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Evaluating a new method to estimate the rate of leaf respiration in the light by analysis of combined gas exchange and chlorophyll fluorescence measurements

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Abstract

Day respiration (R_d) is an important parameter in leaf ecophysiology. It is difficult to measure directly and is indirectly estimated from gas exchange (GE) measurements of the net photosynthetic rate (A), commonly using the Laisk method or the Kok method. Recently a new method was proposed to estimate R_d indirectly from combined GE and chlorophyll fluorescence (CF) measurements across a range of low irradiances. Here this method is tested for estimating R_d in five C_3 and one C_4 crop species. Values estimated by this new method agreed with those by the Laisk method for the C_3 species. The Laisk method, however, is only valid for C_3 species and requires measurements at very low CO_2 levels. In contrast, the new method can be applied to both C_3 and C_4 plants and at any CO_2 level. The R_d estimates by the new method were consistently somewhat higher than those by the Kok method, because using CF data corrects for errors due to any non-linearity between A and irradiance of the used data range. Like the Kok and Laisk methods, the new method is based on the assumption that R_d varies little with light intensity, which is still subject to debate. Theoretically, the new method, like the Kok method, works best for non-photorespiratory conditions. As CF information is required, data for the new method are usually collected using a small leaf chamber, whereas the Kok and Laisk methods use only GE data, allowing the use of a larger chamber to reduce the noise-to-signal ratio of GE measurements.

Key words: Kok effect, mitochondrial respiration in the light, photosynthesis models.

Introduction

Non-photorespiratory CO_2 release in the light, also known as 'day respiration' (R_d; Azcon-Bieto *et al.*, 1981), is an important parameter in modelling net rate of leaf photosynthesis. Unlike the respiratory CO_2 release in the dark (R_{dk}), R_d is difficult to measure directly *in vivo* because of the flux from simultaneous photosynthetic carbon fixation and photorespiration (Ribas-Carbo *et al.*, 2010). Direct measurement of R_d requires sophisticated methodologies, exploiting the different time course of labelling by carbon isotopes of photosynthetic, photorespiratory, and respiratory pathways (e.g. Haupt-Herting *et al.*, 2001; Loreto *et al.*, 2001; Pinelli and Loreto, 2003; Pärnik and Keerberg,

2007). For leaf ecophysiological studies, usually R_d is indirectly estimated from gas exchange (GE) measurements for net photosynthetic rate (A) by extrapolating the linear relationship between A and light intensity (Kok, 1948) or by identifying the intersection of the linear relationships of A versus the intercellular CO_2 concentration (C_i) assessed at several levels of irradiance (Laisk, 1977). Other indirect methods based on GE data have also been described (e.g. Laisk and Loreto, 1996; Peisker and Apel, 2001).

The Kok method (Kok, 1948) utilizes the fact that the response of A to light is generally linear at low irradiances. However, in the vicinity of the light compensation point

there might be a break in the linear relationship, with a markedly higher slope of the response curve below than above the break point—the so-called 'Kok-effect' (Kok, 1948; Sharp *et al.*, 1984; Brooks and Farquhar, 1985; Kirschbaum and Farquhar, 1987; Villar *et al.*, 1994). Sharp *et al.* (1984) explained that the higher slope below the break was attributable to the effect of the suppression of dark respiration by light (see also Ribas-Carbo *et al.*, 2010). To avoid the influence of the Kok effect, data of the linear range above the break point are analysed, and the extrapolation of that particular linear section of the curve to the zero irradiance gives an estimate of R_d (Brooks and Farquhar, 1985; Villar *et al.*, 1994; Wang *et al.*, 2001; Shapiro *et al.*, 2004). The method can be applied to any CO_2 level, and might be used to examine whether or not R_d varies with a change of the CO_2 levels. Obviously, the method assumes that R_d does not vary with light within the range of light levels used.

The second method, described by Laisk (1977), analyses the response curves of A to low C_i that are obtained at several light intensities. It aims to identify the intercellular CO_2 level (C_{i*}) at which the rate of CO_2 fixation by photosynthesis equals the rate of CO_2 release from photo-respiration. At this C_{i*} (i.e. C_i-based CO_2 compensation point in the absence of R_d), all of the fixed CO_2 is consumed in photorespiration, and the rate of CO_2 release should represent R_d. The values of C_{i*} and R_d are identified as the coordinates of the common intersection point of A versus C_i at two or more light intensities (Fig. 1a). Obviously, the Laisk method also assumes that R_d does not vary with irradiance within the irradiance ranges used. However, by using a wide array of irradiances, the method can be used to explore any effect of light intensity on the value of R_d (Villar *et al.*, 1994). The main disadvantage of the Laisk method is that the measurements must be performed at very low CO_2 levels and are therefore under far from normal environmental conditions, especially given that a change in R_d with CO_2 level has been reported (Villar *et al.*, 1994). Nevertheless, the Laisk method has been widely used as a standard method to estimate R_d (e.g. Brooks and Farquhar, 1985; von Caemmerer *et al.*, 1994; Atkin *et al.*, 1997, 2000; Peisker and Apel, 2001; Priault *et al.*, 2006; Flexas *et al.*, 2007b).

