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## Short- and long-term responses of leaf day respiration to elevated atmospheric CO<sub>2</sub>

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1 **Short- and long-term responses of leaf day respiration to elevated**  
2 **atmospheric CO<sub>2</sub>**

3  
4 **Running title:** CO<sub>2</sub> response of leaf respiration in the light

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23 article in accordance with the policy described in the Instructions for Authors

24 (<https://academic.oup.com/plphys/pages/General-Instructions>) is Xiao Ying Gong.

25

## 1 AUTHOR CONTRIBUTIONS

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

## 6 ABSTRACT

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

25  
26 **Keywords:** leaf day respiration, Kok method, atmospheric  $CO_2$ , photosynthesis, climate change,  
27 carbon balance, mesophyll conductance, chlorophyll fluorescence

## 1 INTRODUCTION

2 Terrestrial vegetation assimilates ca. 120 pg carbon via photosynthesis but releases about half of  
3 assimilated carbon via respiration (Gifford, 2003; Dusenge et al., 2019). The balance between  
4 plant respiration and photosynthesis is therefore essential for plant productivity and global  
5 carbon balance. Despite considerable variations depending on N fertilisation and climatic  
6 conditions, the ongoing increase in atmospheric CO<sub>2</sub> mole fraction ([CO<sub>2</sub>]) promotes leaf  
7 photosynthesis and primary production, which is referred to as the “CO<sub>2</sub> fertilization effect”  
8 (Drake et al., 1997; Cramer et al., 2001). Although the CO<sub>2</sub> fertilization effect on biomass (but  
9 not necessarily yield) is evident from greenhouse and field experiments (Ainsworth and Long,  
10 2005; Norby et al., 2005; Walker et al., 2021), the response of plant respiration to [CO<sub>2</sub>] is rather  
11 uncertain, limiting our ability to predict future climate change-driven modifications of plant  
12 physiology.

13 The respiratory response is complicated by the fact that leaf respiration takes place not  
14 only in darkness (the respiration rate is denoted as  $R_{Dk}$ ), but also in the light. In illuminated  
15 leaves, respiration is referred to as "respiration in the light" or "day respiration" (denoted as  $R_L$ ;  
16 here we refer to CO<sub>2</sub> evolution rather than O<sub>2</sub> consumption). Leaf respiration has been shown to  
17 be partially inhibited by the light although the magnitude of inhibition varies broadly, with  
18 reported  $R_L/R_{Dk}$  values ranging from 0.2 to 1.3 (Ayub et al., 2011; Griffin and Turnbull, 2013;  
19 Crous et al., 2017; Gong et al., 2018; Way et al., 2019). Given the longer light periods during the  
20 growing season and higher temperature during the day than at night in most ecosystems,  $R_L$  is a  
21 key component of plant- and community-scale carbon budgets (Atkin et al., 2007; Gong et al.,  
22 2017). Experimental results revealed that the inhibition of respiration by light (i.e.,  $1-R_L/R_{Dk}$ )  
23 also occurs at the stand scale (Gong et al., 2017). Neglecting respiration inhibition might have  
24 led to considerable errors in estimated gross primary production (Wehr et al., 2016; Gong et al.,  
25 2017). Furthermore, the response of  $R_L$  to environmental cues are essential to predict carbon  
26 balance, carbon use efficiency and improve land surface models (Wehr et al., 2016; Atkin et al.,  
27 2017; Tcherkez et al., 2017b; Keenan et al., 2019).

28 So far, there is no consensus on the response of  $R_L$  to long-term [CO<sub>2</sub>] increase. Some  
29 studies have shown that  $R_L$  is stimulated by elevated growth [CO<sub>2</sub>] (Wang et al., 2001; Shapiro et  
30 al., 2004; Crous et al., 2012; Griffin and Turnbull, 2013), and this effect may be related to higher  
31 carbohydrate concentrations in leaves (Rogers et al., 2004; Gong et al., 2017). Also, increased

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

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

23 The decrease of  $R_L$  at elevated  $[\text{CO}_2]$  has also been suggested to be linked to nitrogen  
24 metabolism. It has been observed in many free air  $\text{CO}_2$  enrichment (FACE) experiments that  
25 elevated  $[\text{CO}_2]$  reduces leaf N content, which is accompanied by a down-regulation of  
26 photosynthetic capacity (Long et al., 2004; Ainsworth and Long, 2005). It is believed that  
27 elevated  $[\text{CO}_2]$  inhibits N assimilation in leaves via the potential link between photorespiration  
28 and nitrate assimilation (Bloom et al., 2010; Bloom et al., 2014; Busch et al., 2018). Given that N  
29 assimilation in leaves is energy demanding and thus a driving factor for leaf respiration (Amthor,  
30 2000; Reich et al., 2008), it would be important to know whether  $[\text{CO}_2]$  affected  $R_L$  and  $R_{Dk}$  via

1 leaf N content. All in all, the response of  $R_L$  to elevated  $[\text{CO}_2]$  appears to be highly variable and  
2 mechanisms behind are unclear.

