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 (RD) 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 RD indirectly from combined GE and chlorophyll fluorescence (CF) measurements across a range of low irradiances. Here this method is tested for estimating RD in five C3 and one C4 crop species. Values estimated by this new method agreed with those by the Laisk method for the C3 species. The Laisk method, however, is only valid for C3 species and requires measurements at very low CO2 levels. In contrast, the new method can be applied to both C3 and C4 plants and at any CO2 level. The RD 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 RD 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 CO2 release in the light, also known as ‘day respiration’ (RD; Azcon-Bieto et al., 1981), is an important parameter in modelling net rate of leaf photosynthesis. Unlike the respiratory CO2 release in the dark (RDk), RD 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 RD 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; Parnik and Keerberg, 2007). For leaf ecophysiological studies, usually RD 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 CO2 concentration (C𝑖) 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 CO₂ level, and might be used to examine whether or not \( R_d \) varies with a change of the CO₂ 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 CO₂ level (\( C_{ir} \)) at which the rate of CO₂ fixation by photosynthesis equals the rate of CO₂ release from photorespiration. At this \( C_{ir} \) (i.e. \( C_{ir} \)-based CO₂ compensation point in the absence of \( R_d \)), all of the fixed CO₂ is consumed in photorespiration, and the rate of CO₂ release should represent \( R_d \). The values of \( C_{ir} \) 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 CO₂ levels and are therefore under far from normal environmental conditions, especially given that a change in \( R_d \) with CO₂ 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 (\( \Phi_e \)) 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 CO₂/O₂ 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 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 C₃ 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 \( \Phi_e \) 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:

\[
A = J_2 \left( 1 - f_{\text{pseudo}} \right) \frac{C_e - \Gamma_s}{4(C_e + 2\Gamma_s)} - R_d
\]

where \( J_2 \) is the total rate of \( e^- \) transport passing PSII, \( f_{\text{pseudo}} \) and \( f_{\text{cyt}} \) represent fractions of the total \( e^- \) flux passing PSII that follow cyclic and pseudocyclic pathways, respectively, \( C_e \) is the CO2 level at the carboxylation sites of Rubisco, and \( \Gamma_s \) is the \( e^- \)-based CO2 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:

\[
A = J - \frac{C_e - \Gamma_s}{4(C_e + 2\Gamma_s)} - R_d
\]

where \( J \) is the PSII \( e^- \) transport rate that is used for CO2 fixation and photorespiration.

By definition, the variable \( J_2 \) in Equation (1) can be replaced by \( \rho Cb_{\text{inc}} \Phi_s \), where \( b_{\text{inc}} \) is the level of incident irradiance, \( \beta \) is the absorptance by leaf photosynthetic pigments, and \( \rho_c \) is the fraction of absorbed irradiance partitioned to PSII. Substituting this term into Equation (1) gives:

\[
A = \rho Cb_{\text{inc}} \Phi_s \left( 1 - f_{\text{pseudo}} \right) \frac{C_e - \Gamma_s}{4(C_e + 2\Gamma_s)} - R_d
\]

For non-photorespiratory conditions where \( C_e \) approaches infinity and/or \( \Gamma_s \) approaches zero, Equation (3) becomes:

\[
A = \rho Cb_{\text{inc}} \Phi_s \left( 1 - f_{\text{pseudo}} \right) / 4 - R_d = \left( I_{\text{inc}} \Phi_s / 4 \right) - R_d
\]

where the lumped transport parameter \( = \rho Cb_{\text{inc}} \Phi_s / 4 - R_d \), in which \( \Phi_s \) is based on CF measurements. The slope of the regression will yield the estimate of a lumped parameter \( \rho \), 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 irradiiances, yet above the Kok break point if the Kok effect occurs. However, the Kok method has an additional assumption that \( \Phi_s \) is constant within the range of limiting lights. As will be shown later, this assumption is not true.

