying Gong^{1*}

¹ Key Laboratory for Subtropical Mountain Ecology, College of Geographical Sciences, Fujian Normal University, Fuzhou, 350007, China

² Research School of Biology, ANU College of Science, Australian National University, Canberra ACT 0200, Australia

³ Institut de Recherche en Horticulture et Semences, INRAE, Université d'Angers, 42 rue Georges Morel, 49070 Beaucouzé, France

***Corresponding author:**

Xiao Ying Gong, Tel: +86 59183465214, Fax: +86 59183465397, Email: xgong@fjnu.edu.cn

ORCIDs: Xiao Ying Gong (0000-0002-4983-5645); Guillaume Tcherkez (0000-0002-3339-956X); Xuming Wang (0000-0001-5291-9332)

Twitter accounts: Xiao Ying Gong (@gong_xiaoying); Guillaume Tcherkez (@IsoSeed)

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (<https://academic.oup.com/plphys/pages/General-Instructions>) is Xiao Ying Gong.

AUTHOR CONTRIBUTIONS

X.Y.G. designed and planned the research; Y.R.S. and Y.N.X. performed the experiment, Y.R.S. and W.T.M. analyzed the data and wrote the first draft, and all authors discussed the results and implications and contributed to the revision.

ABSTRACT

Evaluating leaf day respiration rate (R_L), which is believed differ from that in the dark (R_{Dk}), is essential for predicting global carbon cycles under climate change. Several studies have suggested that atmospheric CO_2 impacts R_L . However, the magnitude of such an impact and associated mechanisms remain uncertain. To explore the CO_2 effect on R_L , wheat (*Triticum aestivum*) and sunflower (*Helianthus annuus*) plants were grown under ambient (410 ppm) and elevated (820 ppm) CO_2 mole fraction ($[CO_2]$). R_L was estimated from combined gas exchange and chlorophyll fluorescence measurements using the Kok method, the Kok-Phi method, and a revised Kok method (Kok- C_c method). We found that elevated growth $[CO_2]$ led to an 8.4% reduction in R_L and a 16.2% reduction in R_{Dk} in both species, in parallel to decreased leaf N and chlorophyll contents at elevated growth $[CO_2]$. We also looked at short-term CO_2 effects during gas exchange experiments. Increased R_L or R_L/R_{Dk} at elevated measurement $[CO_2]$ were found using the Kok and Kok-Phi methods, but not with the Kok- C_c method. This discrepancy was attributed to the unaccounted changes in C_c in the former methods. We found that the Kok and Kok-Phi methods underestimate R_L and overestimate the inhibition of respiration under low irradiance conditions of the Kok curve, and the inhibition of R_L was only 6%, representing 26% of the apparent Kok effect. We found no significant long-term CO_2 effect on R_L/R_{Dk} , originating from concurrent reduction in R_L and R_{Dk} at elevated growth $[CO_2]$, and likely mediated by acclimation of nitrogen metabolism.

Keywords: leaf day respiration, Kok method, atmospheric CO_2 , photosynthesis, climate change, carbon balance, mesophyll conductance, chlorophyll fluorescence

INTRODUCTION

Terrestrial vegetation assimilates ca. 120 pg carbon via photosynthesis but releases about half of assimilated carbon via respiration (Gifford, 2003; Dusenage et al., 2019). The balance between plant respiration and photosynthesis is therefore essential for plant productivity and global carbon balance. Despite considerable variations depending on N fertilisation and climatic conditions, the ongoing increase in atmospheric CO₂ mole fraction ([CO₂]) promotes leaf photosynthesis and primary production, which is referred to as the “CO₂ fertilization effect” (Drake et al., 1997; Cramer et al., 2001). Although the CO₂ fertilization effect on biomass (but not necessarily yield) is evident from greenhouse and field experiments (Ainsworth and Long, 2005; Norby et al., 2005; Walker et al., 2021), the response of plant respiration to [CO₂] is rather uncertain, limiting our ability to predict future climate change-driven modifications of plant physiology.

The respiratory response is complicated by the fact that leaf respiration takes place not only in darkness (the respiration rate is denoted as R_{Dk}), but also in the light. In illuminated leaves, respiration is referred to as “respiration in the light” or “day respiration” (denoted as R_L ; here we refer to CO₂ evolution rather than O₂ consumption). Leaf respiration has been shown to be partially inhibited by the light although the magnitude of inhibition varies broadly, with reported R_L/R_{Dk} values ranging from 0.2 to 1.3 (Ayub et al., 2011; Griffin and Turnbull, 2013; Crous et al., 2017; Gong et al., 2018; Way et al., 2019). Given the longer light periods during the growing season and higher temperature during the day than at night in most ecosystems, R_L is a key component of plant- and community-scale carbon budgets (Atkin et al., 2007; Gong et al., 2017). Experimental results revealed that the inhibition of respiration by light (i.e., $1-R_L/R_{Dk}$) also occurs at the stand scale (Gong et al., 2017). Neglecting respiration inhibition might have led to considerable errors in estimated gross primary production (Wehr et al., 2016; Gong et al., 2017). Furthermore, the response of R_L to environmental cues are essential to predict carbon balance, carbon use efficiency and improve land surface models (Wehr et al., 2016; Atkin et al., 2017; Tcherkez et al., 2017b; Keenan et al., 2019).

So far, there is no consensus on the response of R_L to long-term [CO₂] increase. Some studies have shown that R_L is stimulated by elevated growth [CO₂] (Wang et al., 2001; Shapiro et al., 2004; Crous et al., 2012; Griffin and Turnbull, 2013), and this effect may be related to higher carbohydrate concentrations in leaves (Rogers et al., 2004; Gong et al., 2017). Also, increased

leaf respiration at elevated $[\text{CO}_2]$ has been suggested to be associated with a larger mitochondrial number per mesophyll cell (Griffin et al., 2001), indicating cellular and transcriptional (gene regulation) mechanisms of respiratory control (Leakey et al., 2009). Other studies have reported a decrease in R_L in plants grown under elevated $[\text{CO}_2]$ compared with that grown under ambient $[\text{CO}_2]$ (Ayub et al., 2011; Ayub et al., 2014).

The decrease in R_L at elevated $[\text{CO}_2]$ has been suggested to be linked to either photorespiration or nitrogen metabolism. Under elevated CO_2 , there is a reduction in photorespiration rate (and the rate of oxygenation of RuBP, v_o), and this could cause an alteration in R_L , as suggested by results obtained on short-term changes in respiratory metabolism under varying CO_2 mole fraction. In effect, using ^{13}C -enriched substrates to trace decarboxylation processes, Tcherkez *et al.* (2008) found that decarboxylation decreased when leaves were exposed to elevated $[\text{CO}_2]$ for short periods. Likewise, results obtained using the Kok method suggested there was a linear relationship between photorespiration rate and R_L (Griffin and Turnbull, 2013). However, the mechanism behind this relationship is still unclear. In particular, the Kok effect itself has been shown not to be fully caused by changes in respiration rate (Gauthier et al., 2020) and thus, the relationships between photorespiration and Kok method-based R_L are presently uncertain. In addition, R_L has been reported to either decrease (Pinelli and Loreto, 2003; Tcherkez et al., 2008; Griffin and Turnbull, 2013), increase (Yin et al., 2020; Fang et al., 2022) or remain unaffected (Sharp et al., 1984; Tcherkez et al., 2012), in the short-term using gas-exchange experiments at elevated $[\text{CO}_2]$. Thus, conclusions drawn from short-term changes in R_L caused by instantaneous elevation of $[\text{CO}_2]$ might not be relevant to long-term changes in R_L .

The decrease of R_L at elevated $[\text{CO}_2]$ has also been suggested to be linked to nitrogen metabolism. It has been observed in many free air CO_2 enrichment (FACE) experiments that elevated $[\text{CO}_2]$ reduces leaf N content, which is accompanied by a down-regulation of photosynthetic capacity (Long et al., 2004; Ainsworth and Long, 2005). It is believed that elevated $[\text{CO}_2]$ inhibits N assimilation in leaves via the potential link between photorespiration and nitrate assimilation (Bloom et al., 2010; Bloom et al., 2014; Busch et al., 2018). Given that N assimilation in leaves is energy demanding and thus a driving factor for leaf respiration (Amthor, 2000; Reich et al., 2008), it would be important to know whether $[\text{CO}_2]$ affected R_L and R_{Dk} via

leaf N content. All in all, the response of R_L to elevated $[CO_2]$ appears to be highly variable and mechanisms behind are unclear.

Another uncertainty associated with R_L and how it varies is technological. In fact, there are several methods to estimate R_L , but none of them can measure R_L directly (for a review see (Tcherkez et al., 2017b)). The Kok method (Kok, 1949) and the Laisk method (Laisk, 1977), the two most commonly used methods, require manipulation of net CO_2 assimilation rates (A) at low irradiances ($I_{inc} < 150 \mu mol m^{-2} s^{-1}$) (Kok) or low CO_2 (Laisk). Another method, the ^{13}C isotopic disequilibrium method, uses two CO_2 sources with different $\delta^{13}C$ values to disentangle R_L and photosynthesis under physiologically relevant environmental conditions without the need to manipulate A (Gong et al., 2015; Gong et al., 2018). The ^{13}C disequilibrium method is valuable since it does not require the use of low irradiance or low CO_2 and can be performed at any CO_2 mole fraction, and therefore, is suitable to study CO_2 effects on R_L . It is, however, technically demanding (isotopic CO_2 sources, mass spectrometers). The Laisk method is, by definition, not suitable for studying CO_2 effects because it manipulates $[CO_2]$ at sub-ambient levels. So far, the response of R_L to $[CO_2]$ has mainly been estimated using the Kok method. However, as mentioned above, the Kok method has been questioned since the Kok effect is not exclusively caused by a decrease in respiration rates (Gauthier et al., 2020). Several studies showed that the Kok method has conceptual uncertainties (Farquhar and Busch, 2017; Tcherkez et al., 2017a; Tcherkez et al., 2017b; Yin et al., 2020). First, the Kok method assumes a constant photochemical efficiency of PS II (Φ_2) along the $A-I_{inc}$ curve (i.e. the Kok curve, see *Theory*). To address this issue, Yin et al. (2009) suggested to use measured Φ_2 to improve the R_L estimation. Second, the Kok method usually disregards variation in chloroplastic $[CO_2]$ (C_c) along the $A-I_{inc}$ curve, which could bias the estimates of R_L according to recent studies based on model analysis (Farquhar and Busch, 2017; Yin et al., 2020). Estimating C_c along the $A-I_{inc}$ curve requires measurements of mesophyll conductance (g_m). Measuring g_m is challenging and this is particularly true when measurements are performed at low irradiance (Pons et al., 2009; Gu and Sun, 2014; Gong et al., 2015). So far, the uncertainty associated with C_c has not been fully solved.