3 Another uncertainty associated with  $R_L$  and how it varies is technological. In fact, there are  
4 several methods to estimate  $R_L$ , but none of them can measure  $R_L$  directly (for a review see  
5 (Tcherkez et al., 2017b)). The Kok method (Kok, 1949) and the Laisk method (Laisk, 1977), the  
6 two most commonly used methods, require manipulation of net  $\text{CO}_2$  assimilation rates ( $A$ ) at low  
7 irradiances ( $I_{\text{inc}} < 150 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) (Kok) or low  $\text{CO}_2$  (Laisk). Another method, the  $^{13}\text{C}$  isotopic  
8 disequilibrium method, uses two  $\text{CO}_2$  sources with different  $\delta^{13}\text{C}$  values to disentangle  $R_L$  and  
9 photosynthesis under physiologically relevant environmental conditions without the need to  
10 manipulate  $A$  (Gong et al., 2015; Gong et al., 2018). The  $^{13}\text{C}$  disequilibrium method is valuable  
11 since it does not require the use of low irradiance or low  $\text{CO}_2$  and can be performed at any  $\text{CO}_2$   
12 mole fraction, and therefore, is suitable to study  $\text{CO}_2$  effects on  $R_L$ . It is, however, technically  
13 demanding (isotopic  $\text{CO}_2$  sources, mass spectrometers). The Laisk method is, by definition, not  
14 suitable for studying  $\text{CO}_2$  effects because it manipulates  $[\text{CO}_2]$  at sub-ambient levels. So far, the  
15 response of  $R_L$  to  $[\text{CO}_2]$  has mainly been estimated using the Kok method. However, as  
16 mentioned above, the Kok method has been questioned since the Kok effect is not exclusively  
17 caused by a decrease in respiration rates (Gauthier et al., 2020). Several studies showed that the  
18 Kok method has conceptual uncertainties (Farquhar and Busch, 2017; Tcherkez et al., 2017a;  
19 Tcherkez et al., 2017b; Yin et al., 2020). First, the Kok method assumes a constant  
20 photochemical efficiency of PS II ( $\Phi_2$ ) along the  $A$ - $I_{\text{inc}}$  curve (i.e. the Kok curve, see *Theory*). To  
21 address this issue, Yin et al. (2009) suggested to use measured  $\Phi_2$  to improve the  $R_L$  estimation.  
22 Second, the Kok method usually disregards variation in chloroplastic  $[\text{CO}_2]$  ( $C_c$ ) along the  $A$ - $I_{\text{inc}}$   
23 curve, which could bias the estimates of  $R_L$  according to recent studies based on model analysis  
24 (Farquhar and Busch, 2017; Yin et al., 2020). Estimating  $C_c$  along the  $A$ - $I_{\text{inc}}$  curve requires  
25 measurements of mesophyll conductance ( $g_m$ ). Measuring  $g_m$  is challenging and this is  
26 particularly true when measurements are performed at low irradiance (Pons et al., 2009; Gu and  
27 Sun, 2014; Gong et al., 2015). So far, the uncertainty associated with  $C_c$  has not been fully  
28 solved.

29 Taken as a whole, neither long-term nor short-term responses of  $R_L$  to  $\text{CO}_2$  mole fraction  
30 are well-known, and technologies used to measure  $R_L$  may be problematic. Here, we intend to  
31 address the following questions: 1) how do short-, medium- and long-term  $\text{CO}_2$  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  $\Phi_2$  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).

31

1 *CO<sub>2</sub> response of R<sub>L</sub> estimated by different methods*

2  $\Phi_2$ ,  $C_c$ , and  $\gamma$ , the key parameters associated with assumptions in both original and revised Kok  
3 methods, were found to decrease along the Kok curve in all species and treatments (Fig. 2). With  
4 the increase of  $I_{inc}$  from 40 to 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $\Phi_2$  decreased by 3.1% for wheat and 2.4% for  
5 sunflower and this trend was not substantially influenced by growth  $[\text{CO}_2]$  (long term effect) and  
6 measurement  $[\text{CO}_2]$  (short-term effect). A short-term  $\text{CO}_2$  effect on  $\gamma$  was detected, i.e.,  $\gamma$   
7 decreased more strongly at measurement  $[\text{CO}_2]$  of 410 ppm (by 5.2%) than that at measurement  
8  $[\text{CO}_2]$  of 820 ppm (by 3.0%, averaged across species) with the increase in  $I_{inc}$  (Fig. S1). That is,  
9 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  
10 Kok curve, causing errors in  $R_L$  estimated by the Kok and the Kok-Phi method, respectively.

11 Applying the Kok, the Kok-Phi and the Kok- $C_c$  method,  $A$  was plotted against  $I_{inc}$ ,  $\Phi_2 I_{inc}$ ,  
12 and  $\gamma \Phi_2 I_{inc}$ , respectively (Fig. 3). Both growth  $[\text{CO}_2]$  and measurement  $[\text{CO}_2]$  had impacts on  $A$ -  
13  $I_{inc}$  and  $A-\Phi_2 I_{inc}$  response curves (Fig. 3A-D). As a result, growth at elevated  $[\text{CO}_2]$  led to a  
14 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  
15 only significant with a  $p$ -value of 0.06 (Fig. 4, Table 2). There was a clear, although statistically  
16 insignificant ( $p > 0.05$ ), tendency for elevated measurement  $[\text{CO}_2]$  to increase both  $R_{L \text{ Kok}}$  and  $R_{L \text{ Kok-Phi}}$   
17 (Fig. 3 and Fig. 4) in both species. By contrast,  $A-\gamma \Phi_2 I_{inc}$  curves obtained under different  
18 measurement  $[\text{CO}_2]$  seemed to coincide perfectly (Fig. 3EF), in agreement with the insignificant  
19 effect of measurement  $[\text{CO}_2]$  on  $R_{L \text{ Kok-}C_c}$ .

20  
21 *CO<sub>2</sub> response of R<sub>L</sub>/R<sub>DK</sub> estimated by different methods*

22 We found no significant long-term  $\text{CO}_2$  effect on  $R_L/R_{DK}$  estimated via all three methods (Table  
23 3). There is a tendency that  $R_L/R_{DK}$  of wheat increased with the growth  $[\text{CO}_2]$  for all three  
24 methods (comparing a $\text{CO}_2$ -410 and e $\text{CO}_2$ -820), while that tendency was not found in sunflower.  
25 That is, the long-term  $\text{CO}_2$  effect on  $R_L/R_{DK}$  is not conclusive. Under elevated measurement  
26  $[\text{CO}_2]$ , significant increases in  $R_{L \text{ Kok}}/R_{DK}$  and  $R_{L \text{ Kok-Phi}}/R_{DK}$  were observed, but this short-term  
27 response was not observed using the Kok- $C_c$  method (Fig. 5). These results indicate that short-  
28 term  $\text{CO}_2$  effect on  $R_{L \text{ Kok}}$  and  $R_{L \text{ Kok-Phi}}$  could result from a technical bias simply due to  
29 neglecting the change in  $C_c$  along the Kok curve.

30 When pooling all data across species and treatments together,  $R_{DK}$  was positively correlated  
31 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).

1 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  
 2  $0.85 \pm 0.04$ , and  $R_L \text{ Kok-Cc}/R_{Dk}$  of  $0.94 \pm 0.04$ . That is, the Kok-Cc method showed a small light-  
 3 induced inhibition of respiration, of 6% only, thus much lower than inhibition values from the  
 4 other two methods (22%, 15%).