Assuming the variation of the term \( (C_e - \Gamma_s)/(C_e + 2\Gamma_s) \) in Equation (3) is negligible across an \( A-\text{I}_{\text{inc}} \) 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 \( \text{I}_{\text{inc}} \Phi_s \) 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 CO2 level across an \( A-\text{I}_{\text{inc}} \) 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 \( g_{\text{mv}} \), 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 \( g_{\text{mv}} \) in addition to the estimation of \( \Gamma_s \). No correction was therefore made in using the CF method for the purpose of simplicity.

Plant material and measurements

Five C3 crop species, wheat (cv. L’Avett), rice (Oryza sativa, cv. ‘IR64’), potato (Solanum tuberosum, cv. ‘Bintje’), tomato (an indred line from a cross between Solanum lycoceastrum cv. ‘Moneyberg’ and Solanum chmielewskii), and rose (Rosa hybrida cv. ‘Akito’), and one C4 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 glasshouse [CO2] 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 cm² chamber) were used. While the Laisk and Kok methods require only GE measurements, data would be collected by using the larger 6 cm² chamber to reduce GE measurement noises. However, for comparison of the three methods, all the data were collected using the 2 cm² chamber. All measurements were carried out at a leaf temperature of 25°C and a leaf-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 CO2 level (\( C_i \)) was increased step-wise from 50 μmol mol⁻¹ up to a maximum of 150 μmol mol⁻¹ in six steps while keeping \( I_{\text{inc}} \) 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 \( I_{\text{inc}} \) response curves as required by the Kok method and the new method, \( I_{\text{inc}} \) was in a series 1, 5, 10, 15, 20, 30, 50, 70, 100, 150, and 200 μmol mol⁻¹, while keeping \( C_i \) at 350 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 CO2 was controlled. Extra SON-T light was switched on when solar light pulse). The apparent PSII \( e^- \) transport efficiency was calculated as:

\[
\Delta F/F_m = \frac{F_{m(\text{act})} - F_{m(\text{inc})}}{F_{m(\text{act})}}
\]

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The value of \( \Delta F/F_m \) was measured 15–20 min after leaves had been placed in darkness. For measurements at each irradiance or CO2 step, \( A \) was allowed to reach steady state, after which \( F_i \) (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:

\[
\frac{\Delta F/F_m}{\text{inc.(in the presence of a true PSII e- transport efficiency, \( \Phi_s \), because the ratio \( \Delta F/F_m \Phi_s \), 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 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 $A$–$I_{inc}$ $\Phi_m$/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 $A$–$I_{inc}$ $\Phi_m$/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 smaller, were also identified at 2% O$_2$ and in maize. This abrupt change of the slope value was clearly shown in the $A$–$I_{inc}$ $\Phi_m$/2 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 $\Phi_2$ 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 $\Phi_2$ by analysing the $A$–$I_{inc}$ $\Phi_m$/4 relationships (Fig. 3). For this reason, the $R_d$ values estimated by the CF method were consistently higher.
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 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \) for rice to 1.52 \( \text{mol} \text{ m}^{-2} \text{s}^{-1} \) for potato (Table 1). For the common 21% \( O_2 \), the overall trend for the variation of \( R_d \) among the C3 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 method was mostly lower (Fig. 5) and, on average, was \( \sim 87\% \) 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
A intercept value at the respiration rate in darkness $R_{dk}$ for by the new CF method (filled circles), compared with the estimates relationship.

less apparent (Figs 2, 3, 6). As expected, 2% O$_2$ (compared level, a change of the slope for the Kok effect was relatively
and 2% O$_2$ levels were implemented for some crops; so for For the Kok method and the new CF method, both 21%
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 for the C$_3$ species rice and potato (Table 1).

In terms of the estimates of $R_{dk}$, there was no apparent difference between the two O$_2$ levels for the C$_3$ species rice and potato (Table 1). However, $R_{dk}$ did not differ significantly ($P > 0.10$) between increasing and decreasing irradiance series.

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.

