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Comparison of Methods to Estimate Dark Respiration in the Light in Leaves of Two Woody Species[1]

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Dark respiration in the light was estimated in leaves of two woody species (*Heteromeles arbutifolia* Ait. and *Lepechinia fragans* Greene) using two different approaches based on gas-exchange techniques: the Kok method and the Laisk method. In all cases, dark respiration in the light was lower ($P < 0.05$) than respiration in darkness, indicating that dark respiration was inhibited in the light. Rates of dark respiration in the light estimated by the Laisk method were 52% higher ($P < 0.05$) than those estimated by the Kok method. Differences between the methods could be explained by the low ambient CO_2 concentrations required by the Laisk approach. The mean value of the inhibition of respiration by light for the two species, corrected for the ambient CO_2 concentration effect, was 55%. Despite the differences in leaf characteristics between the species, values of the CO_2 photocompensation point, at which the rate of photosynthetic CO_2 uptake equaled that of photorespiratory CO_2 evolution, were very constant, suggesting an excellent consistency in the results obtained with the Laisk approach.

In most studies of the carbon balance of plants or plant organs it is assumed that dark respiration in the light continues at the same rate as in darkness. However, there is evidence that light inhibits dark respiration in photosynthetic tissue (Kok, 1948; Sharp et al., 1984; Kirschbaum and Farquhar, 1987). This inhibition appears to be caused by metabolites from photosynthesis (ATP, NADPH) acting on the respiratory enzymes as respiratory regulators (Graham, 1980; McCashing et al., 1988). However, the mechanism of inhibition is not clear and appears to be complex (Gardeström and Wigge, 1988; Krömer and Heldt, 1991).

The instantaneous evolution of CO_2 in the light is a result of at least four processes, which take place at different rates: photosynthesis, photorespiration, dark respiration in the light (R_d), and refixing of CO_2 from respiration (Graham, 1980). Thus, techniques for estimating R_d are complicated.

To estimate R_d by analysis of gas exchange, two main methods are used. The first, described by Kok (1948), analyzes the response of **A** to light at low intensities. The response is linear at low levels of irradiation, but near the light compensation point there is a break in the linear response, increasing markedly the slope of the light curve due to a decrease in **A**. This has been interpreted as a result of an increase in the respiration rate due to a progressive disappearance of the light-induced inhibition of dark respiration (Kok, 1948; Sharp et al., 1984). Extrapolation of the linear section of the light curve before the change of slope to a light intensity of zero gives an estimate of R_d.

One of the main pitfalls of this method is that the decrease of PPFD during the construction of the light curves induces a gradual increase of c_i and in turn a relative increase in the value of **A**. As a result, the slope of the regression of **A** versus PPFD decreases. This underestimates the true value of R_d, yielding an R_d^a. To cope with this problem in the estimation of the true value of R_d, we corrected the value of R_d^a following the approach of Kirschbaum and Farquhar (1987). With this approach, the value of R_d^a obtained from the light-assimilation curve is corrected by considering the relationships between both the rate of ribulose-1,5-bisphosphate regeneration and the **A** with the PPFD over the range of the measurement and by assuming that the dark respiration in the light is independent of both c_i and PPFD.

The second method, described by Laisk (1977) and extended by Brooks and Farquhar (1985), analyzes **A** at low c_i values and varying light intensities. **A** can be expressed as:

$$\mathbf{A} = v_c - 0.5\ v_o - R_d \qquad (1)$$

where v_c and v_o are the rate of carboxylation and oxygenation, respectively. The CO_2 concentration at which CO_2 uptake from carboxylation and CO_2 loss from photorespiration are equal is that at which $v_c = 0.5\ v_o$. This CO_2 concentration has been called Γ_*, which is related to the CO_2/O_2 specificity of Rubisco. From Farquhar and von Caemmerer (1981), Equation 1 becomes:

$$\mathbf{A} = v_c\ (1 - \Gamma_*/[CO_2]) - R_d \qquad (2)$$

Equation 2 shows that, at a CO_2 concentration equal to Γ_*, **A** is equal to $-R_d$. Analysis by the Laisk method is aimed at

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Abbreviations: **A**, net CO_2 assimilation rate; c_i, intercellular CO_2 concentration; Γ_*, CO_2 photocompensation point at which photosynthetic CO_2 uptake equals photorespiratory CO_2 evolution; R_d, rate of dark respiration in the light; R_d^a, apparent value of R_d; R_n, rate of respiration in darkness.

finding the internal concentration of CO_2 (Γ_*) at which the rate of photosynthesis equals that of photorespiration. Given that at this internal CO_2 concentration all of the CO_2 from photorespiration is consumed in the photosynthetic process, the rate of CO_2 evolution under these conditions represents R_d. The main disadvantage of this method is that the experiments must be performed at very low CO_2 concentrations and are therefore under far from normal environmental conditions. In addition, this method assumes that the CO_2/O_2 specificity of Rubisco and R_d are independent of the light intensity. However, changes in the value of R_d with light intensity have been reported (Brooks and Farquhar, 1985).