## 6 DISCUSSION

### 7 Growth at elevated $\text{CO}_2$ leads to reduction in $R_L$

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

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

### 25 The long-term response of $R_L$ to $\text{CO}_2$ is associated with changes in leaf N status

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

1 It has often been found in FACE or growth cabinet experiments that leaf N content was  
 2 lower at elevated  $[\text{CO}_2]$ . This has been explained by different mechanisms. For example,  
 3 elevated  $[\text{CO}_2]$  was shown to cause a decrease in stomatal conductance of leaves, leading to  
 4 decreasing transpiration rates (Ainsworth and Rogers, 2007) and thus, lower transpiration-driven  
 5 mass flow of soil N to roots and stems (so-called transpiration mechanism (McGrath and Lobell,  
 6 2013; Feng et al., 2015)). Another mechanism is associated with photorespiration. Generally N  
 7 assimilation is believed to be lower due to lower photorespiration (Bloom et al., 2010), which is  
 8 accompanied by the reduced reductant supplied via photorespiration at elevated  $[\text{CO}_2]$  (Taub and  
 9 Wang, 2008). Furthermore, a ‘dilution effect’ could occur whereby N uptake does not increase  
 10 proportionally to the increase of biomass at elevated  $[\text{CO}_2]$  (Feng et al., 2015).

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

## 21 **Changes in $\Phi_2$ and $C_c$ are involved in the Kok effect and impact on $R_L$ estimates**

22 Our study found a short-term  $\text{CO}_2$  effect on  $R_L$  estimated using the Kok and Kok-Phi method, but  
 23 no effect using the Kok- $C_c$  method. In fact, both Kok and Kok-Phi methods showed an increase  
 24 in  $R_L$  when measured at elevated  $[\text{CO}_2]$ . This short-term response was in agreement with the  
 25 finding of Yin et al. (2020) and Fang et al. (2021), but is not supported by the findings of other  
 26 studies (Tcherkez et al., 2008; Griffin and Turnbull, 2013). We believe discrepancies in short-  
 27 term  $\text{CO}_2$  effect on  $R_L$  is mostly associated with methodological differences. As shown in the  
 28 *Theory* section, the classical Kok method has conceptual uncertainty with the assumption that  $\Phi_2$   
 29 remains constant across the Kok curve. This assumption must be rejected as  $\Phi_2$  decreases with  
 30 increasing  $I_{\text{inc}}$  (Fig. 2). However, this short-term  $\text{CO}_2$  effect on  $R_L$  cannot be explained by  
 31 changes in  $\Phi_2$  because (i) the decrease in  $\Phi_2$  along Kok curve was similar at both measurement

1 [CO<sub>2</sub>] and (ii) the effect persisted when the Kok-Phi method was used to account for variation in  
2  $\Phi_2$ .

3 Another assumption that has been made for both the Kok and Kok-Phi method is that  $\gamma$   
4 (determined by  $\Gamma^*/C_c$ ) remains constant throughout the Kok curve. This assumption has also  
5 been challenged in recent model analyses (Buckley et al., 2017; Farquhar and Busch, 2017), but  
6 the question is how to quantify the change in  $C_c$  as this requires  $g_m$  estimates. Here, we used  
7 species-specific  $g_{sc}/g_m$  ratios to calculate  $C_c$ , suggesting that  $C_c$  and  $\gamma$  decreased with increasing  
8  $I_{inc}$ . Importantly, measurement CO<sub>2</sub> influenced the trend of  $\gamma$  with increasing  $I_{inc}$ , which might be  
9 the origin of this short-term CO<sub>2</sub> effect on  $R_{L\text{ Kok}}$  and  $R_{L\text{ Kok-Phi}}$ . When changes in  $\gamma$  (or  $\Gamma^*/C_c$ ) are  
10 accounted for, the apparent short-term effect of CO<sub>2</sub> on  $R_L$ , as found with the Kok and Kok-Phi  
11 methods, became insignificant (see also Fig. 3, 4).

12

### 13 **Kok- and Kok-Phi based estimates of $R_L$ suppression are overestimates**

14 The inhibition of  $R_L$  by light is supported by biochemical evidence. Utilizing <sup>13</sup>C labelling, flux  
15 calculations suggest that decarboxylation rates associated to glucose catabolism and activation of  
16 malic enzyme increase with decreasing irradiance in the irradiance region where the Kok effect  
17 occurs (Gauthier et al., 2020). Recently, how much of the Kok effect is associated with  
18 respiration has been under debate (Farquhar and Busch, 2017; Gauthier et al., 2020; Yin et al.,  
19 2020). Indeed, the methods used in the present study show different levels of inhibition of  
20 respiration by light. The average  $R_L/R_{Dk}$  was 0.74 for the Kok method, 0.81 for the Kok-Phi  
21 method and 0.93 for the Kok- $C_c$  method. That is, the change in  $\Phi_2$ ,  $\gamma$  (or  $\Gamma^*/C_c$ ), and real light  
22 inhibition of  $R_L$  explained *c.* 27, 46 and 27% of the apparent Kok effect (i.e. the apparent 26%-  
23 inhibition of  $R_L$  found with the classical Kok method), respectively. This is in agreement with the  
24 results of previous model analyses which show that the Kok effect is not purely respiratory  
25 (Farquhar and Busch, 2017; Yin et al., 2020), and both the Kok method and the Kok-Phi method  
26 underestimated  $R_L$  and overestimated the inhibition of  $R_L$  (Yin et al., 2020).

27 The real light inhibition of  $R_L$  (as revealed by the Kok- $C_c$  method) was only 6%, which is  
28 close to the mean inhibition of 8% of several herbaceous species determined using the <sup>13</sup>C  
29 disequilibrium method (Gong et al., 2018) and the mean inhibition of 10% in wheat leaves  
30 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  $\gamma$  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  $\pm 0.4$  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  $\pm 0.4$  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  $g_{sc}/g_m$  ( $\pm 0.4$  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  $\gamma$  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%.