Taken as a whole, neither long-term nor short-term responses of R_L to CO_2 mole fraction are well-known, and technologies used to measure R_L may be problematic. Here, we intend to address the following questions: 1) how do short-, medium- and long-term CO_2 enrichment

1 affect R_L in C_3 leaves? 2) do the original- and revised Kok methods provide similar estimations
 2 of R_L ? To this end, we combine gas exchange and chlorophyll fluorescence (ChF) measurements
 3 to study the response of R_L of wheat (*Triticum aestivum* L.) and sunflower (*Helianthus annuus*
 4 L.) plants grown under ambient (410 ppm) and elevated $[CO_2]$ (820 ppm). We assessed the
 5 medium-to-long term CO_2 response (days to months) by comparing parameters of plants at
 6 different growth $[CO_2]$, and the short-term CO_2 response (minutes) by measuring the same leaves
 7 at 410 and 820 ppm of $[CO_2]$. We compared R_L estimated by the Kok method, the Yin method
 8 (i.e. the Kok-Phi method) and a revised Kok method (i.e. the Kok- C_c method) which takes the
 9 influence of Φ_2 and C_c into account.

11 RESULTS

12 *Effects of growth CO_2 on photosynthetic parameters and leaf traits*

13 Growth at elevated $[CO_2]$ led to a reduction in net CO_2 assimilation (A) for both species, when A
 14 values were compared at the same intercellular CO_2 concentration (C_i) levels (Fig. 1AB).
 15 Sunflower plants grown at elevated $[CO_2]$ exhibited lower E and g_{sw} compared with that grown
 16 at ambient CO_2 (Fig. 1DF). This effect on water vapour exchange was minor in wheat (Fig.
 17 1CE). In order to assess the long-term growth CO_2 effect on common grounds, gas exchange
 18 parameters of leaves were compared at their respective growth $[CO_2]$ (indicated by the subscript
 19 ‘growth’). Net CO_2 assimilation rate (A_{growth}), intrinsic water-use efficiency ($iWUE_{growth}$) and leaf
 20 carbon-use efficiency (CUE_L) of plants grown under elevated $[CO_2]$ were significantly higher
 21 than those of plants grown under ambient $[CO_2]$ in both species (Table 1). Averaged across
 22 species, growth at elevated $[CO_2]$ led to 5.6% reduction in A_{max} , 7.9% reduction in V_{cmax} , and
 23 8.0% in J , indicating a decline in photosynthesis capacity. The ratio of g_{sc} to g_m was not
 24 significantly affected by growth $[CO_2]$ or species. R_{Dk} of both species was lower at elevated
 25 $[CO_2]$ but this decrease differed between species (20% for wheat and 11% for sunflower).

26 Leaf chlorophyll content was significantly lower at elevated $[CO_2]$ compared with ambient
 27 $[CO_2]$. Similarly, elevated $[CO_2]$ led to 6.7% reduction (averaged across species) in nitrogen
 28 elemental content (N%) and 12% reduction in nitrogen content per surface area (N_{area}) on
 29 average, but the effect of CO_2 was not significant at a p -level of 0.05. SLA was significantly
 30 different between species but not affected by growth $[CO_2]$ (Table 1).

CO₂ response of R_L estimated by different methods

Φ_2 , C_c , and γ , the key parameters associated with assumptions in both original and revised Kok methods, were found to decrease along the Kok curve in all species and treatments (Fig. 2). With the increase of I_{inc} from 40 to 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, Φ_2 decreased by 3.1% for wheat and 2.4% for sunflower and this trend was not substantially influenced by growth [CO₂] (long term effect) and measurement [CO₂] (short-term effect). A short-term CO₂ effect on γ was detected, i.e., γ decreased more strongly at measurement [CO₂] of 410 ppm (by 5.2%) than that at measurement [CO₂] of 820 ppm (by 3.0%, averaged across species) with the increase in I_{inc} (Fig. S1). That is, under our conditions, terms ($\gamma f_{act} \Phi_2 \rho_2 \alpha$) in Eqn 3 and ($\gamma f_{act} \rho_2 \alpha$) in Eqn 4 were not constant along a Kok curve, causing errors in R_L estimated by the Kok and the Kok-Phi method, respectively.

Applying the Kok, the Kok-Phi and the Kok- C_c method, A was plotted against I_{inc} , $\Phi_2 I_{inc}$, and $\gamma \Phi_2 I_{inc}$, respectively (Fig. 3). Both growth [CO₂] and measurement [CO₂] had impacts on A , I_{inc} and $A - \Phi_2 I_{inc}$ response curves (Fig. 3A-D). As a result, growth at elevated [CO₂] led to a significant decrease in $R_{L \text{ Kok}}$ and $R_{L \text{ Kok-Phi}}$. The same was true for $R_{L \text{ Kok-}C_c}$ on average but it was only significant with a p -value of 0.06 (Fig. 4, Table 2). There was a clear, although statistically insignificant ($p > 0.05$), tendency for elevated measurement [CO₂] to increase both $R_{L \text{ Kok}}$ and $R_{L \text{ Kok-Phi}}$ (Fig. 3 and Fig. 4) in both species. By contrast, $A - \gamma \Phi_2 I_{inc}$ curves obtained under different measurement [CO₂] seemed to coincide perfectly (Fig. 3EF), in agreement with the insignificant effect of measurement [CO₂] on $R_{L \text{ Kok-}C_c}$.

CO₂ response of R_L/R_{Dk} estimated by different methods

We found no significant long-term CO₂ effect on R_L/R_{Dk} estimated via all three methods (Table 3). There is a tendency that R_L/R_{Dk} of wheat increased with the growth [CO₂] for all three methods (comparing aCO₂-410 and eCO₂-820), while that tendency was not found in sunflower. That is, the long-term CO₂ effect on R_L/R_{Dk} is not conclusive. Under elevated measurement [CO₂], significant increases in $R_{L \text{ Kok}}/R_{Dk}$ and $R_{L \text{ Kok-Phi}}/R_{Dk}$ were observed, but this short-term response was not observed using the Kok- C_c method (Fig. 5). These results indicate that short-term CO₂ effect on $R_{L \text{ Kok}}$ and $R_{L \text{ Kok-Phi}}$ could result from a technical bias simply due to neglecting the change in C_c along the Kok curve.

When pooling all data across species and treatments together, R_{Dk} was positively correlated to $R_{L \text{ Kok}}$ ($r^2=0.82$, $p<0.05$), $R_{L \text{ Kok-Phi}}$ ($r^2=0.82$, $p<0.05$) and $R_{L \text{ Kok-}C_c}$ ($r^2=0.77$, $p<0.05$) (Fig. 6).

These linear regressions yielded an average $R_L \text{ Kok}/R_{Dk}$ of $0.78 \pm 0.04(\text{SE})$, $R_L \text{ Kok-Phi}/R_{Dk}$ of 0.85 ± 0.04 , and $R_L \text{ Kok-Cc}/R_{Dk}$ of 0.94 ± 0.04 . That is, the Kok-Cc method showed a small light-induced inhibition of respiration, of 6% only, thus much lower than inhibition values from the other two methods (22%, 15%).

DISCUSSION

Growth at elevated CO_2 leads to reduction in R_L

This study showed that R_L of plants grown at elevated $[\text{CO}_2]$ was lower than that at ambient $[\text{CO}_2]$, and this result was confirmed by all three methods: Kok, Kok-Phi and Kok-Cc. On average, elevated $[\text{CO}_2]$ led to an 8.4% reduction in R_L , regardless of the method. This is in agreement with previous findings that leaf R_L of plants grown at elevated $[\text{CO}_2]$ is lower (Ayub et al., 2014) despite opposite findings (Wang et al., 2001, Shapiro et al., 2004).

Interestingly, although a long-term CO_2 effect on R_L was evident, elevated $[\text{CO}_2]$ had no influence on the R_L/R_{Dk} ratio, because R_{Dk} was also significantly lower at elevated $[\text{CO}_2]$. Similar to our study, there was no significant long-term CO_2 effect on R_L/R_{Dk} in Sydney blue gum (*Eucalyptus saligna*) (Ayub et al., 2011; Crous et al., 2012). However, Wang et al. (2001), Shapiro et al. (2004) and (Gong et al., 2017) found that non proportional changes in R_L and R_{Dk} led to higher R_L/R_{Dk} ratio in common cocklebur (*Xanthium strumarium*) leaves and sunflower stands grown at elevated $[\text{CO}_2]$. By contrast, R_L/R_{Dk} was reduced by elevated growth $[\text{CO}_2]$ in wheat because R_L declined (Ayub et al., 2014) or R_{Dk} increased (Griffin and Turnbull, 2013). Presumably, variations in the response to growth CO_2 between species and conditions might be linked to differences in nutrient content, metabolism, protein content, etc. which are all related to respiration.

The long-term response of R_L to CO_2 is associated with changes in leaf N status

Leaf N has long been suggested to be a key parameter influencing respiration rate, and used to estimate leaf respiration in vegetation models (Atkin et al., 2017). In our study, the reduction in R_L and R_{Dk} was associated with a decrease in N_{area} and chlorophyll content, suggesting that leaf N effectively drives the respiration rate. Nitrate reduction and maintenance of proteins are energy consuming (Wullschlegel et al., 1997). Lower N content implies lower energy requirements and thus lower growth and maintenance respiration.

It has often been found in FACE or growth cabinet experiments that leaf N content was lower at elevated $[\text{CO}_2]$. This has been explained by different mechanisms. For example, elevated $[\text{CO}_2]$ was shown to cause a decrease in stomatal conductance of leaves, leading to decreasing transpiration rates (Ainsworth and Rogers, 2007) and thus, lower transpiration-driven mass flow of soil N to roots and stems (so-called transpiration mechanism (McGrath and Lobell, 2013; Feng et al., 2015)). Another mechanism is associated with photorespiration. Generally N assimilation is believed to be lower due to lower photorespiration (Bloom et al., 2010), which is accompanied by the reduced reductant supplied via photorespiration at elevated $[\text{CO}_2]$ (Taub and Wang, 2008). Furthermore, a ‘dilution effect’ could occur whereby N uptake does not increase proportionally to the increase of biomass at elevated $[\text{CO}_2]$ (Feng et al., 2015).

The decreased leaf N content at elevated $[\text{CO}_2]$ has also consequences on photosynthetic capacity (i.e. V_{cmax}). It was reported that species grown under elevated $[\text{CO}_2]$ had lower maximum apparent carboxylation velocity (V_{cmax}) and carboxylation efficiency (Ainsworth and Long, 2005). Finally, elevated $[\text{CO}_2]$ significantly increased CUE_L by enhancing photosynthetic rate and reducing dark respiration. Gong et al. (2017) reported that CUE of sunflower stands was higher at 200 ppm growth $[\text{CO}_2]$ than that of 1000 ppm growth $[\text{CO}_2]$. This results thus could not be explained by the response of CUE_L itself since at the leaf level, CUE_L likely increased at elevated growth $[\text{CO}_2]$. We speculate that the reduction of whole plant CUE in their study was mainly due to enhanced respiration of heterotrophic organs or exudation.