Like GE measurements, chlorophyll fluorescence (CF) measurements have increasingly been used as a non-invasive tool in leaf ecophysiological studies. In particular when the two types of measurements are combined to assess both A and photosystem II (PSII) electron (e^-) transport efficiency (Φ_2) simultaneously, a number of photosynthesis parameters underlying physiological responses to environmental variables can be estimated (e.g. Laisk and Loreto, 1996). For example, combined GE and CF measurements have been used to estimate mesophyll conductance g_m (Harley *et al.*, 1992; Yin and Struik, 2009), relative CO_2/O_2 specificity of Rubisco (Peterson, 1989), inter-photosystem excitation partitioning factor, and alternative e^- transport (Makino *et al.*, 2002; Yin *et al.*, 2006). However, combined GE and CF measurements have hardly been used to
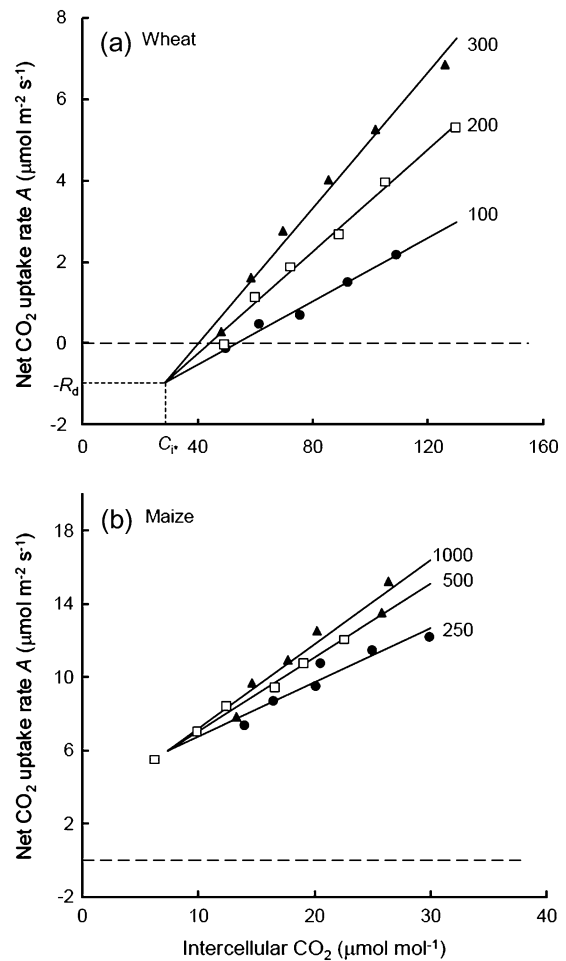
Fig. 1. Net CO_2 assimilation rate (A) as a function of intercellular CO_2 concentration (C_i). Numbers indicate the three incident irradiances (I_{inc}) under which measurements were carried out (in µmol m^{-2} s^{-1}). Regression lines at these I_{inc}, fitted to data points that each represents the mean of measurements from four replicated plants, were forced to join at the common intersection point (C_{i*}, $-R_d$), where R_d is the estimated leaf respiration rate in the light (Laisk method) and C_{i*} is the C_i-based CO_2 compensation point in the absence of R_d. The dashed horizontal line is the line of $A=0$. The estimated R_d is negative for maize (b), indicating that the Laisk method does not work for C$_4$ species. Note that the scales in the two panels are different.

estimate R_d. The only report is a recent integrated method of using these combined data to estimate photosynthesis parameters (including R_d) of a biochemical C$_3$ photosynthesis model (Yin *et al.*, 2009). Like the Kok method, this method utilizes the response of A to irradiance at low light intensities. However, this method also utilizes the CF information on the response of Φ_2 to light. Preliminary results for wheat (*Triticum aestivum*) leaves have shown that the new CF-based method allows a better estimate of R_d than the Kok method does (Yin *et al.*, 2009).

In the present work, this novel CF-based method is compared not only with the Kok method but also with the more widely used Laisk method, in estimating R_d of leaves in various crop species. The specific emphasis is placed on

examining whether the CF-based method is generally applicable.

Materials and methods

Theoretical considerations

The method of Yin *et al.* (2009) to estimate R_d is based on the fact that at low values of irradiance A is limited by the light-dependent e$^-$ transport rate. Building upon the well-known model of Farquhar *et al.* (1980), Yin *et al.* (2004) described a generalized equation for A within the e$^-$ transport-limited range as:

$$A = J_2 \left(1 - \frac{f_{pseudo}}{1 - f_{cyc}}\right) \frac{C_c - \Gamma_*}{4(C_c + 2\Gamma_*)} - R_d \qquad (1)$$

where J_2 is the total rate of e$^-$ transport passing PSII, f_{cyc} and f_{pseudo} represent fractions of the total e$^-$ flux passing PSI that follow cyclic and pseudocyclic pathways, respectively, C_c is the CO_2 level at the carboxylation sites of Rubisco, and Γ_* is the C_c-based CO_2 compensation point in the absence of R_d. A special case of Equation (1) is the e$^-$ transport-limited equation of the Farquhar *et al.* (1980) model:

$$A = J \frac{C_c - \Gamma_*}{4(C_c + 2\Gamma_*)} - R_d \qquad (2)$$

where J is the PSII e$^-$ transport rate that is used for CO_2 fixation and photorespiration.

By definition, the variable J_2 in Equation (1) can be replaced by $\rho_2\beta I_{inc}\Phi_2$, where I_{inc} is the level of incident irradiance, β is the absorptance by leaf photosynthetic pigments, and ρ_2 is the fraction of absorbed irradiance partitioned to PSII. Substituting this term into Equation (1) gives:

$$A = \rho_2 I_{inc} \Phi_2 \left(1 - \frac{f_{pseudo}}{1 - f_{cyc}}\right) \left(\frac{C_c - \Gamma_*}{C_c + 2\Gamma_*}\right)/4 - R_d \qquad (3)$$

For non-photorespiratory conditions where C_c approaches infinity and/or Γ_* approaches zero, Equation (3) becomes:

$$A = \rho_2\beta I_{inc}\Phi_2 \left(1 - \frac{f_{pseudo}}{1 - f_{cyc}}\right)/4 - R_d = s\left(I_{inc}\Phi_2/4\right) - R_d \qquad (4)$$

where the lumped parameter $s=\rho_2\beta[1-f_{pseudo}/(1-f_{cyc})]$. So, using data of the e$^-$ transport-limited range under non-photorespiratory conditions, a simple linear regression can be performed for the observed A against ($I_{inc}\Phi_2/4$), in which Φ_2 is based on CF measurements. The slope of the regression will yield the estimate of a lumped parameter s, and the intercept will give an estimate of R_d (Yin *et al.*, 2009). Clearly, this CF-based method is very similar to the Kok method; therefore, it should apply to the range of limiting irradiances, yet above the Kok break point if the Kok effect occurs. However, the Kok method has an additional assumption that Φ_2 is constant within the range of limiting lights. As will be shown later, this assumption is not true.

Assuming the variation of the term $(C_c-\Gamma_*)/(C_c+2\Gamma_*)$ in Equation (3) is negligible across an A–I_{inc} curve, Yin *et al.* (2009) showed that the simple regression procedure can also be used to estimate R_d for photorespiratory conditions, although it is then less certain that the relationship between A and $I_{inc}\Phi_2/4$ will be linear. This assumption is in fact also used implicitly in applying the Kok method to estimate R_d or quantum yield under photorespiratory conditions. To correct for small differences of CO_2 level across an A–I_{inc} curve when estimating R_d, a procedure as proposed by Kirschbaum and Farquhar (1987) would need to be implemented. However, their correction procedure was based on an assumption of infinite g_m, which is now known to be unlikely to

be true (Harley *et al.*, 1992; Flexas *et al.*, 2007b; Yin and Struik, 2009). A full correction would require a pre- or simultaneous estimation of g_m, in addition to the estimation of Γ_*. No correction was therefore made in using the CF method for the purpose of simplicity.