Effect of O$_2$, and comparison between $R_d$ and $R_{dk}$

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important parameter probably also because it generates an estimate of another Apel, 2001; Priault where regression lines to the zero virtually overlaps with the solid line. The extrapolation of these (filled circles) and decreasing (open circles) light series, respec-
tively. The dotted regression line is invisible in (b) because it virtu-
ally overlaps with the solid line. The extrapolation of these regression lines to the zero $I_{inc}/4$ 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 $C_{i\ast}$. 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 $C_i=\Gamma_i$ (equivalently when $C_i=C_{i\ast}$). So, strictly speaking, one must use $\Gamma_i$, instead of $C_{i\ast}$, in the Laisk method, although few have done so because $\Gamma$ and $C_{i\ast}$ 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 CO2-concentrating mechanism plays such an important role in determining the $C_4$ photosyn-	hetic rate at low CO2 levels, the simple Equation (1) or (2), valid for $C_3$ photosynthesis, does not suit for $C_4$ photosyn-
thesis. 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 CO2 concentra-
tions, far below normal ambient CO2 levels (Villar et al., 1994, 1995). When a large gradient exists between the set CO2 concentration and that in the ambient air, it is hard to avoid CO2 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 CO2 concentration (e.g. Villar et al., 1994; Atkin et al., 2000), seemingly explained by CO2 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 CO2 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 CO2 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 CO2 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 CO2 assimilation ($\Phi_{CO2}$) 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 \( \beta \) and \( \rho_2 \), \( \Phi_2 \) does not vary with \( I_{\text{inc}} \) within the used data range. However, data from CF measurements reveal that the loss of \( \Phi_2 \), 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 \( A - I_{\text{inc}} \) 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 \( \Phi_2 \) over low irradiances, thereby accounting for any pitfall caused by possible non-linearity, undetectable by visual or statistical inspection (Fig. 2), between \( A \) and \( I_{\text{inc}} \) 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 \( \Phi_{\text{CO}_2} \) 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 \( \Phi_{\text{CO}_2} \) 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 \( \Phi_{\text{CO}_2} \) 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 have to be obtained from a small (e.g. 2 cm\(^2\)) leaf chamber. |
| 1. Data used could be obtained from a large (e.g. 6 cm\(^2\)) leaf chamber. | 1. Low \( C_3 \) 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 \( \text{CO}_2 \) 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. |
| **The Kok method** | 4. The method is sensitive to errors of the system in measuring transpiration that affects \( C_i \). |
| 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 \( \Phi_2 \) 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 \( \text{CO}_2 \) levels for normal plant growth; so it is possible that no correction for \( \text{CO}_2 \) leakage during measurement is required. | 3. Theoretically, the method works best for the non-photorrespiratory condition. |
| 4. The method provides additional estimate for \( \Phi_{\text{CO}_2} \). | **The new CF method** |
| 5. The method is insensitive to errors in measuring transpiration. | 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). |
| 6. The method could be used to check if \( R_d \) varies with \( \text{CO}_2 \) levels. | 2. Generally low irradiance levels are used, which may not represent the light level for normal plant growth. |
| **The new CF method** | 3. Theoretically, the method works best for the non-photorrespiratory condition. |
| 1. Using CF information, the method corrects for the error of the Kok method assuming a constant \( \Phi_2 \) 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 \( \text{CO}_2 \) levels for normal plant growth; so it is possible that no correction for \( \text{CO}_2 \) leakage during measurement is required. | 3. Theoretically, the method works best for the non-photorrespiratory condition. |
| 4. The method provides additional estimate for parameter \( s \), that lumps a number of useful physiological parameters (see text). | 4. The method is sensitive to errors of the system in measuring transpiration that affects \( C_i \). |
| 5. The method is insensitive to errors in measuring transpiration. | 1. The method is based on the assumption that \( \Phi_2 \) is constant within used irradiances, which is highly unlikely; as a result, it may underestimate \( R_d \). |
| 6. The method could be used to check if \( R_d \) varies with \( \text{CO}_2 \) levels. | 2. Low irradiance levels have to be used, which may not represent the light level for normal plant growth. |

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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 $C_a$, 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 C3 crops, and not higher in C3 than in C4 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

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 C4 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 C4, albeit to a lesser extent compared with 21% $O_2$ or C3 crops (Fig. 2), and that the Kok effect did not disappear when values of $A$ were plotted against $I_{inc}$/$O_2$/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; Parnik 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.

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