Changes in Φ_2 and C_c are involved in the Kok effect and impact on R_L estimates

Our study found a short-term CO_2 effect on R_L estimated using the Kok and Kok-Phi method, but no effect using the Kok- C_c method. In fact, both Kok and Kok-Phi methods showed an increase in R_L when measured at elevated $[\text{CO}_2]$. This short-term response was in agreement with the finding of Yin et al. (2020) and Fang et al. (2021), but is not supported by the findings of other studies (Tcherkez et al., 2008; Griffin and Turnbull, 2013). We believe discrepancies in short-term CO_2 effect on R_L is mostly associated with methodological differences. As shown in the *Theory* section, the classical Kok method has conceptual uncertainty with the assumption that Φ_2 remains constant across the Kok curve. This assumption must be rejected as Φ_2 decreases with increasing I_{inc} (Fig. 2). However, this short-term CO_2 effect on R_L cannot be explained by changes in Φ_2 because (i) the decrease in Φ_2 along Kok curve was similar at both measurement

[CO₂] and (ii) the effect persisted when the Kok-Phi method was used to account for variation in Φ_2 .

Another assumption that has been made for both the Kok and Kok-Phi method is that γ (determined by Γ^*/C_c) remains constant throughout the Kok curve. This assumption has also been challenged in recent model analyses (Buckley et al., 2017; Farquhar and Busch, 2017), but the question is how to quantify the change in C_c as this requires g_m estimates. Here, we used species-specific g_{sc}/g_m ratios to calculate C_c , suggesting that C_c and γ decreased with increasing I_{inc} . Importantly, measurement CO₂ influenced the trend of γ with increasing I_{inc} , which might be the origin of this short-term CO₂ effect on R_L Kok and R_L Kok-Phi. When changes in γ (or Γ^*/C_c) are accounted for, the apparent short-term effect of CO₂ on R_L , as found with the Kok and Kok-Phi methods, became insignificant (see also Fig. 3, 4).

Kok- and Kok-Phi based estimates of R_L suppression are overestimates

The inhibition of R_L by light is supported by biochemical evidence. Utilizing ¹³C labelling, flux calculations suggest that decarboxylation rates associated to glucose catabolism and activation of malic enzyme increase with decreasing irradiance in the irradiance region where the Kok effect occurs (Gauthier et al., 2020). Recently, how much of the Kok effect is associated with respiration has been under debate (Farquhar and Busch, 2017; Gauthier et al., 2020; Yin et al., 2020). Indeed, the methods used in the present study show different levels of inhibition of respiration by light. The average R_L/R_{Dk} was 0.74 for the Kok method, 0.81 for the Kok-Phi method and 0.93 for the Kok- C_c method. That is, the change in Φ_2 , γ (or Γ^*/C_c), and real light inhibition of R_L explained *c.* 27, 46 and 27% of the apparent Kok effect (i.e. the apparent 26%-inhibition of R_L found with the classical Kok method), respectively. This is in agreement with the results of previous model analyses which show that the Kok effect is not purely respiratory (Farquhar and Busch, 2017; Yin et al., 2020), and both the Kok method and the Kok-Phi method underestimated R_L and overestimated the inhibition of R_L (Yin et al., 2020).

The real light inhibition of R_L (as revealed by the Kok- C_c method) was only 6%, which is close to the mean inhibition of 8% of several herbaceous species determined using the ¹³C disequilibrium method (Gong et al., 2018) and the mean inhibition of 10% in wheat leaves determined using a nonrectangular hyperbolic model to interactively solve g_m and R_L (Fang et al.,

2022). In line with these results, a break point in the linear section of the photosynthetic response curve could hardly be seen in the Kok- C_c plots (Fig. 3EF).

The Kok- C_c method developed here requires g_{sc}/g_m to estimate C_c along a Kok curve since C_c cannot be directly measured. Estimating g_m under low light remains technically very challenging. We used species specific g_{sc}/g_m values measured under the growth condition to estimate g_m at each step of Kok curves. Similar approach has been applied to estimate C_c to improve the Laisk method (Gong et al., 2018; Way et al., 2019). These calculations assume that g_{sc}/g_m was the same under the measurement condition of the Kok method and the growth condition. In another word, g_{sc} and g_m should decrease similarly with the decrease of PPFD. This assumption is supported by experimental results (Flexas et al., 2008; Douthe et al., 2011; Xiong et al., 2015). Estimating g_m from species specific g_{sc}/g_m ratio is supported by the robust relationship between g_{sc} and g_m observed in different species under manipulated CO_2 , irradiance, and drought stress (Flexas et al., 2008; Ma et al., 2021; Gong et al., 2022). Although the g_{sc}/g_m ratio estimated here could have a certain level of uncertainty due to methodological issues associated with g_m estimation (Pons et al., 2009; Gu and Sun, 2014; Gong et al., 2015), R_L Kok- C_c was not very sensitive to g_{sc}/g_m . Importantly, the factor that directly influences R_L Kok- C_c estimation is the decreasing rate of γ with the increase of I_{inc} ($d\gamma/dI_{inc}$) but not absolute values of g_m or C_c . Varying g_{sc}/g_m by ± 0.4 or assuming a constant g_m has little effect on $d\gamma/dI_{inc}$ and a negative $d\gamma/dI_{inc}$ was evident in all cases (Fig. S1, S2). In effect, our sensitivity tests showed that varying g_{sc}/g_m by ± 0.4 has a minor influence on both R_L estimates and the CO_2 effect (Fig. S3). However, R_L/R_{Dk} is sensitive to small variations in R_L and thus is affected by g_{sc}/g_m (Fig. S4). Adjusting g_{sc}/g_m (± 0.4 units) leads to changes of mean light inhibition from 4% to 10%. These results highlighted that accounting for $d\gamma/dI_{inc}$ is essential for estimating R_L (Farquhar and Busch, 2017), and the uncertainty associated with the accuracy of $d\gamma/dI_{inc}$ is much less than assuming a constant γ along a Kok curve. The Kok- C_c based estimates of R_L suppression could be further improved if a new method is developed to precisely estimate g_m at very low light. Taken as a whole, neither the Kok nor Kok-Phi method seem suitable to quantify the inhibition of respiration by light (as also suggested by Yin et al. 2020 and Tcherkez et al. 2017a, 2017b), and the inhibition of R_L at the operating PPFDs of this study should be lower than 10%.

Conclusions and Perspectives

This study showed that elevated growth [CO₂] reduced R_L and R_{DK} likely as a result of decreasing leaf N status and chlorophyll content. We found no significant long-term CO₂ effect on R_L/R_{DK} , indicating a concurrent response of R_L and R_{DK} to elevated growth [CO₂], mediated by the adjustment of nitrogen metabolism in leaves. These results shed light into the incorporation of R_L into the carbon cycling models. We revisited the theoretical basis of the Kok method, revised Kok methods and discussed their respective limitations. Using Kok and Kok-Phi methods, we found that R_L were stimulated by short-term CO₂ enrichment, while the effect was not supported by the data of the Kok- C_c method. We attributed this short-term CO₂ effect to methodological uncertainty associated with unaccounted changes in γ (or Γ^*/C_c) along a Kok curve. Accounting for those effects, we found that the Kok and Kok-Phi method underestimate R_L and overestimate the inhibition of respiration under low irradiance conditions of the Kok method, and the inhibition of R_L is only $6\pm 4\%$, which represents 26% of the Kok effect (i.e. of the apparent inhibition of R_L found using the classical Kok method). Although the Kok- C_c method has less theoretical uncertainty and is thus in principle more reliable, we are aware that all three methods have operating PPFD much lower than usual, ambient irradiance encountered by plants. However, estimated R_L could vary with irradiance. Earlier studies have showed a decrease of R_L with the increase of operating PPFD (Brooks and Farquhar, 1985; Atkin et al., 1998; Atkin et al., 2000) by using the Laisk method which also has the uncertainty associated with the unaccounted changes in C_c (Farquhar and Busch 2017). To date, the effect of irradiance on R_L is poorly known and this should be addressed in subsequent studies.

MATERIALS AND METHODS

Theory

When estimating R_L with the Kok method, A should be measured at low irradiance, where A is limited by the light-dependent electron transport rate. According to the equation of the electron transport-limited photosynthesis (Farquhar et al., 1980), A at low light is described as:

$$A = J \frac{1 - \Gamma^*/C_c}{4 + 8\Gamma^*/C_c} - R_L \quad \text{Eqn 1}$$

where J is the electron transport rate that is used for CO₂ fixation and photorespiration, Γ^* is the C_c -based CO₂ compensation point in the absence of mitochondrial respiration ($37.4 \mu\text{mol mol}^{-1}$ at 25°C, (Silva-Perez et al., 2017)). According to the theoretical evaluations of Yin et al. (2011,

2020), Eqn 1 forms the theoretical basis of the Kok method, and is useful for evaluating methodological uncertainties.

In this equation, J can be replaced by $f_{\text{aet}}\Phi_2\rho_2\alpha I_{\text{inc}}$, where f_{aet} is the fraction of electron transport for photosynthesis, ρ_2 is the fraction of absorbed irradiance partitioned to PS II, α is the absorptance by leaf photosynthetic pigments and I_{inc} is incident irradiance (Yin et al., 2011). Here, we define the term $\frac{1-\Gamma^*/C_c}{4+8\Gamma^*/C_c}$ as γ , so that Equation (1) becomes:

$$A = \gamma f_{\text{aet}}\Phi_2\rho_2\alpha I_{\text{inc}} - R_L \quad \text{Eqn 2}$$

With the Kok method, net CO_2 assimilation rates are plotted against I_{inc} and datapoints that fall above the breakpoint are used to extrapolate A up the y-axis and thereby estimate R_L . In fact, if the term $\gamma f_{\text{aet}}\Phi_2\rho_2\alpha$ is assumed to be constant, thus the intercept of this linear relation provides the estimate of $R_{L \text{ Kok}}$. In terms of equation, this can be written as:

$$A = (\gamma f_{\text{aet}}\Phi_2\rho_2\alpha) I_{\text{inc}} - R_{L \text{ Kok}} \quad \text{Eqn 3}$$

However, it has been shown that Φ_2 could decrease with increasing I_{inc} even within the range of low irradiance (Genty and Harbinson, 1996; Yin et al., 2020). Alternatively, Φ_2 can be obtained from chlorophyll fluorescence measurements. Yin et al. (2009) thus suggested to plot A against $\Phi_2 I_{\text{inc}}$ as:

$$A = (\gamma f_{\text{aet}}\rho_2\alpha)\Phi_2 I_{\text{inc}} - R_{L \text{ Kok-Phi}} \quad \text{Eqn 4}$$

The Yin et al. (2011) method can be considered as a revised Kok method with variation in Φ_2 accounted for, and thus it is renamed as the 'Kok-Phi' method here to highlight the modification. This method assumes that γ is constant across Kok curve, which is obviously not true under photorespiratory conditions, i.e. under ambient conditions where O_2 mole fraction is about 21% (Yin et al., 2014). Theoretically, the Kok-Phi method is applicable for measuring C_3 leaves at nonphotorespiratory conditions or C_4 leaves (Yin et al., 2011; 2020; Fang et al., 2021).