Plant material and measurements

Five C_3 crop species, wheat (cv. 'Lavett'), rice (*Oryza sativa*, cv. 'IR64'), potato (*Solanum tuberosum*, cv. 'Bintje'), tomato (an inbred line from a cross between *Solanum lycopersicum* cv. 'Moneyberg' and *Solanum chmielewskii*), and rose (*Rosa hybrida* cv. 'Akito'), and one C_4 species (*Zea mays*, experimental hybrid '2-05R00061') were chosen for this study. Plants were grown in a glasshouse complex, in pot soil (wheat, rice, potato, and maize) or on rock-wool hydroponics (tomato and rose), without water or nutrient stress. Climatic conditions in the glasshouses were semi-controlled. Extra SON-T light was switched on when solar radiation outside the glasshouses was <400 W m^{-2}. The glass-house [CO_2] was ~370 µmol mol^{-1}, relative humidity was 60–80%, and temperature was 25±5 °C during measurements.

An open GE system (Li-Cor 6400; Li-Cor Inc., Lincoln, NE, USA) and an integrated fluorescence chamber head (i.e. the 2 cm^2 chamber) were used. While the Laisk and Kok methods require only GE measurements, data would be collected by using the larger 6 cm^2 chamber to reduce GE measurement noises. However, for comparison of the three methods, all the data were collected using the 2 cm^2 chamber. All measurements were carried out at a leaf temperature of 25 °C and a leaf-to-air vapour pressure difference of 1.0–1.6 kPa, using a flow rate of 400 µmol s^{-1}. Each measurement was made on four full-grown leaves in replicated plants.

Two sets of measurements were conducted. The first set was to compare the three methods. For C_i response curves required by the Laisk method, ambient CO_2 level (C_a) was increased step-wise from 50 µmol mol^{-1} up to a maximum of 150 µmol mol^{-1} in six steps while keeping I_{inc} at three levels depending on the species. The three light levels chosen for maize were higher than for the other species, following preliminary trials to obtain linear A–C_i relationships. For I_{inc} response curves as required by the Kok method and the new method, I_{inc} was in a serial 5, 10, 15, 20, 30, 50, 70, 100, 150, and 200 µmol mol^{-1}, while keeping C_a at 370 µmol mol^{-1} and O_2 at 21% O_2. For rice, potato, and maize, the light response of the same leaves was also measured at 2% O_2. Leaf photosynthesis and respiration may acclimate to incident light conditions during measurement. To test whether the estimated R_d by the new method is affected by the direction of changing light levels, a second, separate set of measurements were undertaken for wheat, rice, and maize, in which both increasing and decreasing series of the above light levels were applied for each of the two O_2 levels.

For the measurements at 2% O_2, a gas cylinder containing a mixture of 2% O_2 and 98% N_2 was used. Gas from the cylinder was humidified and supplied to the Li-Cor 6400 where CO_2 was blended with the gas. CO_2 exchange data where the set C_a values were lower than the ambient air value (i.e. those measurements required for the Laisk method) were corrected for leakage of CO_2 into the leaf cuvette, using measurements with heat-killed leaves (Flexas *et al.*, 2007a).

The value of R_{dk} was measured 15–20 min after leaves had been placed in darkness. For measurements at each irradiance or CO_2 step, A was allowed to reach steady state, after which F_s (the steady-state fluorescence) was recorded from the leaf, and then a saturating light pulse (>8500 µmol m^{-2} s^{-1} for 0.8 s) was applied to determine F'_m (the maximum fluorescence during the saturating light pulse). The apparent PSII e$^-$ transport efficiency was calculated as: $\Delta F/F'_m=(F'_m-F_s)/F'_m$ (Genty *et al.*, 1989). This $\Delta F/F'_m$ was treated in the present analysis as a true PSII e$^-$ transport efficiency Φ_2, because the ratio $\Delta F/F'_m$:Φ_2, if not equal to 1, has an

impact on the value of parameter *s* but not on the estimated R_d (Yin *et al.*, 2009).

Analysis methods

Regression was performed on the mean values of measurements across four replicated leaves. For all methods, data points at high ends that apparently deviated from the required linear pattern were dropped. For the Kok method and the new method, only data of the linear range at light levels above the Kok break point, if the Kok effect occurred significantly, were used to estimate R_d, by the simple linear regression procedure in MS-Excel. As actual values of irradiance may deviate slightly from the I_{inc} set values, the I_{inc} values incident on a leaf assessed by the in-chamber quantum sensor of Li-Cor 6400 were used for analysis. For the Laisk method, the three linear regression lines were forced to intersect at the same point to obtain a single estimate of R_d from each data set, although the lines might not have joined exactly at the same point if regression was carried out separately for the three light levels. Therefore, the PROC NLIN of SAS (SAS Institute Inc., Cary, NC, USA) was used to fit data for the Laisk method. The SAS codes can be obtained upon request.

Results

Comparison of the estimates by the three methods

Data from the first set of measurements were analysed to compare R_d estimated by the three methods. The measured A–C_i curves at three light intensities for the five C_3 species confirmed a general linear pattern with a common intersection as required by the Laisk method. An example of the curves is shown in Fig. 1a for wheat. This common intersection was found for all C_3 species below the line $A=0$; therefore, the estimated R_d was positive for all these species. For the C_4 species maize, however, the identified intersection point was well above the line $A=0$ (Fig. 1b), suggesting a negative R_d. C_4 plants have a CO_2-concentrating mechanism that allows a high CO_2 concentration at Rubisco active sites in bundle sheath cells even if C_i is low, thereby requiring higher irradiances to obtain linear A–C_i relationships and yielding quite high values of A at low C_i commonly applied (Fig. 1b). Since the negative R_d is highly unlikely, the Laisk method cannot be applied to estimate R_d in leaves of C_4 plants.