30  
31

## 1 **Conclusions and Perspectives**

2 This study showed that elevated growth [CO<sub>2</sub>] reduced  $R_L$  and  $R_{DK}$  likely as a result of decreasing  
 3 leaf N status and chlorophyll content. We found no significant long-term CO<sub>2</sub> effect on  $R_L/R_{DK}$ ,  
 4 indicating a concurrent response of  $R_L$  and  $R_{DK}$  to elevated growth [CO<sub>2</sub>], mediated by the  
 5 adjustment of nitrogen metabolism in leaves. These results shed light into the incorporation of  $R_L$   
 6 into the carbon cycling models. We revisited the theoretical basis of the Kok method, revised  
 7 Kok methods and discussed their respective limitations. Using Kok and Kok-Phi methods, we  
 8 found that  $R_L$  were stimulated by short-term CO<sub>2</sub> enrichment, while the effect was not supported  
 9 by the data of the Kok- $C_c$  method. We attributed this short-term CO<sub>2</sub> effect to methodological  
 10 uncertainty associated with unaccounted changes in  $\gamma$  (or  $\Gamma^*/C_c$ ) along a Kok curve. Accounting  
 11 for those effects, we found that the Kok and Kok-Phi method underestimate  $R_L$  and overestimate  
 12 the inhibition of respiration under low irradiance conditions of the Kok method, and the  
 13 inhibition of  $R_L$  is only  $6\pm 4\%$ , which represents 26% of the Kok effect (i.e. of the apparent  
 14 inhibition of  $R_L$  found using the classical Kok method). Although the Kok- $C_c$  method has less  
 15 theoretical uncertainty and is thus in principle more reliable, we are aware that all three methods  
 16 have operating PPFD much lower than usual, ambient irradiance encountered by plants.  
 17 However, estimated  $R_L$  could vary with irradiance. Earlier studies have showed a decrease of  $R_L$   
 18 with the increase of operating PPFD (Brooks and Farquhar, 1985; Atkin et al., 1998; Atkin et al.,  
 19 2000) by using the Laisk method which also has the uncertainty associated with the unaccounted  
 20 changes in  $C_c$  (Farquhar and Busch 2017). To date, the effect of irradiance on  $R_L$  is poorly  
 21 known and this should be addressed in subsequent studies.

22

## 23 **MATERIALS AND METHODS**

### 24 **Theory**

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

$$28 \quad A = J \frac{1 - \Gamma^*/C_c}{4 + 8\Gamma^*/C_c} - R_L \quad \text{Eqn 1}$$

29 where  $J$  is the electron transport rate that is used for CO<sub>2</sub> fixation and photorespiration,  $\Gamma^*$  is the  
 30  $C_c$ -based CO<sub>2</sub> compensation point in the absence of mitochondrial respiration ( $37.4 \mu\text{mol mol}^{-1}$   
 31 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  $f_{\text{aet}}$  is the fraction of electron transport for photosynthesis,  $\rho_2$  is the fraction of absorbed irradiance partitioned to PS II,  $\alpha$  is the absorptance by leaf photosynthetic pigments and  $I_{\text{inc}}$  is incident irradiance (Yin et al., 2011). Here, we define the term  $\frac{1-\Gamma^*/C_c}{4+8\Gamma^*/C_c}$  as  $\gamma$ , 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  $\text{CO}_2$  assimilation rates are plotted against  $I_{\text{inc}}$  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  $\Phi_2$  could decrease with increasing  $I_{\text{inc}}$  even within the range of low irradiance (Genty and Harbinson, 1996; Yin et al., 2020). Alternatively,  $\Phi_2$  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  $\Phi_2$  accounted for, and thus it is renamed as the 'Kok-Phi' method here to highlight the modification. This method assumes that  $\gamma$  is constant across Kok curve, which is obviously not true under photorespiratory conditions, i.e. under ambient conditions where  $\text{O}_2$  mole fraction is about 21% (Yin et al., 2014). Theoretically, the Kok-Phi method is applicable for measuring  $\text{C}_3$  leaves at nonphotorespiratory conditions or  $\text{C}_4$  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  $\gamma$  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  $R_L$  ( $R_{L \text{ Kok-Cc}}$ ):

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

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

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

### 4 **Plant material and growth conditions**

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

### 15 **Gas exchange and chlorophyll fluorescence measurements**

16 Photosynthetic gas exchange and ChF parameters were measured when there were 4 fully  
17 expanded leaves in each plant (sunflower) or tiller (wheat). Using a portable gas exchange  
18 system (LI-6800; Li-Cor Inc., Lincoln, NE, USA), measurements were undertaken on the second  
19 youngest fully developed leaves. Light response curves and ChF parameters were measured to  
20 estimate  $R_L$ . When stable gas exchange rates were achieved, we measured  $A$  starting at  $120 \mu mol$   
21  $m^{-2} s^{-1}$ , and the PPFD was sequentially reduced to 100, 80, 60, 40, 20 and 0 (i.e., with light  
22 source switched off)  $\mu mol m^{-2} s^{-1}$ . ChF measurements were done at PPFD of 120, 100, 80, 60 and  
23  $40 \mu mol m^{-2} s^{-1}$  using the multi-phase flash method.  $\Phi_2$  was calculated as

$$24 \quad \Phi_2 = (F_m' - F_s) / F_m' \quad \text{Eqn 6}$$

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

29 All gas exchange parameters have been corrected for the leak effect (i.e.  $CO_2$  diffusion  
30 across gaskets of leaf chamber) using the measured leak coefficients of intact leaves (Gong et al.,  
31 2015; Gong et al., 2018).  $R_{Dk}$  measured at 410 and 820 ppm [ $CO_2$ ] was used to calculate the

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

### 10 Estimation of day respiration and $C_c$

11 For the Kok method, the data of the linear range of the  $A: I_{inc}$  curve at PPFD levels above the  
 12 Kok breakpoint (kink) were used to estimate  $R_L$  according to Eqn 3. Each  $A: I_{inc}$  curve was  
 13 visually inspected to identify the irradiance at the Kok breakpoint, which was 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$   
 14 (Fig. S6). The data measured at PPFD of 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  deviated from the linear relation (i.e.  
 15 the linear domain of assimilation response curve to light between 40-100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), thus they  
 16 were excluded from the dataset used for the estimation of  $R_L$  via all methods. Linear regressions  
 17 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  
 18 methods, with the exception of 5 out of 45 curves in which a point that deviated from the linear  
 19 relation was excluded for the estimation of  $R_L$ . For the Kok-Phi method, the data from the same  
 20 PPFD range were used to estimate  $R_L$  by plotting  $A$  against  $\Phi_2 I_{inc}$  according to Eqn 4. We have  
 21 not intensively measured  $A$  at very low PPFD levels to accurately identify the breakpoint.  
 22 However, the data at 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD seem to be above the Kok breakpoint and in the  
 23 linear domain of  $A: I_{inc}$  curves. Our approach is similar to recent studies which compared the Kok  
 24 and the Kok-Phi method (Yin et al., 2011; Fang et al., 2021).