On the basis of these two methods, we propose a revised Kok method, named 'Kok- C_c ' method, accounting for variations in γ caused by the decrease in C_c along the Kok curve. In the Kok- C_c method, A should be plotted against $\gamma\Phi_2 I_{\text{inc}}$, the intercept of the linear relation yields the estimation of R_L ($R_{L \text{ Kok-}C_c}$):

$$A = (f_{\text{aet}}\rho_2\alpha)\gamma\Phi_2 I_{\text{inc}} - R_{L \text{ Kok-}C_c} \quad \text{Eqn 5}$$

This method requires estimates of C_c at each step of the A - I_{inc} curve (see below the section dedicated to C_c estimation). It is worth noting that in practice all 'Kok type' methods, assume

that R_L is not sensitive to changes in C_c along the Kok curve, as they rely on linear extrapolations. To our knowledge, this assumption has not been verified (see *Introduction*).

Plant material and growth conditions

Sunflower (*Helianthus annuus* L.) and wheat (*Triticum aestivum* L.) plants were grown from seed in plastic pots with garden soil and thinned to one plant per pot. Initial nutrient composition of the garden soil (Scotts Miracle-Gro, USA) was 0.68% N, 0.27% P_2O_5 , and 0.36% K_2O . Plants were randomly placed in two growth chambers, where CO_2 mole fraction was 410 ppm (ambient) and 820 ppm (elevated $[CO_2]$) respectively. In both chambers, air temperature was maintained at 25°C and the relative humidity of the air was 70% for both light and dark periods. The photosynthetic photon flux density (PPFD) was $700 \mu mol m^{-2} s^{-1}$ for 16-h photoperiod. All plants were watered every 2-3 days to prevent water stress. This experiment had six replicates per treatment, and in total 24 plants were used for measurements.

Gas exchange and chlorophyll fluorescence measurements

Photosynthetic gas exchange and ChF parameters were measured when there were 4 fully expanded leaves in each plant (sunflower) or tiller (wheat). Using a portable gas exchange system (LI-6800; Li-Cor Inc., Lincoln, NE, USA), measurements were undertaken on the second youngest fully developed leaves. Light response curves and ChF parameters were measured to estimate R_L . When stable gas exchange rates were achieved, we measured A starting at $120 \mu mol m^{-2} s^{-1}$, and the PPFD was sequentially reduced to 100, 80, 60, 40, 20 and 0 (i.e., with light source switched off) $\mu mol m^{-2} s^{-1}$. ChF measurements were done at PPFD of 120, 100, 80, 60 and $40 \mu mol m^{-2} s^{-1}$ using the multi-phase flash method. Φ_2 was calculated as

$$\Phi_2 = (F_m' - F_s)/F_m' \quad \text{Eqn 6}$$

where F_s is the steady-state fluorescence in the light conditions and F_m' is maximal fluorescence during short saturating pulses of light. For each leaf, the irradiance response of photosynthesis rates was determined at two atmospheric $[CO_2]$ (410 and 820 ppm) to assess short-term CO_2 response of R_L .

All gas exchange parameters have been corrected for the leak effect (i.e. CO_2 diffusion across gaskets of leaf chamber) using the measured leak coefficients of intact leaves (Gong et al., 2015; Gong et al., 2018). R_{Dk} measured at 410 and 820 ppm $[CO_2]$ was used to calculate the

cuvette leak coefficient for CO₂ (K_{CO_2}) with the leaf present in the leaf chamber using the equations in (Gong et al., 2015). K_{CO_2} was not significantly different between species and growth [CO₂], with a mean K_{CO_2} of 0.21 for wheat and 0.30 for sunflower (Fig. S5). Thereafter, the response of A to [CO₂] (i.e. A - C_i curve) was determined under an irradiance of 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and varying CO₂, using a [CO₂] sequence of 410, 200, 150, 100, 50, 410, 800 and 1600 $\mu\text{mol mol}^{-1}$. ChF parameters were acquired at 200, 410, 800 and 1600 $\mu\text{mol mol}^{-1}$ CO₂. Leaf temperature was maintained at 25°C for all gas exchange measurements, there is thus no temperature correction needed to compare R_L and R_{Dk} .

Estimation of day respiration and C_c

For the Kok method, the data of the linear range of the A : I_{inc} curve at PPFD levels above the Kok breakpoint (kink) were used to estimate R_L according to Eqn 3. Each A : I_{inc} curve was visually inspected to identify the irradiance at the Kok breakpoint, which was 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. S6). The data measured at PPFD of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ deviated from the linear relation (i.e. the linear domain of assimilation response curve to light between 40-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$), thus they were excluded from the dataset used for the estimation of R_L via all methods. Linear regressions were performed using data of the PPFD levels of 40, 60, 80, and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for all three methods, with the exception of 5 out of 45 curves in which a point that deviated from the linear relation was excluded for the estimation of R_L . For the Kok-Phi method, the data from the same PPFD range were used to estimate R_L by plotting A against $\Phi_2 I_{inc}$ according to Eqn 4. We have not intensively measured A at very low PPFD levels to accurately identify the breakpoint. However, the data at 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD seem to be above the Kok breakpoint and in the linear domain of A : I_{inc} curves. Our approach is similar to recent studies which compared the Kok and the Kok-Phi method (Yin et al., 2011; Fang et al., 2021).

Estimating R_L from the Kok- C_c method requires estimates of mesophyll conductance (g_m). According to the variable J method of Harley et al. (1992), g_m could be calculated as:

$$g_m = \frac{A}{C_i - \frac{\Gamma^*[J+8(A+R_L)]}{J-4(A+R_L)}} \quad \text{Eqn 7}$$

Here, we used R_L estimated using the Kok-Phi method to calculate g_m , given that this method addresses the issue of decreasing Φ_2 and provides a more reliable estimation of R_L , compared to the Kok method (Yin et al., 2011). Furthermore, using $R_{L \text{ Kok}}$ or $R_{L \text{ Kok-Phi}}$ has minor influence on

g_{sc}/g_m , thus should have no influence on our conclusions (see the discussion on the uncertainty associated with g_{sc}/g_m). We chose data in a reliable range of dC_c/dA between 10 and 50 for estimating g_m as suggested by Harley et al. (1992). dC_c/dA was calculated as:

$$dC_c/dA = 12\Gamma^* J / (J - 4(A + R_L))^2 \quad \text{Eqn 8}$$

Most of the data obtained with sunflower met this empirical criterion of dC_c/dA , while dC_c/dA of wheat exceeded this range ($dC_c/dA > 100$) in most cases. Therefore, the $A-C_i$ curve-fitting method was used to estimate the g_m value of each leaf in wheat. Based on the FvCB photosynthesis model (Farquhar et al., 1980), the $A-C_i$ curve fitting tool developed by Sharkey et al. (2007) was used to estimate g_m by minimizing the sum of squared deviations between the observed and modelled data.

Recently, it has been found that g_m and stomatal conductance to CO_2 (g_{sc}) are strongly related (Flexas et al., 2012; Ma et al., 2021). A nearly fixed g_{sc}/g_m ratio across different environments and plant functional groups was shown by Ma et al. (2021), offering a useful solution to estimate g_m . We first obtained species- and treatment-specific g_{sc}/g_m using Equation 7 (sunflower) or curve fitting (wheat), and then g_m along the Kok curve was estimated from measured g_{sc} and previously estimated g_{sc}/g_m . C_c was calculated from g_m as:

$$C_c = C_i - A/g_m \quad \text{Eqn 9}$$

With C_c , γ could be calculated and thus $R_{L \text{ Kok-Cc}}$ could be estimated by plotting A against $\gamma\Phi_2 I_{inc}$ using data of the PPFD range of 40-100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ according to Eqn 5. We also tested the sensitivity of $R_{L \text{ Kok-Cc}}$ to g_{sc}/g_m by adjusting obtained species- and treatment-specific g_{sc}/g_m (± 0.4).

The daily carbon-use efficiency of leaves, the ratio of net carbon gain to assimilated carbon (integrated photosynthesis) was calculated as:

$$\text{CUE}_L = (\int A - \int R_{Dk}) / (\int A + \int R_{L \text{ Kok-Cc}}) \quad \text{Eqn 10}$$

Since plants were grown in controlled environments, the daily carbon fluxes were calculated as $\int A = A \times \text{light hours}$, $\int R_{L \text{ Kok-Cc}} = R_{L \text{ Kok-Cc}} \times \text{light hours}$, and $\int R_{Dk} = R_{Dk} \times \text{dark hours}$.

Plant sampling and leaf trait parameters

After gas exchange and ChF measurements, the measured leaves were harvested. We measured leaf area and fresh weight, and the chlorophyll content (Chl) was determined by a chlorophyll meter (SPAD-502 Plus; Konica Minolta Inc., Tokyo, Japan). The chlorophyll content was

1 calculated from the observed SPAD values as $\text{Chl} = (99 \text{ SPAD}) / (144 - \text{SPAD})$ (Cerovic et al.,
2 2012). All leaves were dried at 70 °C to constant mass after drying to stop enzymatic activity at
3 105 °C for 1 hour. We measured dry mass of individual leaves, and then the leaves were ground
4 with a ball mill (Tissuelyser-24, Jingxin Ltd., Shanghai, China). Leaf N content was measured
5 using an elemental analyzer (VARIO EL III, Elementar Analysensysteme GmbH, Hanau,
6 Germany).

8 **Statistical analysis**

9 Statistical analysis was performed using SPSS (v. 25.0, SPSS, Chicago, IL, USA). Leaf traits and
10 photosynthetic parameters were analyzed with two-way ANOVAs to determine the influence of
11 growth $[\text{CO}_2]$, species and their interaction. Besides, ANOVAs were carried out to clarify the
12 effect of growth $[\text{CO}_2]$, measurement $[\text{CO}_2]$, their interaction and species on R_L and R_L/R_{Dk} . A p -
13 value lower than 0.05 is considered statistically significant.

15 **FUNDING**

16 This work was supported by the National Natural Science Foundation of China (NSFC
17 31870377, 32120103005).

19 **DATA AVAILABILITY**

20 All data that support the findings of this study are included in the published article and its
21 Supplementary Information.