In contrast to the Laisk method, both the Kok method and the new CF method can be applied to estimate R_d of both C_3 and C_4 leaves, utilizing the linear part beyond the Kok break point of the A–I_{inc} and A–$I_{inc}\Phi_2/4$ relationships, respectively (Figs 2, 3). There were apparent deviations from linearity at the high end of the A–I_{inc} relationship in some plants, for example tomato (result not shown), and this deviation was only partially corrected when the A–$I_{inc}\Phi_2/4$ relationship was applied. These deviated points, therefore, were excluded in linear regression to estimate R_d for the two methods. At 21% O_2, the slope of the A–I_{inc} relationships at the lower end when I_{inc} was around the light compensation point or lower was clearly higher, although for wheat and maize the change of the slope value was small, suggesting the occurrence of a significant Kok effect in most C_3 species. Similar changes in the slope, albeit
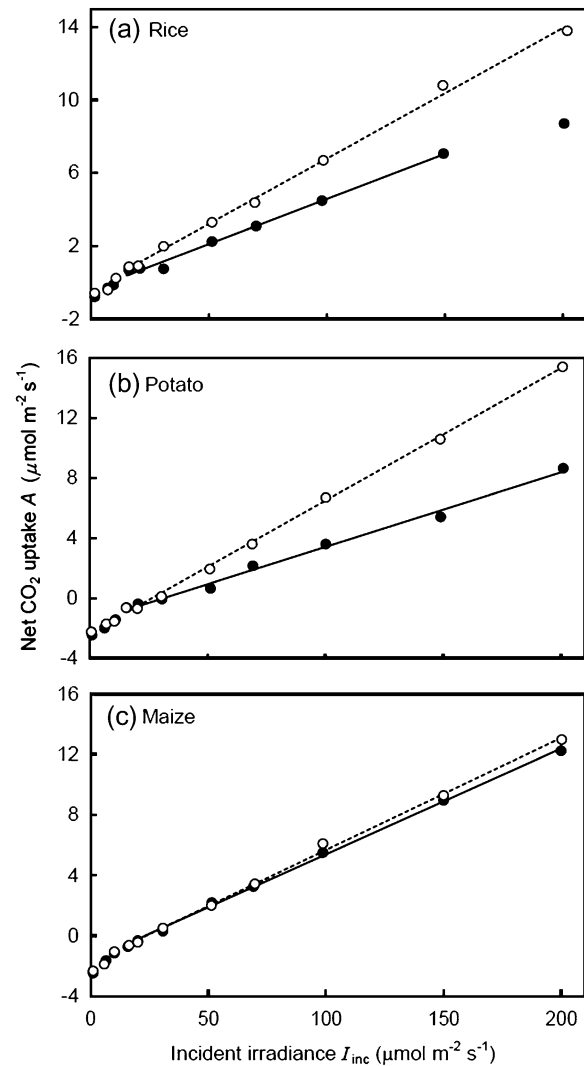
Fig. 2. Net CO_2 assimilation rate (A) as a function of limiting incident irradiances (I_{inc}) at ambient air CO_2 with 21% (filled circles) and 2% (open circles) O_2 levels. Each data point represents the mean of measurements from four replicated plants. Solid and dotted lines represent regressions for data within the linear range from irradiance levels higher than the Kok break point at 21% and 2% O_2, respectively. The extrapolation of these regression lines to the zero light level gives an estimation of $-R_d$, where R_d is the estimated respiration rate in the light (Kok method). The regression lines below the break point are not shown.

smaller, were also identified at 2% O_2 and in maize. This abrupt change of the slope value was clearly shown in the A–$I_{inc}\Phi_2/4$ relationship as well (Fig. 3).

The Kok method requires a linear A–I_{inc} relationship beyond the Kok break point (Fig. 2). Such a linear relationship assumes that Φ_2 is constant within the range of I_{inc} used. However, CF measurements showed that the apparent quantum efficiency of PSII e$^-$ transport ($\Delta F/F'_m$) decreased continuously with increasing I_{inc}, even within the limiting irradiance range (Fig. 4). The new CF method for R_d estimation accounts for such a decline of Φ_2 by analysing the A–$I_{inc}\Phi_2/4$ relationships (Fig. 3). For this reason, the R_d values estimated by the CF method were consistently higher
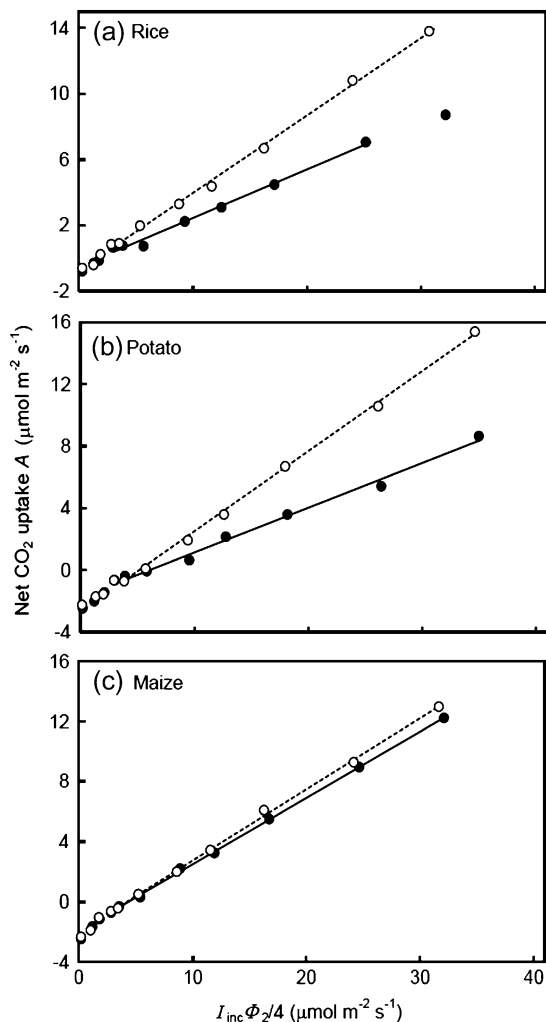
Fig. 3. Net CO_2 assimilation rate (A) as a function of the variable $I_{inc}\Phi_2/4$ (where I_{inc} is the incident irradiance and Φ_2 is the quantum efficiency of PSII electron transport) at ambient air CO_2 with 21% (filled circles) and 2% (open circles) O_2 levels. Each data point represents the mean of measurements from four replicated plants. Solid and dotted lines represent regressions for data within the linear range from irradiance levels higher than the break point at 21% and 2% O_2, respectively. The extrapolation of these regression lines to the zero $I_{inc}\Phi_2/4$ level gives an estimation of -R_d, where R_d is the estimated respiration rate in the light (new CF method). The regression lines below the break point are not shown.

than those estimated by the Kok method (Table 1), on average, by 20%.