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

$$27 \quad g_m = \frac{A}{C_i \frac{\Gamma^*[J+8(A+R_L)]}{J-4(A+R_L)}} \quad \text{Eqn 7}$$

28 Here, we used  $R_L$  estimated using the Kok-Phi method to calculate  $g_m$ , given that this method  
 29 addresses the issue of decreasing  $\Phi_2$  and provides a more reliable estimation of  $R_L$ , compared to  
 30 the Kok method (Yin et al., 2011). Furthermore, using  $R_{L \text{ Kok}}$  or  $R_{L \text{ Kok-Phi}}$  has minor influence on

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

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

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

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

$$17 \quad C_c = C_i - A/g_m \quad \text{Eqn 9}$$

18 With  $C_c$ ,  $\gamma$  could be calculated and thus  $R_{L \text{ Kok-Cc}}$  could be estimated by plotting  $A$  against  $\gamma\Phi_2 I_{inc}$   
 19 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  
 20 sensitivity of  $R_{L \text{ Kok-Cc}}$  to  $g_{sc}/g_m$  by adjusting obtained species- and treatment-specific  $g_{sc}/g_m$  ( $\pm$   
 21 0.4).

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

$$24 \quad \text{CUE}_L = (\int A - \int R_{Dk}) / (\int A + \int R_{L \text{ Kok-Cc}}) \quad \text{Eqn 10}$$

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

27

## 28 **Plant sampling and leaf trait parameters**

29 After gas exchange and ChF measurements, the measured leaves were harvested. We measured  
 30 leaf area and fresh weight, and the chlorophyll content (Chl) was determined by a chlorophyll  
 31 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 ELIII, Elementar Analysensysteme GmbH, Hanau,  
6 Germany).

7

### 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.

14

### 15 **FUNDING**

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18

### 19 **DATA AVAILABILITY**

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

22

1 **TABLES**

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

	<i>T. aestivum</i>		<i>H. annuus</i>		Significance		
	aCO <sub>2</sub>	eCO <sub>2</sub>	aCO <sub>2</sub>	eCO <sub>2</sub>	<i>spe</i>	CO <sub>2</sub>	<i>spe</i> ×CO <sub>2</sub>
$A_{\text{growth}}$	28.42±0.73	32.79±2.01	27.27±2.57	31.43±2.06	0.529	<b>0.042</b>	0.956
$A_{\text{max}}$	37.68±1.29	34.04±2.22	32.32±2.40	31.83±1.73	0.068	0.304	0.430
$R_{\text{Dk}}$	2.26±0.14	1.81±0.17	1.48±0.11	1.32±0.14	<b>&lt;0.001</b>	<b>0.046</b>	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	<b>0.013</b>	0.057
SLA	0.24±0.01	0.27±0.02	0.22±0.02	0.23±0.02	<b>0.038</b>	0.252	0.511
N%	6.61±0.17	6.39±0.11	3.51±0.67	3.16±0.56	<b>&lt;0.001</b>	0.527	0.882
N <sub>area</sub>	2.70±0.06	2.37±0.10	1.53±0.18	1.35±0.16	<b>&lt;0.001</b>	0.062	0.586
Chl	0.69±0.02	0.56±0.03	0.49±0.05	0.48±0.02	<b>&lt;0.001</b>	<b>0.04</b>	0.083
$V_{\text{cmax}}$	160.9±3.2	142.7±13.0	123.0±11.7	117.4±3.3	<b>0.002</b>	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 <sub>L</sub>	0.89±0.01	0.92±0.01	0.92±0.02	0.94±0.01	<b>0.011</b>	<b>0.004</b>	0.547

12

13

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

Source	df	$R_{L\text{ Kok}}$		$R_{L\text{ Kok-Phi}}$		$R_{L\text{ Kok-Cc}}$	
		$F$	$P$	$F$	$P$	$F$	$P$
Species	1	28.36	<b>&lt;0.001</b>	29.74	<b>&lt;0.001</b>	20.06	<b>&lt;0.001</b>
Growth $\text{CO}_2$	1	6.681	<b>0.013</b>	5.777	<b>0.021</b>	3.921	0.055
Measurement $\text{CO}_2$	1	1.227	0.275	1.714	0.198	0.198	0.658
Growth $\text{CO}_2^*$	1	0.092	0.763	0.107	0.745	0.072	0.790
Measurement $\text{CO}_2$	1						

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

Source	df	$R_{L\text{ Kok}}/R_{Dk}$		$R_{L\text{ Kok-Phi}}/R_{Dk}$		$R_{L\text{ Kok-Cc}}/R_{Dk}$	
		$F$	$P$	$F$	$P$	$F$	$P$
Species	1	2.824	0.101	1.638	0.208	0.095	0.759
Growth $\text{CO}_2$	1	2.216	0.144	0.799	0.377	0.389	0.536
Measurement $\text{CO}_2$	1	7.480	<b>0.009</b>	10.410	<b>0.003</b>	1.140	0.292
Growth $\text{CO}_2^*$	1	0.328	0.570	0.441	0.511	0.243	0.625
Measurement $\text{CO}_2$	1						

6  
7 **FIGURE LEGENDS**

8 **Figure 1.** Net  $\text{CO}_2$  assimilation rate ( $A$ ), transpiration rate ( $E$ ) and stomatal conductance for  
9 water vapor ( $g_{sw}$ ) in response to short-term variation of intercellular  $\text{CO}_2$  concentration ( $C_i$ ) for  
10 wheat (*T. aestivum*) and sunflower (*H. annuus*). Blue circles refer to ambient ( $410 \mu\text{mol mol}^{-1}$ )  
11 growth  $\text{CO}_2$ , and red squares refer to elevated ( $820 \mu\text{mol mol}^{-1}$ ) growth  $\text{CO}_2$ . Data are shown as  
12 mean  $\pm$  SE ( $n=6$ ).

13 **Figure 2.** Photochemical efficiency of photosystem II ( $\Phi_2$ ), chloroplastic  $\text{CO}_2$  concentration  
14 ( $C_c$ ) and  $\gamma$  (the lumped parameter in Eqn 2) in response to incident irradiance ( $I_{inc}$ ) for wheat (*T.*  
15 *aestivum*) and sunflower (*H. annuus*). Plants grown under ambient  $\text{CO}_2$  (a $\text{CO}_2$ , blue circles) or  
16 elevated  $\text{CO}_2$  (e $\text{CO}_2$ , red squares) were measured at gaseous conditions of  $410 \mu\text{mol mol}^{-1}$  (open  
17 symbols) or  $820 \mu\text{mol mol}^{-1}$  (closed symbols)  $\text{CO}_2$  in the leaf chamber. Data are shown as mean  
18  $\pm$  SE ( $n=6$ ).