TABLES

Table 1. Leaf traits and photosynthetic parameters of wheat (*T. aestivum*) and sunflower (*H. annuus*) grown under ambient or elevated CO₂ (aCO₂ or eCO₂). Leaf trait parameters include: specific leaf area (SLA, cm² mg⁻¹), leaf nitrogen content per dry mass (N%), leaf nitrogen content per area (N_{area}, g m⁻²), chlorophyll content (Chl, g m⁻²). Photosynthetic parameters include net CO₂ assimilation rate at the growth CO₂ (A_{growth} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), maximum CO₂ assimilation rate (A_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), respiration rate in the dark (R_{Dk} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), intrinsic water-use efficiency ($i\text{WUE}_{\text{growth}}$, $\mu\text{mol mol}^{-1}$), maximum carboxylation rates by Rubisco (V_{cmax} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), electron transport rate (J , $\mu\text{mol m}^{-2} \text{s}^{-1}$), ratio of stomatal conductance for CO₂ to mesophyll conductance ($g_{\text{sc}}/g_{\text{m}}$), leaf carbon-use- efficiency (CUE_L). Data are mean \pm SE ($n=6$); significant treatment effects ($P<0.05$) tested with two-way ANOVAs are shown in bold.

	<i>T. aestivum</i>		<i>H. annuus</i>		Significance		
	aCO ₂	eCO ₂	aCO ₂	eCO ₂	<i>spe</i>	CO ₂	<i>spe</i> ×CO ₂
A_{growth}	28.42±0.73	32.79±2.01	27.27±2.57	31.43±2.06	0.529	0.042	0.956
A_{max}	37.68±1.29	34.04±2.22	32.32±2.40	31.83±1.73	0.068	0.304	0.430
R_{Dk}	2.26±0.14	1.81±0.17	1.48±0.11	1.32±0.14	<0.001	0.046	0.327
$i\text{WUE}_{\text{growth}}$	52.64±1.30	62.25±3.94	37.30±10.25	102.14±24.99	0.380	0.013	0.057
SLA	0.24±0.01	0.27±0.02	0.22±0.02	0.23±0.02	0.038	0.252	0.511
N%	6.61±0.17	6.39±0.11	3.51±0.67	3.16±0.56	<0.001	0.527	0.882
N _{area}	2.70±0.06	2.37±0.10	1.53±0.18	1.35±0.16	<0.001	0.062	0.586
Chl	0.69±0.02	0.56±0.03	0.49±0.05	0.48±0.02	<0.001	0.04	0.083
V_{cmax}	160.9±3.2	142.7±13.0	123.0±11.7	117.4±3.3	0.002	0.203	0.493
J	183.4±3.3	160.1±9.3	164.2±10.4	158.9±8.6	0.234	0.101	0.293
$g_{\text{sc}}/g_{\text{m}}$	0.95±0.03	1.16±0.07	1.74±0.55	1.97±0.42	0.087	0.222	0.859
CUE _L	0.89±0.01	0.92±0.01	0.92±0.02	0.94±0.01	0.011	0.004	0.547

Table 2. ANOVA tests for R_L estimated by the Kok, Kok-Phi and Kok- C_c methods. Significant treatment effects ($P < 0.05$) are shown in bold.

Source	df	R_L Kok		R_L Kok-Phi		R_L Kok- C_c	
		F	P	F	P	F	P
Species	1	28.36	<0.001	29.74	<0.001	20.06	<0.001
Growth CO_2	1	6.681	0.013	5.777	0.021	3.921	0.055
Measurement CO_2	1	1.227	0.275	1.714	0.198	0.198	0.658
Growth CO_2^*	1	0.092	0.763	0.107	0.745	0.072	0.790
Measurement CO_2	1						

Table 3. ANOVA tests for R_L/R_{Dk} estimated by the Kok, Kok-Phi and Kok- C_c methods. Significant treatment effects ($P < 0.05$) are shown in bold.

Source	df	R_L Kok/ R_{Dk}		R_L Kok-Phi/ R_{Dk}		R_L Kok- C_c / R_{Dk}	
		F	P	F	P	F	P
Species	1	2.824	0.101	1.638	0.208	0.095	0.759
Growth CO_2	1	2.216	0.144	0.799	0.377	0.389	0.536
Measurement CO_2	1	7.480	0.009	10.410	0.003	1.140	0.292
Growth CO_2^*	1	0.328	0.570	0.441	0.511	0.243	0.625
Measurement CO_2	1						

FIGURE LEGENDS

Figure 1. Net CO_2 assimilation rate (A), transpiration rate (E) and stomatal conductance for water vapor (g_{sw}) in response to short-term variation of intercellular CO_2 concentration (C_i) for wheat (*T. aestivum*) and sunflower (*H. annuus*). Blue circles refer to ambient ($410 \mu\text{mol mol}^{-1}$) growth CO_2 , and red squares refer to elevated ($820 \mu\text{mol mol}^{-1}$) growth CO_2 . Data are shown as mean \pm SE ($n=6$).

Figure 2. Photochemical efficiency of photosystem II (Φ_2), chloroplastic CO_2 concentration (C_c) and γ (the lumped parameter in Eqn 2) in response to incident irradiance (I_{inc}) for wheat (*T. aestivum*) and sunflower (*H. annuus*). Plants grown under ambient CO_2 (a CO_2 , blue circles) or elevated CO_2 (e CO_2 , red squares) were measured at gaseous conditions of $410 \mu\text{mol mol}^{-1}$ (open symbols) or $820 \mu\text{mol mol}^{-1}$ (closed symbols) CO_2 in the leaf chamber. Data are shown as mean \pm SE ($n=6$).

Figure 3. Net CO_2 assimilation rate (A) in response to I_{inc} (incident irradiance), $\Phi_2 I_{inc}$ (Φ_2 , photochemical efficiency of photosystem II) or $\gamma \Phi_2 I_{inc}$ (γ , the lumped parameter in Eqn 2) for wheat (*T. aestivum*) and sunflower (*H. annuus*). Data are mean \pm SE ($n=6$). Meaning of symbols of different CO_2 treatments and measurement conditions are shown in Fig. 2.

Figure 4. Effects of growth CO_2 treatments (a CO_2 and e CO_2) and measurement conditions (410 and $820 \text{ ppm } CO_2$) on respiration rates in the light (R_L) estimated by three methods for wheat (*T.*

aestivum) and sunflower (*H. annuus*). R_L was measured by the Kok (A and D), Kok-Phi (B and E) and Kok- C_c (C and F) methods. Data are mean \pm SE ($n=5-6$). The results of ANOVA tests are shown in Table 2.

Figure 5. Effects of growth CO_2 treatments (a CO_2 and e CO_2) and measurement conditions (410 and 820 ppm) on ratio of respiration in the light to respiration in the dark (R_L/R_{Dk}) for wheat (*T. aestivum*) and sunflower (*H. annuus*). R_L/R_{Dk} was estimated by the Kok (A and D), Kok-Phi (B and E) and Kok- C_c (C and F) methods. Data are mean \pm SE ($n=5-6$). The results of ANOVA tests are shown in Table 3.

Figure 6. Correlation between respiration in the dark (R_{Dk}) and respiration in the light (R_L). R_L was measured by the Kok (A), Kok-Phi (B) and Kok- C_c (C) methods. The average R_L/R_{Dk} (\pm SE, $n=45$) was calculated by pooling over the data of species (wheat and sunflower) and CO_2 treatments. Grey dashed lines give the 1:1 relationship.

REFERENCES

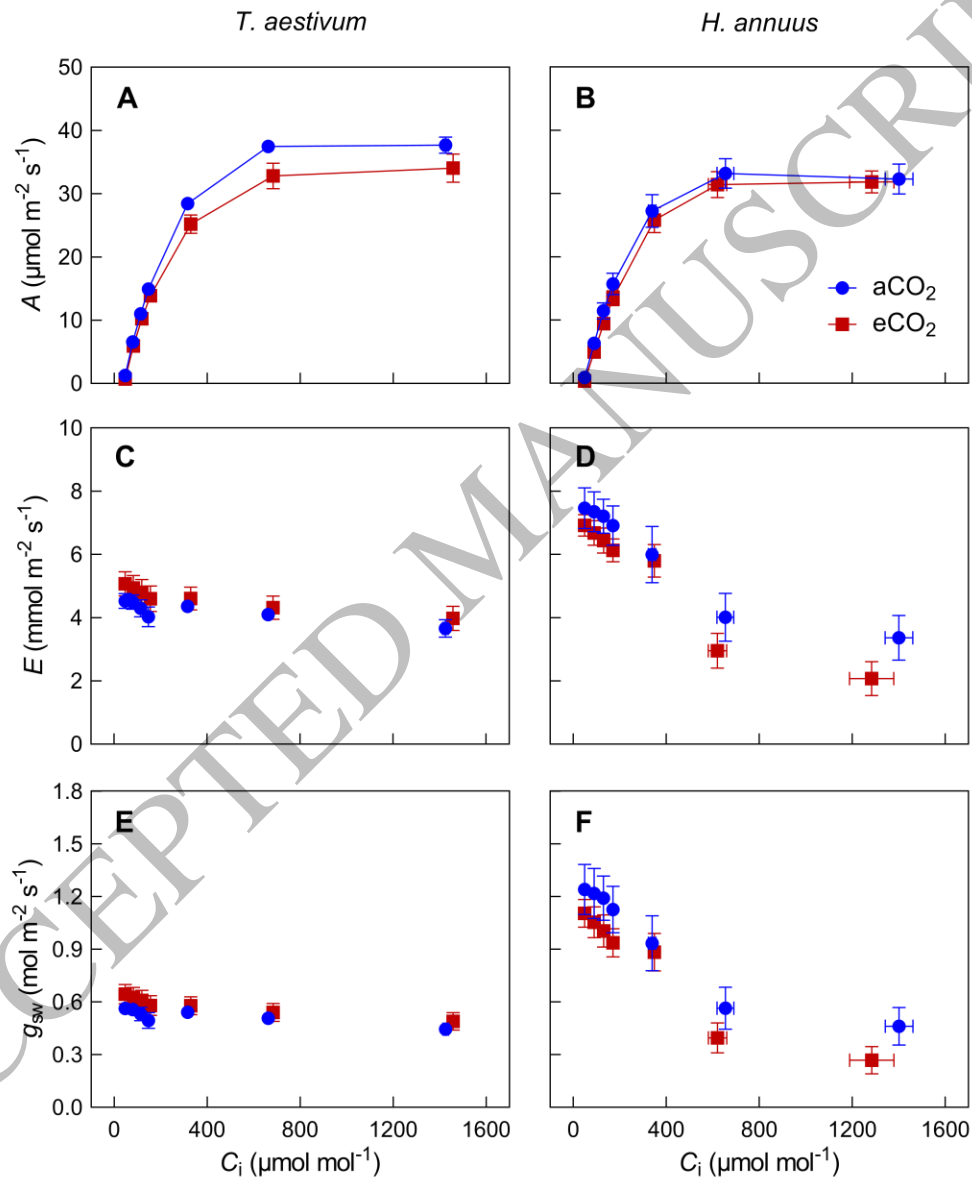
- Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2 . *New Phytologist* **165**: 351-371
- Ainsworth EA, Rogers A (2007) The response of photosynthesis and stomatal conductance to rising $[CO_2]$: mechanisms and environmental interactions. *Plant, Cell and Environment* **30**: 258-270
- Amthor JS (2000) The McCree-de Wit-Penning de Vries-Thornley respiration paradigms: 30 years later. *Annals of Botany* **86**: 1-20
- Atkin OK, Bahar NHA, Bloomfield KJ, Griffin KL, Heskell MA, Huntingford C, de la Torre AM, Turnbull MH (2017) Leaf Respiration in Terrestrial Biosphere Models. *In* *Plant Respiration: Metabolic Fluxes and Carbon Balance*, pp 107-142
- Atkin OK, Evans JR, Ball MC, Lambers H, Pons TL (2000) Leaf respiration of snow gum in the light and dark: interactions between temperature and irradiance. *Plant Physiology* **122**: 915-923
- Atkin OK, Evans JR, Siebke K (1998) Relationship between the inhibition of leaf respiration by light and enhancement of leaf dark respiration following light treatment. *Functional Plant Biology* **25**: 437-443
- Atkin OK, Scheurwater I, Pons TL (2007) Respiration as a percentage of daily photosynthesis in whole plants is homeostatic at moderate, but not high, growth temperatures. *New Phytologist* **174**: 367-380
- Ayub G, Smith RA, Tissue DT, Atkin OK (2011) Impacts of drought on leaf respiration in darkness and light in *Eucalyptus saligna* exposed to industrial-age atmospheric CO_2 and growth temperature. *New Phytologist* **190**: 1003-1018
- Ayub G, Zaragoza-Castells J, Griffin KL, Atkin OK (2014) Leaf respiration in darkness and in the light under pre-industrial, current and elevated atmospheric CO_2 concentrations. *Plant Science* **226**: 120-130
- Bloom AJ, Burger M, Asensio JSR, Cousins AB (2010) Carbon dioxide enrichment inhibits nitrate assimilation in wheat and *Arabidopsis*. *Science* **328**: 899-903
- Bloom AJ, Burger M, Kimball BA, Pinter PJ (2014) Nitrate assimilation is inhibited by elevated CO_2 in field-grown wheat. *Nature Climate Change* **4**: 477-480
- Brooks A, Farquhar GD (1985) Effect of temperature on the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light. *Planta* **165**: 397-406