The values of R_d estimated by the Laisk method, which as usual was applied to ambient O_2 condition (21%) for the measurements, varied from 0.63 µmol m^{-2} s^{-1} for rice to 1.52 µmol m^{-2} s^{-1} for potato (Table 1). For the common 21% O_2, the overall trend for the variation of R_d among the C_3 crops provided by the three methods was consistent. The difference in the R_d estimates may be due to differences in crop type and/or leaf ages. Generally, R_d estimated by the new CF method agreed well with those estimated by the Laisk method (Fig. 5). However, R_d estimated by the Kok
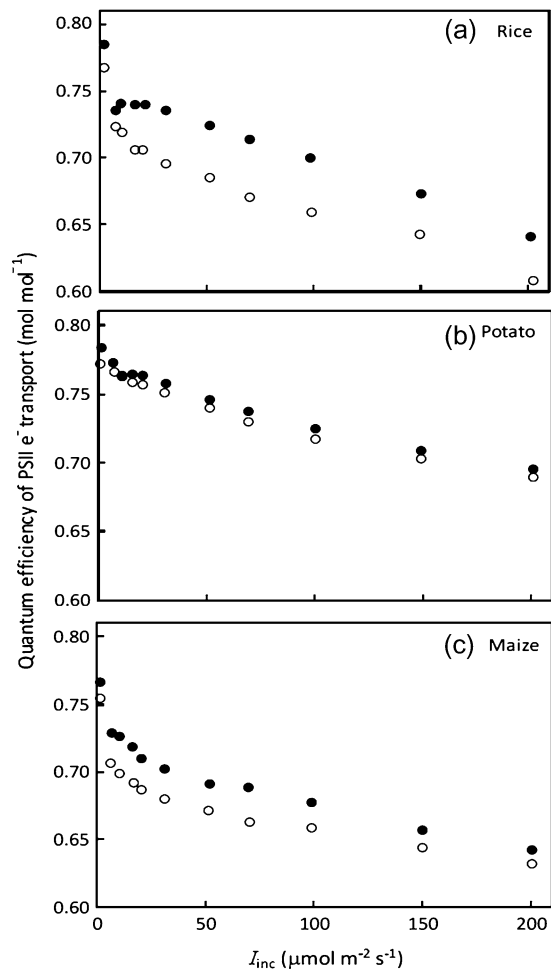
Fig. 4. Quantum efficiency of PSII electron transport (as indicated by chlorophyll fluorescence data for the apparent PSII quantum efficiency $\Delta F/F'_m$) as a function of incident irradiance I_{inc} at ambient air CO_2 with 21% (filled circles) and 2% (open circles) O_2 levels for rice, potato, and maize. Each data point represents the mean of measurements from four replicated plants.

method was mostly lower (Fig. 5) and, on average, was ~87% of R_d obtained from the Laisk method.

Effect of the direction of changing irradiances on R_d estimated by the CF method

For a second set of measurements, the same levels of irradiances but two contrasting directions (increasing versus decreasing) of changing the irradiances were used for wheat, rice, and maize, to test whether the value of R_d estimated by the new CF method is sensitive to the direction of the change. An example of these measurements is given in Fig. 6 for wheat.

Using data points above the Kok break points, values of R_d estimated from measurements of increasing I_{inc} differed slightly from those estimated from measurements of decreasing I_{inc} (Table 2). In most cases, R_d values from increasing I_{inc} were slightly higher than those from decreasing I_{inc}, whereas in other cases the opposite was true. However, in no case was the difference statistically

Table 1. Value of the day respiration R_d (SE of the estimate in parentheses) estimated by three methods (i.e. Laisk, Kok, and CF), the intercept value at the *A*-axis by extrapolating the linear relationship below the break point (see Figs 2 and 3), and the mean value of the respiration rate in darkness R_{dk} across four replications (SE of the mean in parentheses), for leaves in six crop species

Data were from the first set of measurements. The unit of all parameters is µmol m^{-2} s^{-1}.

Crop		O$_2$ (%)	R_d			Intercept at *A*-axis		R_{dk}
			Laisk	Kok	CF	Kok	CF	
C$_3$	Wheat	21	0.972 (0.516)	0.631 (0.151)	1.043 (0.140)	1.436 (0.129)	1.474 (0.142)	1.358 (0.048)
	Rice	21	0.628 (0.511)	0.368 (0.146)	0.522 (0.172)	0.903 (0.218)	0.901 (0.218)	0.806 (0.122)
		2	–	0.369 (0.155)	0.744 (0.149)	0.984 (0.327)	1.015 (0.336)	0.608 (0.120)
	Potato	21	1.522 (0.612)	1.563 (0.209)	1.751 (0.229)	2.725 (0.218)	2.735 (0.220)	2.468 (0.150)
		2	–	2.555 (0.105)	2.890 (0.123)	2.327 (0.384)	2.339 (0.389)	2.250 (0.263)
	Tomato	21	1.310 (0.729)	0.962 (0.081)	1.024 (0.084)	1.790 (0.198)	1.793 (0.201)	1.675 (0.150)
	Rose	21	1.320 (0.976)	1.286 (0.069)	1.503 (0.096)	1.929 (0.340)	1.938 (0.343)	1.930 (0.188)
C$_4$	Maize	21	NA	1.614 (0.126)	1.911 (0.084)	2.213 (0.212)	2.234 (0.209)	2.473 (0.398)
		2	NA	1.740 (0.169)	1.985 (0.133)	2.417 (0.415)	2.441 (0.418)	2.325 (0.368)

NA, not applicable, as the Laisk method does not work for C$_4$ species (see text); –, not measured, as the Laisk method is usually applied under the ambient O$_2$ conditions.
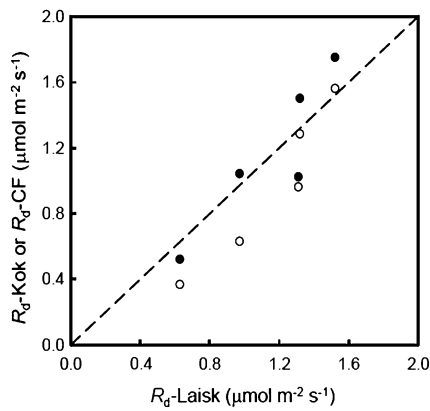
Fig. 5. Values of leaf respiration rate in the light (R_d) for five C$_3$ species at 21% O$_2$, estimated by the Kok method (open circles) or by the new CF method (filled circles), compared with the estimates for R_d by the Laisk method. The dashed line represents the 1:1 relationship.