19 **Figure 3.** Net  $\text{CO}_2$  assimilation rate ( $A$ ) in response to  $I_{inc}$  (incident irradiance),  $\Phi_2 I_{inc}$  ( $\Phi_2$ ,  
20 photochemical efficiency of photosystem II) or  $\gamma \Phi_2 I_{inc}$  ( $\gamma$ , the lumped parameter in Eqn 2) for  
21 wheat (*T. aestivum*) and sunflower (*H. annuus*). Data are mean  $\pm$  SE ( $n=6$ ). Meaning of symbols  
22 of different  $\text{CO}_2$  treatments and measurement conditions are shown in Fig. 2.

23 **Figure 4.** Effects of growth  $\text{CO}_2$  treatments (a $\text{CO}_2$  and e $\text{CO}_2$ ) and measurement conditions ( $410$   
24 and  $820 \text{ ppm CO}_2$ ) on respiration rates in the light ( $R_L$ ) estimated by three methods for wheat (*T.*

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

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

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

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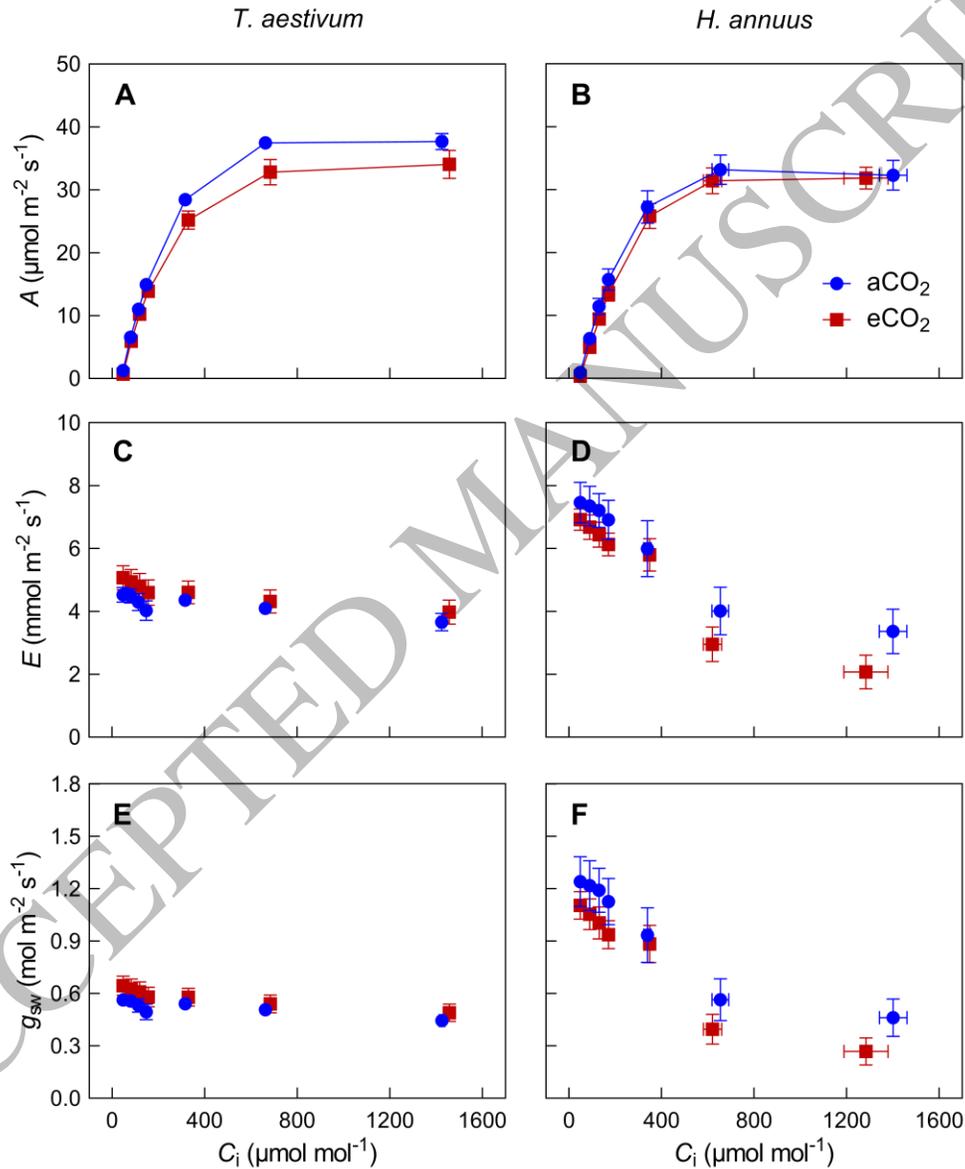
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7  
 8 **Figure 1**  
 9 **159x182 mm (9.1 x DPI)**  
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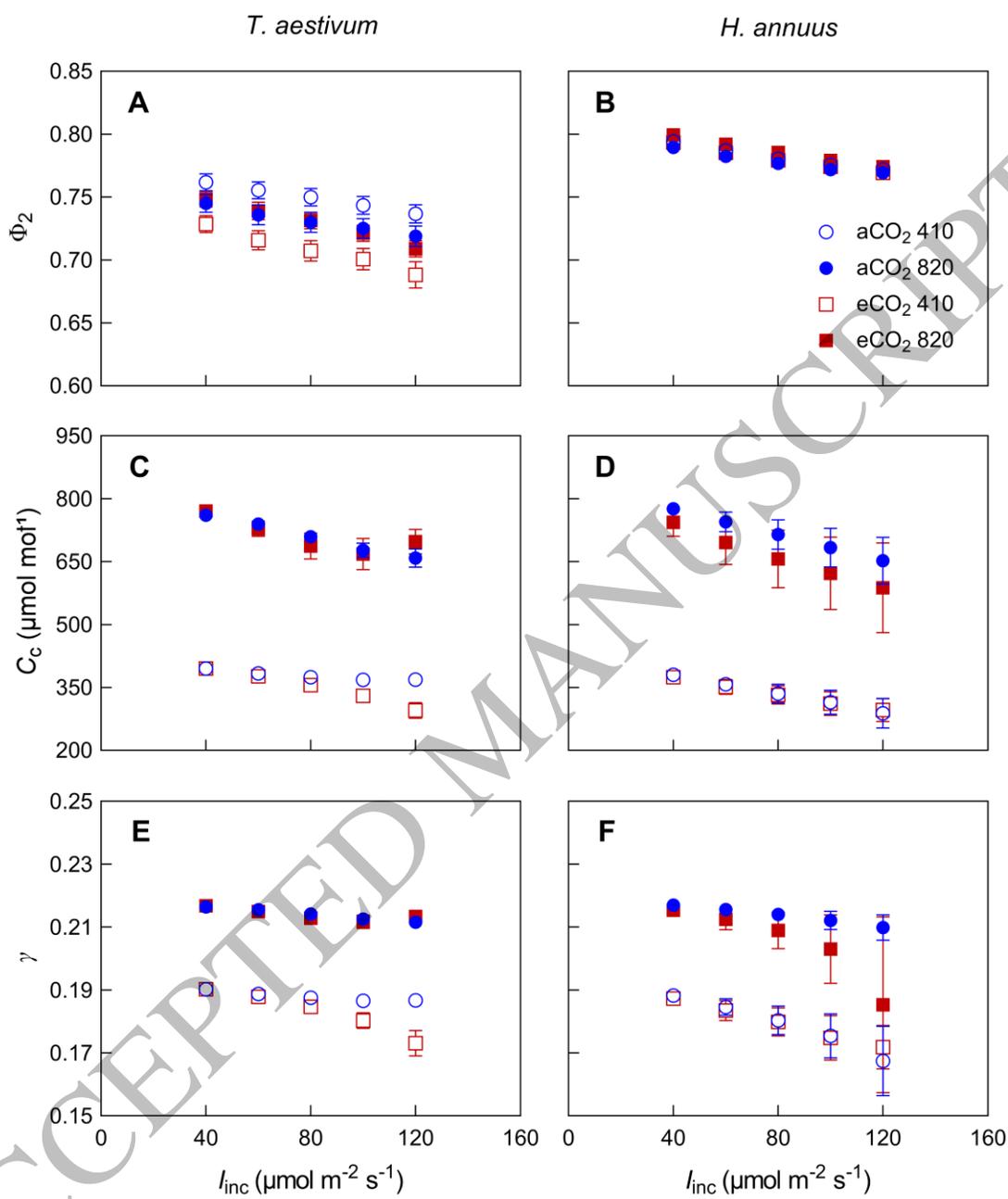
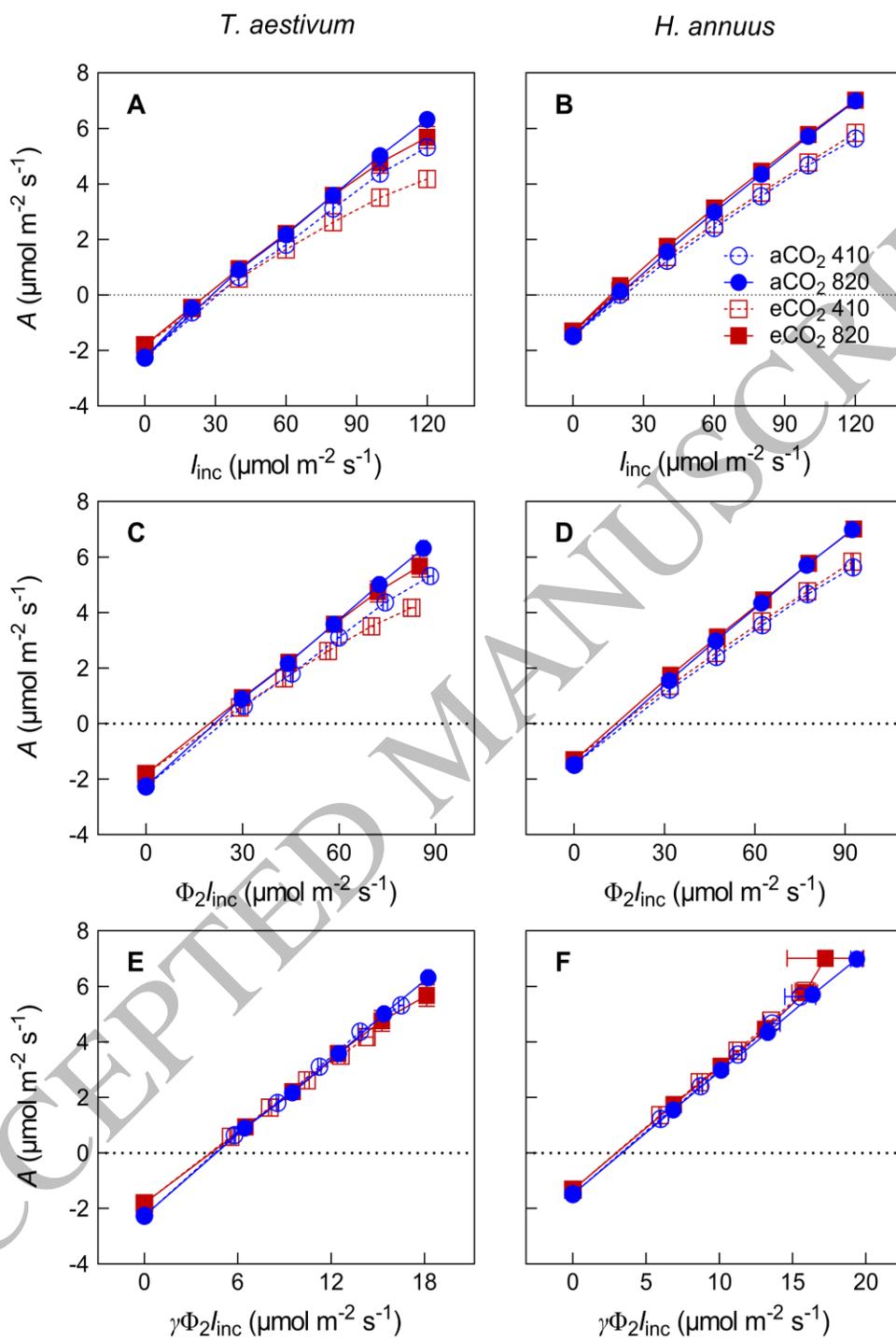


Figure 2  
159x181 mm (9.1 x DPI)