- 1 **Buckley TN, Vice H, Adams MA** (2017) The Kok effect in *Vicia faba* cannot be explained solely by
2 changes in chloroplastic CO₂ concentration. *New Phytologist* **216**: 1064-1071
- 3 **Busch FA, Sage RF, Farquhar GD** (2018) Plants increase CO₂ uptake by assimilating nitrogen via the
4 photorespiratory pathway. *Nature Plants* **4**: 46-54
- 5 **Cerovic ZG, Masdoumier G, Ghozlen NB, Latouche G** (2012) A new optical leaf-clip meter for
6 simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids.
7 *Physiologia Plantarum* **146**: 251-260
- 8 **Cramer W, Bondeau A, Woodward FI, Prentice IC, Betts RA, Brovkin V, Cox PM, Fisher V, Foley**
9 **JA, Friend AD, et al.** (2001) Global response of terrestrial ecosystem structure and function to
10 CO₂ and climate change: results from six dynamic global vegetation models. *Global Change*
11 *Biology* **7**: 357-373
- 12 **Crous KY, Wallin G, Atkin OK, Uddling J, Af Ekenstam A** (2017) Acclimation of light and dark
13 respiration to experimental and seasonal warming are mediated by changes in leaf nitrogen in
14 *Eucalyptus globulus*. *Tree Physiology* **37**: 1069-1083
- 15 **Crous KY, Zaragoza-Castells J, Ellsworth DS, Duursma RA, Low M, Tissue DT, Atkin OK** (2012)
16 Light inhibition of leaf respiration in field-grown *Eucalyptus saligna* in whole-tree chambers
17 under elevated atmospheric CO₂ and summer drought. *Plant, Cell and Environment* **35**: 966-981
- 18 **Douthe C, Dreyer E, Epron D, Warren CR** (2011) Mesophyll conductance to CO₂, assessed from
19 online TDL-AS records of ¹³CO₂ discrimination, displays small but significant short-term
20 responses to CO₂ and irradiance in *Eucalyptus* seedlings. *Journal of Experimental Botany* **62**:
21 5335-5346
- 22 **Drake BG, González-Meler MA, P. LS** (1997) More efficient plants: a consequence of rising
23 atmospheric CO₂? *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 609-639
- 24 **Dusenge ME, Duarte AG, Way DA** (2019) Plant carbon metabolism and climate change: elevated CO₂
25 and temperature impacts on photosynthesis, photorespiration and respiration. *New Phytologist*
26 **221**: 32-49
- 27 **Fang L, Yin X, van der Putten PEL, Martre P, Struik PC** (2022) Drought exerts a greater influence
28 than growth temperature on the temperature response of leaf day respiration in wheat (*Triticum*
29 *aestivum*). *Plant, Cell & Environment* **45**: 2062-2077
- 30 **Farquhar GD, Busch FA** (2017) Changes in the chloroplastic CO₂ concentration explain much of the
31 observed Kok effect: a model. *New Phytologist* **214**: 570-584
- 32 **Farquhar GD, von Caemmerer S, Berry JA** (1980) A biochemical model of photosynthetic CO₂
33 assimilation in leaves of C₃ species. *Planta* **149**: 78-90
- 34 **Feng Z, Rutting T, Pleijel H, Wallin G, Reich PB, Kammann CI, Newton PC, Kobayashi K, Luo Y,**
35 **Uddling J** (2015) Constraints to nitrogen acquisition of terrestrial plants under elevated CO₂.
36 *Global Change Biology* **21**: 3152-3168
- 37 **Flexas J, Barbour MM, Brendel O, Cabrera HM, Carriqui M, Diaz-Espejo A, Douthe C, Dreyer E,**
38 **Ferrio JP, Gago J, et al.** (2012) Mesophyll diffusion conductance to CO₂: an unappreciated
39 central player in photosynthesis. *Plant Science* **193-194**: 70-84
- 40 **Flexas J, Ribas-Carbo M, Diaz-Espejo A, Galmes J, Medrano H** (2008) Mesophyll conductance to
41 CO₂: current knowledge and future prospects. *Plant, Cell and Environment* **31**: 602-621
- 42 **Gauthier PPG, Saenz N, Griffin KL, Way D, Tcherkez G** (2020) Is the Kok effect a respiratory
43 phenomenon? Metabolic insight using ¹³C labeling in *Helianthus annuus* leaves. *New Phytologist*
44 **228**: 1243-1255
- 45 **Genty B, Harbinson J** (1996) Regulation of Light Utilization for Photosynthetic Electron Transport. *In*
46 *Photosynthesis and the Environment*, pp 67-99
- 47 **Gifford RM** (2003) Plant respiration in productivity models: conceptualisation, representation and issues
48 for global terrestrial carbon-cycle research. *Functional Plant Biology* **30**: 171-186
- 49 **Gong XY, Ma WT, Yu YZ, Fang K, Yang Y, Tcherkez G, Adams MA** (2022) Overestimated gains in
50 water-use efficiency by global forests. *Global Change Biology* **28**: 4923-4934

- 1 **Gong XY, Schauffele R, Feneis W, Schnyder H** (2015) $^{13}\text{CO}_2$ / $^{12}\text{CO}_2$ exchange fluxes in a clamp-on leaf
2 cuvette: disentangling artefacts and flux components. *Plant, Cell and Environment* **38**: 2417-2432
- 3 **Gong XY, Schauffele R, Lehmeier CA, Tcherkez G, Schnyder H** (2017) Atmospheric CO_2 mole
4 fraction affects stand-scale carbon use efficiency of sunflower by stimulating respiration in light.
5 *Plant, Cell and Environment* **40**: 401-412
- 6 **Gong XY, Tcherkez G, Wenig J, Schauffele R, Schnyder H** (2018) Determination of leaf respiration in
7 the light: comparison between an isotopic disequilibrium method and the Laisk method. *New*
8 *Phytologist* **218**: 1371-1382
- 9 **Griffin KL, Anderson OR, Gastrich MD, Lewis JD, Lin GH, Schuster W, Seemann JR, Tissue DT,**
10 **Turnbull MH, Whitehead D** (2001) Plant growth in elevated CO_2 alters mitochondrial number
11 and chloroplast fine structure. *Proceedings of the National Academy of Sciences of the United*
12 *States of America* **98**: 2473-2478
- 13 **Griffin KL, Turnbull MH** (2013) Light saturated RuBP oxygenation by Rubisco is a robust predictor of
14 light inhibition of respiration in *Triticum aestivum* L. *Plant Biology* **15**: 769-775
- 15 **Gu LH, Sun Y** (2014) Artefactual responses of mesophyll conductance to CO_2 and irradiance estimated
16 with the variable *J* and online isotope discrimination methods. *Plant, Cell and Environment* **37**:
17 1231-1249
- 18 **Harley PC, Loreto F, Marco GD, Sharkey TD** (1992) Theoretical Considerations when Estimating the
19 Mesophyll Conductance to CO_2 Flux by Analysis of the Response of Photosynthesis to CO_2 .
20 *Plant Physiology* **98**: 1429-1436
- 21 **Keenan TF, Migliavacca M, Papale D, Baldocchi D, Reichstein M, Torn M, Wutzler T** (2019)
22 Widespread inhibition of daytime ecosystem respiration. *Nature Ecology & Evolution* **3**: 407-415
- 23 **Kok B** (1949) On the interrelation of respiration and photosynthesis in green plants. *Biochimica et*
24 *Biophysica Acta* **3**: 625-631
- 25 **Laisk A** (1977) Kinetics of photosynthesis and photorespiration in C_3 plants. Moscow, Russia: Nauka
- 26 **Leakey AD, Ainsworth EA, Bernacchi CJ, Rogers A, Long SP, Ort DR** (2009) Elevated CO_2 effects
27 on plant carbon, nitrogen, and water relations: six important lessons from FACE. *Journal of*
28 *Experimental Botany* **60**: 2859-2876
- 29 **Long SP, Ainsworth EA, Rogers A, Ort DR** (2004) Rising atmospheric carbon dioxide: plants FACE
30 the future. *Annu Rev Plant Biol* **55**: 591-628
- 31 **Ma WT, Tcherkez G, Wang XM, Schauffele R, Schnyder H, Yang Y, Gong XY** (2021) Accounting
32 for mesophyll conductance substantially improves ^{13}C -based estimates of intrinsic water-use
33 efficiency. *New Phytologist* **229**: 1326-1338
- 34 **McGrath JM, Lobell DB** (2013) Reduction of transpiration and altered nutrient allocation contribute to
35 nutrient decline of crops grown in elevated CO_2 concentrations. *Plant, Cell & Environment* **36**:
36 697-705
- 37 **Norby RG, DeLuciac EH, Gielend B, Calfapietra C, Giardinà CP, King J, Ledford J,**
38 **McCarthy HR, Moore DJP, Ceulemans R** (2005) Forest response to elevated CO_2 is
39 conserved across a broad range of productivity. *Proceedings of the National Academy of*
40 *Sciences, USA* **102**: 18052-18056
- 41 **Pinelli P, Loreto F** (2003) $^{12}\text{CO}_2$ emission from different metabolic pathways measured in illuminated
42 and darkened C_3 and C_4 leaves at low, atmospheric and elevated CO_2 concentration. *Journal of*
43 *Experimental Botany* **54**: 1761-1769
- 44 **Pons TL, Flexas J, von Caemmerer S, Evans JR, Genty B, Ribas-Carbo M, Brugnoli E** (2009)
45 Estimating mesophyll conductance to CO_2 : methodology, potential errors, and recommendations.
46 *Journal of Experimental Botany* **60**: 2217-2234
- 47 **Reich PB, Tjoelker MG, Pregitzer KS, Wright IJ, Oleksyn J, Machado J-L** (2008) Scaling of
48 respiration to nitrogen in leaves, stems and roots of higher land plants. *Ecology Letters* **11**: 793-
49 801
- 50 **Rogers A, Allen DJ, Davey PA, Morgan PB, Ainsworth EA, Bernacchi CJ, Cornic G, Dermody O,**
51 **Dohleman FG, Heaton EA, et al.** (2004) Leaf photosynthesis and carbohydrate dynamics of