Table 2. Value of the day respiration R_d (SE of the estimate in parentheses) estimated by the CF method, and the mean value of the respiration rate in darkness R_{dk} across four replications (SE of the mean in parentheses), for leaves in three crop species

Data were from the second set of measurements, where irradiances were changed in either increasing or decreasing order. The unit of R_d and R_{dk} is µmol m^{-2} s^{-1}.

Crop		O$_2$ (%)	R_d			R_{dk}
			Increasing	Decreasing	Pooled	
C$_3$	Wheat	21	1.054 (0.113)a	0.820 (0.112)a	0.936 (0.083)	1.782 (0.173)
		2	0.687 (0.093)a	0.502 (0.093)a	0.594 (0.068)	1.076 (0.055)
	Rice	21	0.628 (0.190)a	0.734 (0.188)a	0.681 (0.130)	0.975 (0.161)
		2	0.552 (0.136)a	0.402 (0.136)a	0.477 (0.095)	0.644 (0.061)
C$_4$	Maize	21	2.629 (0.263)a	2.086 (0.270)a	2.365 (0.199)	2.094 (0.273)
		2	1.284 (0.226)a	1.928 (0.260)a	1.562 (0.192)	1.794 (0.202)

The same letter in a row means that the estimated R_d did not differ significantly (*P* >0.10) between increasing and decreasing irradiance series.

significant (*P* >0.10). Therefore, values of R_d estimated from the pooled data of the two changing series are also shown in Table 2. The differences in R_d among the three crops from this set of measurements agreed generally with those obtained from the first set of measurements (Table 1) —$R_{d,maize} > R_{d,wheat} > R_{d,rice}$.

Effect of O$_2$, and comparison between R$_d$ *and* R$_{dk}$

For the Kok method and the new CF method, both 21% and 2% O$_2$ levels were implemented for some crops; so for these crops, R_d was estimated by the methods for the two O$_2$ levels (Tables 1, 2). For measurements at the 2% O$_2$ level, a change of the slope for the Kok effect was relatively less apparent (Figs 2, 3, 6). As expected, 2% O$_2$ (compared with 21% O$_2$) suppressed photorespiration and thus increased the slope of the relationship above the Kok break point in the C$_3$ crops wheat, rice, and potato, whereas the

difference in the slope between the two O$_2$ levels was very small in the C$_4$ crop maize (Figs 2, 3). Below the Kok break point, there was no apparent difference between the two O$_2$ levels in any species. As a result, the estimated R_d did not differ between the two O$_2$ levels in the C$_4$ species maize, but the CF method showed that it was higher at low than at high O$_2$ levels for the C$_3$ species rice and potato (Table 1). In contrast, from the second set of measurements, the estimated R_d was lower at low than at high O$_2$ levels (Table 2), as the low O$_2$ increased *A* somewhat already at very low irradiances (results not shown). However, this difference in R_d between the O$_2$ levels was not significant in most cases, suggesting that the effect of O$_2$ on R_d, if any, was not consistent.

Except for a few cases, the measured R_{dk} was higher than the R_d estimated by any of the three methods (Tables 1, 2). However, R_{dk} did not differ significantly (*P* >0.05) from the
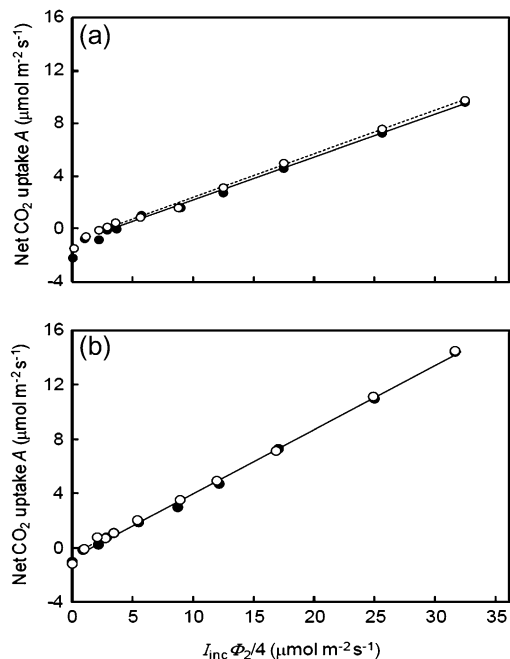
Fig. 6. Net CO_2 assimilation rate (A) of wheat leaves as a function of the variable $I_{inc}\Phi_2/4$ (where I_{inc} is the incident irradiance and Φ_2 is the quantum efficiency of PSII electron transport) at ambient air CO_2 with 21% (a) and 2% (b) O_2 levels. Each data point represents the mean of measurements from four replicated plants. Solid and dotted lines represent regressions for data within the linear range from irradiance levels higher than the break point for increasing (filled circles) and decreasing (open circles) light series, respectively. The dotted regression line is invisible in (b) because it virtually overlaps with the solid line. The extrapolation of these regression lines to the zero $I_{inc}\Phi_2/4$ level gives an estimation of $-R_d$, where R_d is the estimated leaf respiration rate in the light (new CF method). The regression lines below the break point are not shown.

intercept at the A-axis by extrapolating the linear relationship below the Kok break point of the light response (Figs 2, 3, 6; Table 1).