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4



**Figure 3**  
 159x212 mm (9.1 x DPI)

1  
 2  
 3  
 4

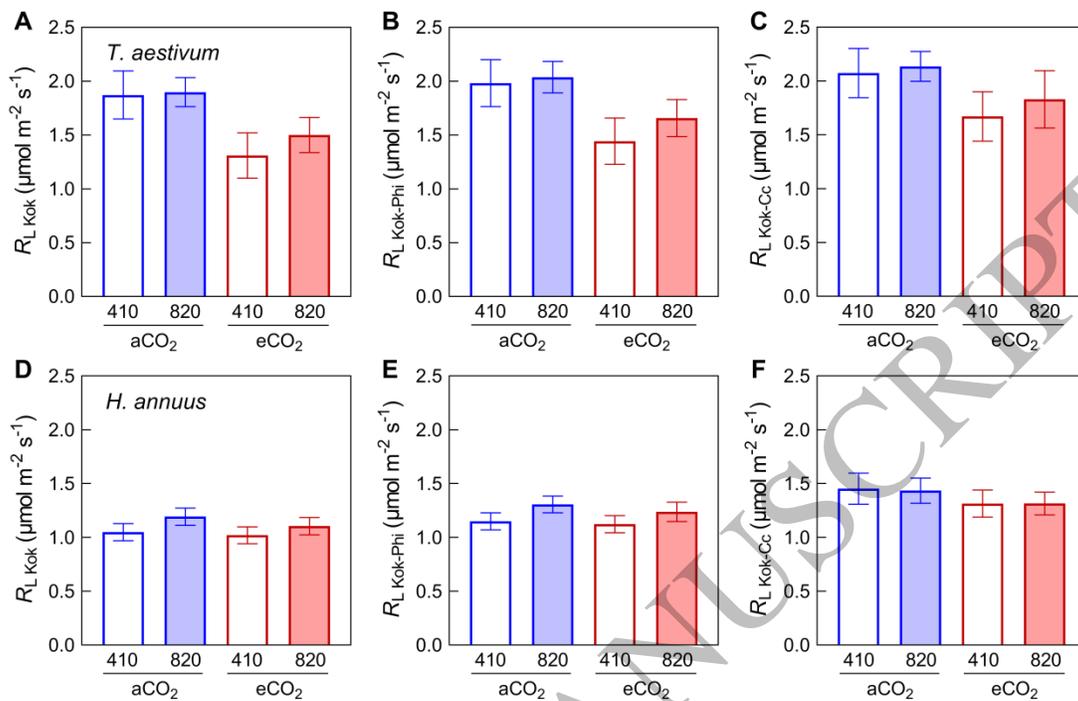


Figure 4  
153x100 mm (9.1 x DPI)

1  
2  
3  
4

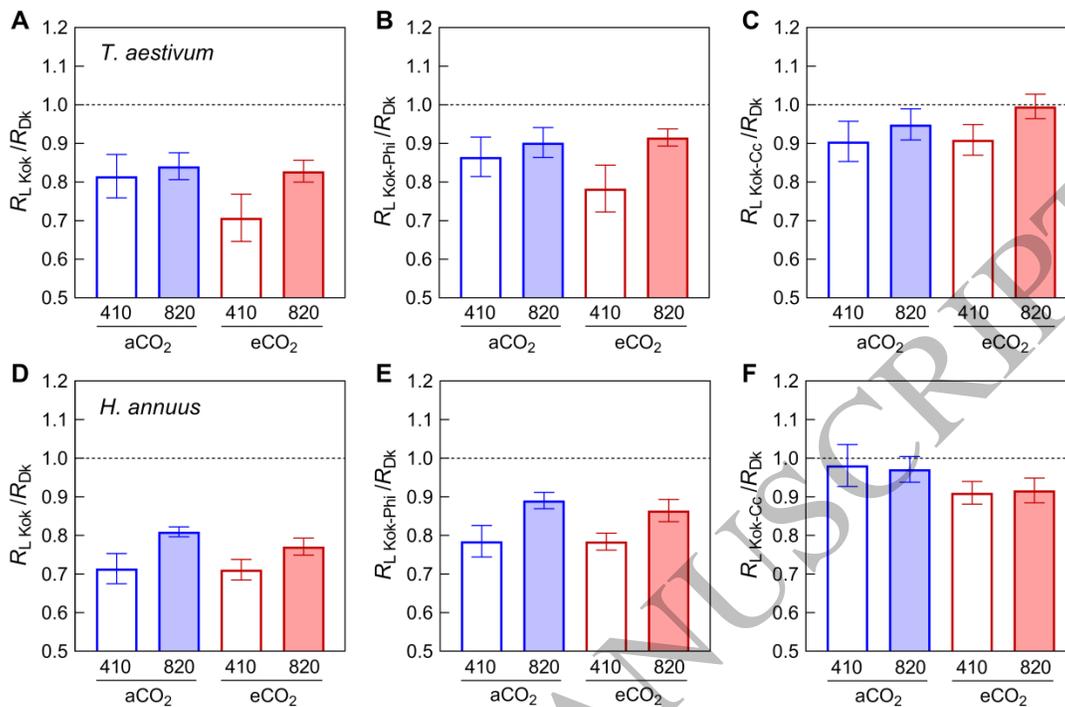


Figure 5  
152x101 mm (9.1 x DPI)

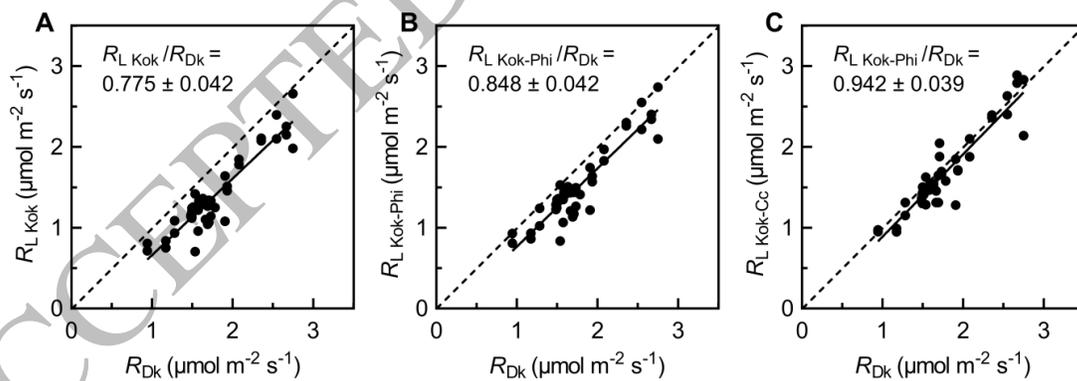


Figure 6  
159x59 mm (9.1 x DPI)

1  
2  
3  
4

5  
6  
7