- soybeans grown throughout their life-cycle under Free-Air Carbon dioxide Enrichment. *Plant Cell and Environment* **27**: 449-458
- Shapiro JB, Griffin KL, Lewis JD, Tissue DT** (2004) Response of *Xanthium strumarium* leaf respiration in the light to elevated CO₂ concentration, nitrogen availability and temperature. *New Phytologist* **162**: 377-386
- Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL** (2007) Fitting photosynthetic carbon dioxide response curves for C₃ leaves. *Plant, Cell and Environment* **30**: 1035-1040
- Sharp RE, Matthews MA, Boyer JS** (1984) Kok Effect and the Quantum Yield of Photosynthesis. *Plant Physiology* **75**: 95-101
- Silva-Perez V, Furbank RT, Condon AG, Evans JR** (2017) Biochemical model of C₃ photosynthesis applied to wheat at different temperatures. *Plant, Cell and Environment* **40**: 1552-1564
- Taub DR, Wang X** (2008) Why are nitrogen concentrations in plant tissues lower under elevated CO₂? A critical examination of the hypotheses. *Journal of Integrative Plant Biology* **50**: 1365-1374
- Tcherkez G, Bligny R, Gout E, Mahé A, Hodges M, Cornic G** (2008) Respiratory metabolism of illuminated leaves depends on CO₂ and O₂ conditions. *Proceedings of the National Academy of Sciences, USA* **105**: 797-802
- Tcherkez G, Gauthier P, Buckley TN, Busch FA, Barbour MM, Bruhn D, Heskell MA, Gong XY, Crous KY, Griffin KL, et al.** (2017a) Tracking the origins of the Kok effect, 70 years after its discovery. *New Phytologist* **214**: 506-510
- Tcherkez G, Gauthier P, Buckley TN, Busch FA, Barbour MM, Bruhn D, Heskell MA, Gong XY, Crous KY, Griffin KL, et al.** (2017b) Leaf day respiration: low CO₂ flux but high significance for metabolism and carbon balance. *New Phytologist* **216**: 986-1001
- Tcherkez G, Mahe A, Guerard F, Boex-Fontvieille ER, Gout E, Lamothe M, Barbour MM, Bligny R** (2012) Short-term effects of CO₂ and O₂ on citrate metabolism in illuminated leaves. *Plant, Cell and Environment* **35**: 2208-2220
- Walker AP, De Kauwe MG, Bastos A, Belmecheri S, Georgiou K, Keeling RF, McMahon SM, Medlyn BE, Moore DJP, Norby RJ, et al.** (2021) Integrating the evidence for a terrestrial carbon sink caused by increasing atmospheric CO₂. *New Phytologist* **229**: 2413-2445
- Wang XZ, Lewis JD, Tissue DT, Seemann JR, Griffin KL** (2001) Effects of elevated atmospheric CO₂ concentration on leaf dark respiration of *Xanthium strumarium* in light and in darkness. *Proceedings of the National Academy of Sciences, USA* **98**: 2479-2434
- Way DA, Aspinwall MJ, Drake JE, Crous KY, Campamy CE, Ghannoum O, Tissue DT, Tjoelker MG** (2019) Responses of respiration in the light to warming in field-grown trees: a comparison of the thermal sensitivity of the Kok and Laisk methods. *New Phytologist* **222**: 132-143
- Wehr R, Munger JW, McManus JB, Nelson DD, Zahniser MS, Davidson EA, Wofsy SC, Saleska SR** (2016) Seasonality of temperate forest photosynthesis and daytime respiration. *Nature* **534**: 680-683
- Wullschlegel SD, Norby RJ, Love JC, Runck C** (1997) Energetic costs of tissue construction in yellow-poplar and white oak trees exposed to long-term CO₂ enrichment. *Annals of Botany* **80**: 289-297
- Xiong D, Liu X, Liu L, Douthe C, Li Y, Peng S, Huang J** (2015) Rapid responses of mesophyll conductance to changes of CO₂ concentration, temperature and irradiance are affected by N supplements in rice. *Plant, Cell & Environment* **38**: 2541-2550
- Yin X, Belay DW, van der Putten PE, Struik PC** (2014) Accounting for the decrease of photosystem photochemical efficiency with increasing irradiance to estimate quantum yield of leaf photosynthesis. *Photosynth Research* **122**: 323-335
- Yin X, Niu Y, van der Putten PEL, Struik PC** (2020) The Kok effect revisited. *New Phytologist* **227**: 1764-1775
- Yin X, Struik PC, Romero P, Harbinson J, Evers JB, Van Der Putten PEL, Vos J** (2009) Using combined measurements of gas exchange and chlorophyll fluorescence to estimate parameters of a biochemical C₃ photosynthesis model: a critical appraisal and a new integrated approach

1 applied to leaves in a wheat (*Triticum aestivum*) canopy. Plant, Cell and Environment **32**: 448-
2 464
3 **Yin X, Sun Z, Struik PC, Gu J** (2011) Evaluating a new method to estimate the rate of leaf respiration
4 in the light by analysis of combined gas exchange and chlorophyll fluorescence measurements.
5 Journal of Experimental Botany **62**: 3489-3499
6



7
8 **Figure 1**
9 159x182 mm (9.1 x DPI)
10

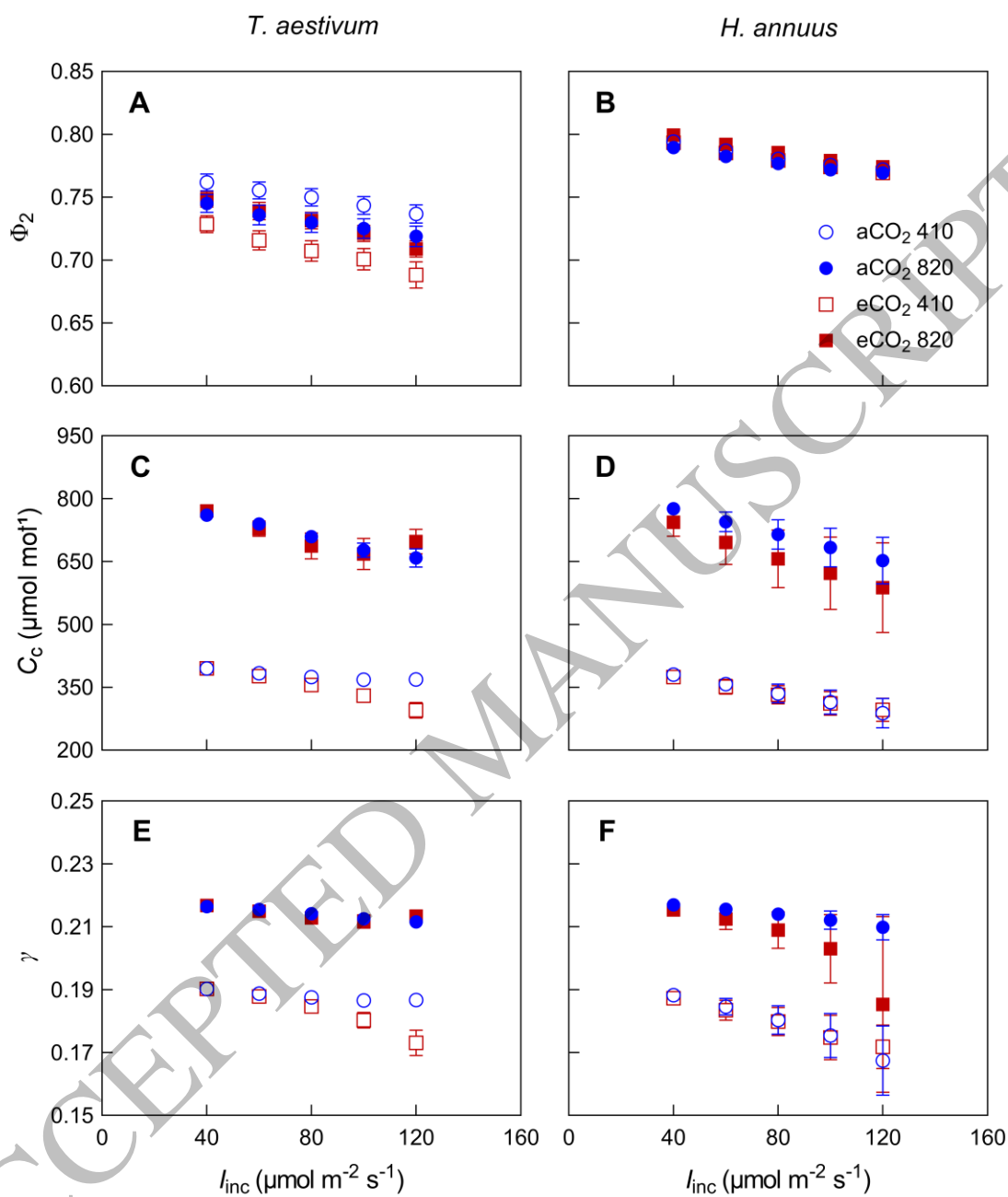


Figure 2
159x181 mm (9.1 x DPI)