Discussion

Comparison of the three methods

The Laisk (1977) method has been widely considered as a standard method to estimate leaf R_d indirectly in ecophysiological studies for C_3 plants (e.g. Brooks and Farquhar, 1985; von Caemmerer et al., 1994; Peisker and Apel, 2001; Priault et al., 2006; Flexas et al., 2007b), probably also because it generates an estimate of another important parameter C_{i*}. Its applicability is confirmed by the present results (e.g. Fig. 1a) for five C_3 species. However, the results for maize (Fig. 1b) suggest that the Laisk method yielded a negative R_d, which is physiologically impossible. Measurements show that there are no obvious differences in respiratory costs between C_3 and C_4

plants of similar habitats (Byrd et al., 1992). The present results are in line with the literature, in which the Laisk method has been used only for C_3 plants. In fact, the theoretical basis of the Laisk method is Equation (1) or (2), which predicts that A has a common value (i.e. $-R_d$) at various light intensities when $C_c=\Gamma_*$ (equivalently when $C_i=C_{i*}$). So, strictly speaking, one must use Γ_*, instead of C_{i*}, in the Laisk method, although few have done so because Γ_* and C_{i*} differ by R_d/g_m, and g_m is difficult to measure (Harley et al., 1992; Flexas et al., 2007b; Yin and Struik, 2009). Since the CO_2-concentrating mechanism plays such an important role in determining the C_4 photosynthetic rate at low CO_2 levels, the simple Equation (1) or (2), valid for C_3 photosynthesis, does not suit for C_4 photosynthesis. It is not surprising, therefore, that the Laisk method does not work for C_4 plants.

Another disadvantage of the Laisk method is that the experiments must be performed at very low CO_2 concentrations, far below normal ambient CO_2 levels (Villar et al., 1994, 1995). When a large gradient exists between the set CO_2 concentration and that in the ambient air, it is hard to avoid CO_2 exchange or leakage between IRGA's leaf chamber of the open GE system and the surrounding air, leading to erroneous measurements of A and C_i (Flexas et al., 2007a). Therefore, a correction of A and C_i for this leakage is necessary (Flexas et al., 2007a; Rodeghiero et al., 2007). If no correction was made, the estimated R_d by the Laisk method would have become, on average, ~50% higher than the values given in Table 1. The reported increase of leaf respiration with a short-term decrease in CO_2 concentration (e.g. Villar et al., 1994; Atkin et al., 2000), seemingly explained by CO_2 acting as an inhibitor of certain enzymes, means a further uncertainty in the estimated R_d by the Laisk method, although such an impact of CO_2 on leaf respiration was not always evident (Brooks and Farquhar, 1985; Kirschbaum and Farquhar, 1987; Tjoelker et al., 2001). Amthor et al. (2001) suggested that earlier reported changes of leaf respiration with the CO_2 level may have been due to small leaks in the GE measurement systems.

The above major disadvantages of the Laisk method can be overcome by the Kok method and the new CF method, which can be implemented under ambient CO_2 conditions and are applicable to both C_3 and C_4 species. For example, the Kok method was used to assess the quantum yield of CO_2 assimilation (Φ_{CO2}) as well as R_d in a large number of C_3, C_4, and intermediate species (Björkman and Demmig, 1987). This is because the Kok and the CF methods use data measured under limiting irradiance, which is the predominant factor determining photosynthesis, so Equation (1) or (2) applies even for C_4 photosynthesis. Furthermore, the present measurements showed that data in the low C_i portions of A–C_i curves required by the Laisk method generally had more noise (were more scattered) than those in the low portions of light response curves required by the Kok method and the new CF method, probably because the former involves use of additional data for transpiration (to calculate C_i), whose measurements are

sensitive to uncontrolled environmental perturbations. This uncertainty is also reflected by the standard errors of R_d estimates which were higher for the Laisk method than for the other two methods (Table 1). In line with the results of Villar *et al.* (1994), the present values of R_d estimated by the Kok method were generally lower than those by the Laisk method (Fig. 5; Table 1). However, values estimated by the new CF method were in better agreement with those estimated by the Laisk method (Fig. 5; Table 1).

The difference between the Kok method and the new method is that not only data from GE measurements on A but also those from CF measurements on $\Delta F/F'_m$ are used in the new method. According to Equation (3), the Kok method implicitly assumes that like coefficients β and ρ_2, Φ_2 does not vary with I_{inc} within the used data range. However, data from CF measurements reveal that the loss of Φ_2, as indicated by $\Delta F/F'_m$, develops as the irradiance increases even within low light ranges (Fig. 4; see also Genty and Harbinson, 1996), implying a non-linear A–I_{inc} relationship. Thus, the new method using the information of CF (in addition to GE information) corrects the error of the Kok method of the constant Φ_2 over low irradiances, thereby accounting for any pitfall caused by possible non-linearity, undetectable by visual or statistical inspection (Fig. 2), between A and I_{inc} of the used data range (Yin *et al.*, 2009). Use of the combined GE and CF data in the new method is justified by a generally observed linear relationship between $\Delta F/F'_m$ and Φ_{CO2} over a wide range of conditions for C_3 (e.g. Genty *et al.*, 1989) and C_4 (Edwards and Baker, 1993) species. The sometimes reported break of the linearity between $\Delta F/F'_m$ and Φ_{CO2} at low light levels (e.g. Seaton and Walker, 1990) may be, at least partly, due to uncertainty in estimating R_d (Edwards and Baker, 1993) since R_d accounts for a large portion of the variation in Φ_{CO2} under low light conditions. It is worth noting that data for the new method have to be obtained from a small (e.g. 2 cm^2) leaf chamber because errors with CF measurements for $\Delta F/F'_m$ are inversely proportional to leaf area, although this limitation does not apply for fluorescence systems based on area-imaging cameras rather than spot measurements. However, the Kok method, like the Laisk method, uses only GE data; therefore, data would be collected with the large chamber (e.g. 6 cm^2) to reduce the noise-to-signal ratio and to represent the whole leaf better.

In short, each method has its own advantages and disadvantages, which are summarized in Table 3. It would be useful to compare the results of these indirect methods with those obtained by one of the methods that directly measure R_d (e.g. those of Haupt-Herting *et al.*, 2001; Loreto *et al.*, 2001; Pärnik and Keerberg, 2007).

Table 3. Evaluation of the three methods to estimate leaf respiration rate in the light R_d

	Advantages	Disadvantages
The Laisk method	1. Data used could be obtained from a large (e.g. 6 cm^2) leaf chamber.	1. Low C_i levels have to be used, which are far from the level for normal plant growth.
	2. The method provides additional estimates on carboxylation efficiencies at various irradiances and on the very useful parameter C_{i*}.	2. It is required to correct for the CO_2 leakage during the gas exchange measurement.
	3. The method could be used to check roughly if R_d varies with irradiance levels.	3. The method is applicable only for C_3, not for C_4 plants.
		4. The method is sensitive to errors of the system in measuring transpiration that affects C_i.
The Kok method	1. Data used could be obtained from a large (e.g. 6 cm^2) leaf chamber.	1. The method is based on the assumption that Φ_2 is constant within used irradiances, which is highly unlikely; as a result, it may underestimate R_d.
	2. The method is applicable for both C_3 and C_4 plants.	2. Low irradiance levels have to be used, which may not represent the light level for normal plant growth
	3. The method could potentially be applied to the CO_2 levels for normal plant growth; so it is possible that no correction for CO_2 leakage during measurement is required.	3. Theoretically, the method works best for the non-photorespiratory condition.
	4. The method provides additional estimate for Φ_{CO2}.	
	5. The method is insensitive to errors in measuring transpiration.	
	6. The method could be used to check if R_d varies with CO_2 levels.	
The new CF method	1. Using CF information, the method corrects for the error of the Kok method assuming a constant Φ_2 with low irradiances; as a result, data of a wider range of irradiance could be useable, relative to the Kok method.	1. Data used have to be obtained from a small (e.g. 2 cm^2) leaf chamber because errors with CF measurements are inversely proportional to leaf area (but note that this limitation does not apply for fluorescence systems based on area-imaging cameras).
	2. The method is applicable for both C_3 and C_4 plants.	2. Generally low irradiance levels are used, which may not represent the light level for normal plant growth.
	3. The method could potentially be applied to the CO_2 levels for normal plant growth; so it is possible that no correction for CO_2 leakage during measurement is required.	3. Theoretically, the method works best for the non-photorespiratory condition.
	4. The method provides additional estimate for parameter s, that lumps a number of useful physiological parameters (see text).	
	5. The method is insensitive to errors in measuring transpiration.	
	6. The method could be used to check if R_d varies with CO_2 levels.	

Little evidence for dependence of R_d on the direction of changing irradiance

Little evidence for dependence of R_d on the direction of changing irradiance

One relevant issue for using the new method is whether or not the direction of changing irradiances has an impact on the estimated R_d, since the CF method, like the Kok method, requires a series of data points across the low light range. As discussed in the section 'Theoretical considerations', both methods are theoretically valid under non-photorespiratory conditions (Table 3). For normal photorespiratory conditions, the methods rely on the assumption that variation of C_c, and therefore C_i, with irradiance is negligible. This assumption is questionable given that at a given C_a, the variation of C_i with irradiance is most apparent in the low I_{inc} range, within which data are collected to estimate R_d by the Kok and CF methods. High irradiances induce stomatal opening, which may have a consequence on GE and C_i at subsequent light levels and, therefore, on the estimated R_d, especially under photorespiratory condition. For this reason, the second set of measurements were conducted using the same light levels but contrasting (increasing versus decreasing) directions of changing irradiances.

The estimated R_d values by the CF method from measurements of increasing and decreasing irradiances were not identical (Table 2). However, the difference was not significant, nor was it consistent or systematic. As discussed above, the effect of the direction of changing irradiance on R_d, if any, is expected to occur under photorespiratory conditions. However, any difference in R_d between the two light series was not higher at 21% than at 2% O_2 levels in two C_3 crops, and not higher in C_3 than in C_4 species (Table 2). Moreover, a 'drifting' in the actual values of R_d may occur with increasing or decreasing light since it is hard to complete low-light series measurements quickly enough to preclude the drifting. Therefore, it is believed that the difference in the estimated R_d between the light series was possibly due to measurement noise or 'drifting', rather than to biological mechanisms.

Effect of light on mitochondrial respiration, and the Kok effect

Effect of light on mitochondrial respiration, and the Kok effect

Values of R_d estimated by all three methods were generally lower than those of R_{dk} (Tables 1, 2), supporting the assertion that leaf respiration can be inhibited by light (Sharp *et al.*, 1984; Brooks and Farquhar, 1985; Villar *et al.*, 1994, 1995; Laisk and Loreto, 1996; Atkin *et al.*, 1997, 2000; Wang *et al.*, 2001; Shapiro *et al.*, 2004). An *in vivo* metabolic study (Tcherkez *et al.*, 2005) indicated that the main inhibited steps were the entrance of hexose molecules into the glycolytic pathway and the Krebs cycle. However, whether this difference between R_d and R_{dk} is due to real inhibition has been challenged (e.g. Loreto *et al.*, 2001) because CO_2 released from respiration during illumination is possibly re-fixed by photosynthesis.

Another uncertainty is the assumption used in all three methods (Laisk, Kok, and CF) that R_d is independent of light intensity, and the assumption seems to be supported by some experimental studies (e.g. Haupt-Herting *et al.*, 2001). Furthermore, both Kok and CF methods implicitly assume that R_d is maximally inhibited by light at the Kok break point. However, it has been shown that the extent to which irradiance inhibits R_d increases with increasing light intensity (Brooks and Farquhar, 1985; Villar *et al.*, 1994, 1995; Laisk and Loreto, 1996; Atkin *et al.*, 2000), well beyond the break point. It has been suggested that the Kok effect is caused by the progressive, light-induced inhibition of leaf respiration (e.g. Sharp *et al.*, 1984; Ribas-Carbo, 2010), which is also in line with the present results that R_{dk} did not differ from the intercept of the line below the Kok break point (Table 1). Previously, the Kok effect was suggested to be associated with photorespiration given the observed absence of the Kok effect under low O_2 conditions or in C_4 species that suppress photorespiration (e.g. Ishii and Murata, 1978). The observation that the Kok effect is present under high CO_2 but absent under low O_2 (Sharp *et al.*, 1984) means that a possible decrease in the ratio of photorespiration to photosynthesis with decreasing irradiance has little relevance to the Kok effect. The present data also showed that the Kok effect occurred at 2% O_2 or in C_4, albeit to a lesser extent compared with 21% O_2 or C_3 crops (Fig. 2), and that the Kok effect did not disappear when values of A were plotted against $I_{inc}\Phi_2/4$ (Fig. 3). A new analytical model hypothesizing that the oxidative pentose phosphate pathway is progressively inhibited by the light-driven increase in thylakoid reducing power can reproduce the abrupt transition point of the Kok effect (Buckley and Adams, 2011). Direct measurements of R_d (with procedures from, for example, Haupt-Herting *et al.*, 2001; Loreto *et al.*, 2001; Pinelli and Loreto, 2003; Pärnik and Keerberg, 2007), combined with a model analysis, might help to understand fully the inter-entangling of the Kok effect, light inhibition of R_d, and photorespiration, and to verify the estimates of R_d by the indirect methods evaluated in this study.

Acknowledgements

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