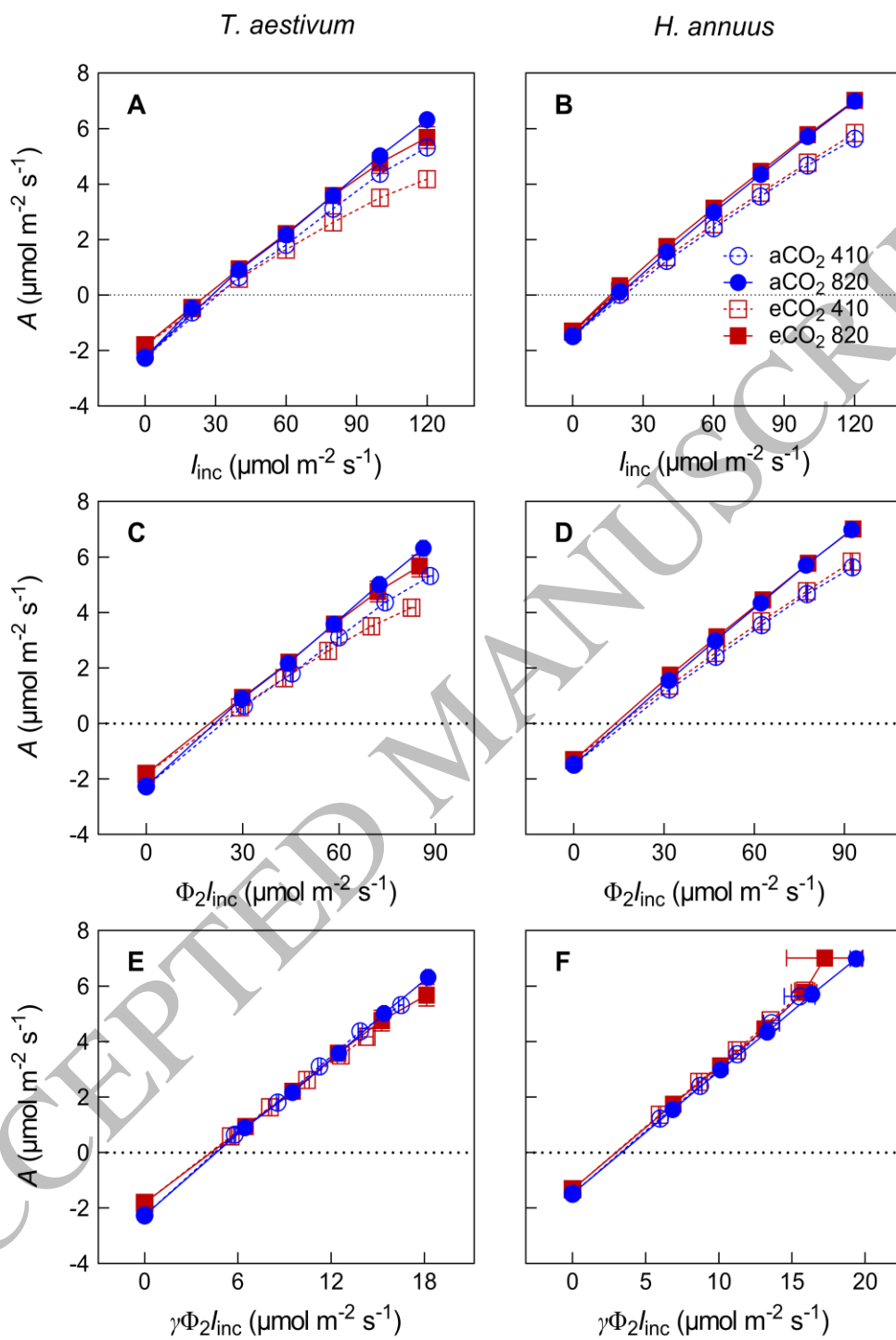


Figure 3
159x212 mm (9.1 x DPI)

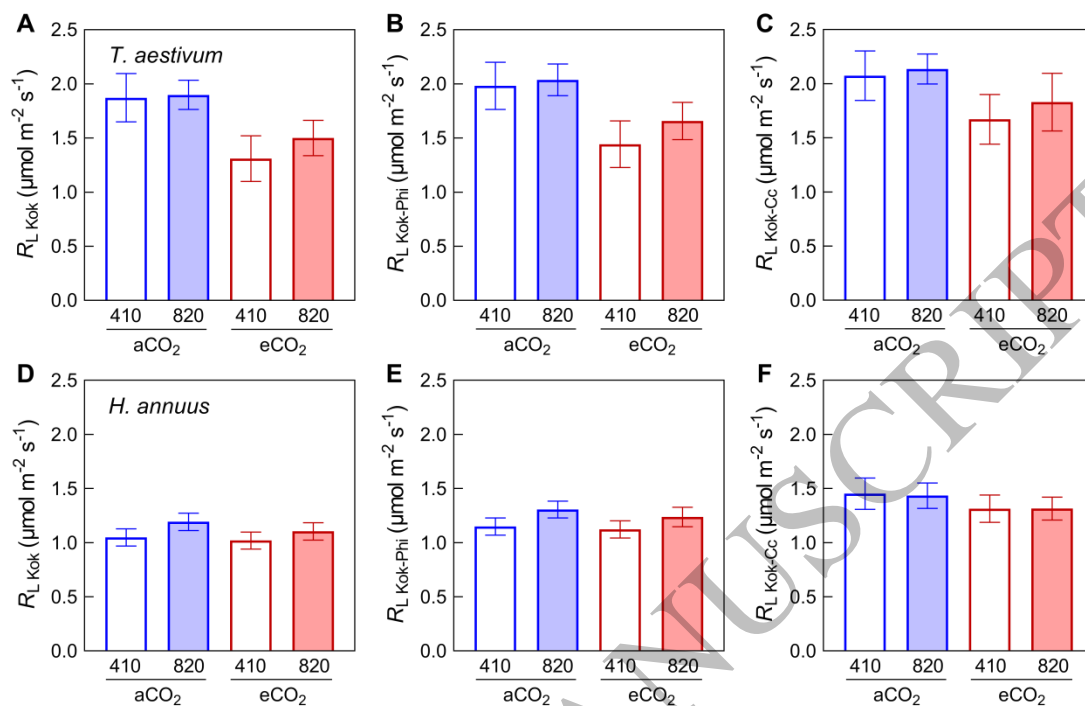


Figure 4
 153x100 mm (9.1 x DPI)

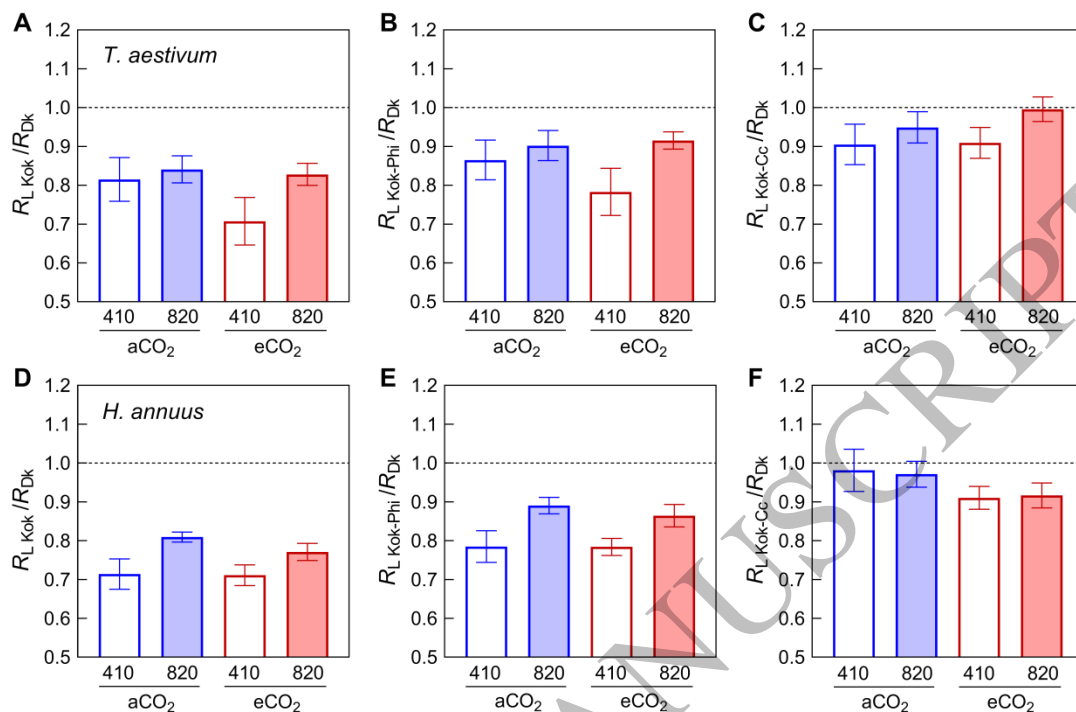


Figure 5
152x101 mm (9.1 x DPI)

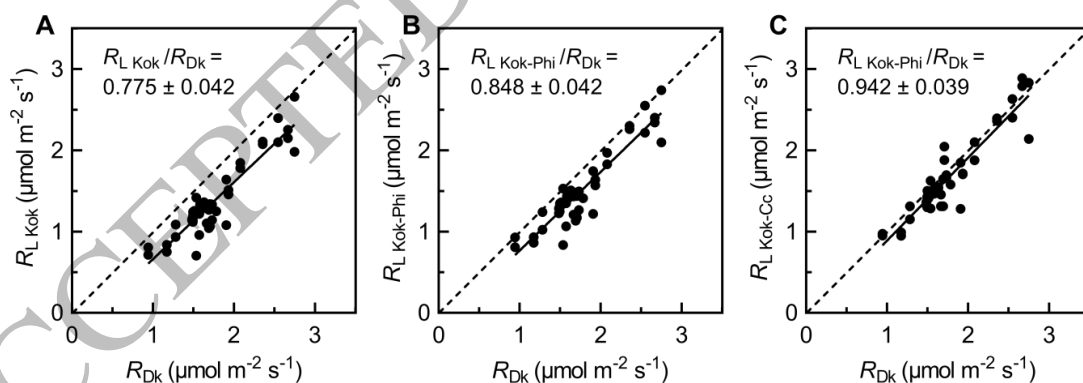


Figure 6
159x59 mm (9.1 x DPI)