Because these methods are based on different approaches and use different experimental conditions, it may be presumed that they provide different estimates of R_d. As far as we know, there is no work comparing the results obtained by the two methods for analysis of the same plant material. The objective of this study was to compare the results of R_d obtained by these two methods.

MATERIALS AND METHODS

Plant Material

Two woody species, typical of the Californian chaparral, were chosen for the study: *Heteromeles arbutifolia* (Ait.), an evergreen shrub, and *Lepechinia fragans* (Greene), a deciduous shrub. Two-year-old plants were grown in pots in an experimental garden. During the 2 months previous to the experiments, temperature ranged from 20 to 25°C during the day and from 10 to 15°C at night, and the PPFD at midday was close to 600 μmol photons m^{-2} s^{-1}. For the analysis, well-developed leaves with similar characteristics (age, size, aspect, and specific leaf area) of each species were used.

Gas-Exchange System

Gas exchange in leaves was measured with an open gas-exchange system as described by Held et al. (1991). Before the experiments, the IRGA (Analytical Development Co., Hoddesdon, United Kingdom; model 225 MK3) was calibrated at the low CO_2 concentrations used in the present study, as recommended by Bloom et al. (1980). IRGA sensitivity was constant over the range of CO_2 concentrations used. The CO_2 concentration was measured in dry air by using a water trap filled with anhydrous magnesium perchlorate. The desired air humidity was obtained by mixing humidified and dry air. Air humidity was measured with RH sensors (Weathermeasure Corp., Sacramento, CA). The vapor pressure deficit during the experiments was approximately 1 kPa. The boundary layer conductance of the chamber was 2 mol m^{-2} s^{-1}. The light source was a metal halide lamp (Sylvania GTE, Danvers, MA) suspended above the chamber. A tray with circulating water was placed between the lamp and the chamber to absorb IR radiation. Different PPFDs were obtained by covering the chamber with nylon filters of different extinction coefficients. The incident PPFD was measured inside the chamber with a gallium arsenide photocell (Hamamatsu, San Jose, CA) previously calibrated with a LI-190S sensor (Lambda Instruments, Lincoln, NE).

Gas-exchange calculations were made according to Ball (1987), and photosynthetic and respiration rates were expressed per unit leaf dry weight. In the calculations, cuticular conductances were assumed to be zero because of the thick cuticle of leaves of the Mediterranean woody species (Lillis, 1991). All measurements were made on individual leaves attached to the plant at a leaf temperature of 20°C. The experiments were started at the same time each day (early in the morning) to avoid variations in the respiration rate due to changes in the concentration of carbohydrates (Azcón-Bieto et al., 1983).

Kok Method

A values were obtained at low PPFD, decreasing in steps from 40 to 0 μmol photons m^{-2} s^{-1}. The partial pressure of O_2 and CO_2 were 21 kPa and 35 Pa, respectively. For each PPFD, the leaf was allowed to stabilize for 1 h to reach a constant value of **A**. The photosynthetic rates recorded before the change in the slope of the light curve were regressed over the corresponding PPFD. The intercept of the regression line with the ordinate (light intensity zero) gave R_d^a. For each leaf, R_d^a was adjusted to a constant c_i using the program reported by Kirschbaum and Farquhar (1987) to obtain a corrected value of R_d.

R_n was measured at the beginning and end of each experiment by covering the chamber with a black cloth at an ambient CO_2 concentration of 350 μL L^{-1}. In most cases, it was unnecessary to wait more than 45 min to achieve stable R_n values. Measurements of **A** at low PPFDs are presented only in those cases in which R_n was similar at the beginning and end of the experiment, as recommended by Sharp et al. (1984).

Laisk Method

On the day following the Kok experiments, R_d was estimated on the same leaves by the method of Laisk (1977). For each leaf, the experiments were repeated at three different low PPFDs (40, 75, and 145 μmol photons m^{-2} s^{-1}). After PPFD had been selected, **A** values were measured at decreasing c_i values (usually in the range 90–30 μL L^{-1}). For each PPFD the linear regression of **A** over c_i was calculated. At the three light intensities considered, the linear regressions crossed at one point where the rate of photosynthesis equals that of photorespiration. The coordinates of this point of **A** and c_i represent R_d and Γ_*, respectively, and were found graphically.

Effect of Light Intensity on R_d

To explore the effect of light intensity on the value of R_d, the Laisk method was used for a medium-aged leaf of *H. arbutifolia*. However, in the present case, instead of a set of only three different low PPFDs (see the above paragraph), a wide array of PPFDs (550, 410, 300, 140, 75, 40, 20, and 9 μmol photons m^{-2} s^{-1}) was considered in the analysis. For each of the selected PPFDs, A at different low c_i values was estimated, and the linear regressions of A versus c_i were calculated. Finally, for each neighboring pair of regression lines, their intersection point (Γ_*, R_d) was determined analyt-

ically and the value of R_d was retained. This procedure gave seven values of R_d, corresponding to the pairs of PPFD: 550–410, 410–300, 300–140, 140–75, 75–40, 40–20, and 20–9 μmol photons m^{-2} s^{-1}.

Effect of CO_2 Concentration on R_n

To analyze the effect of low ambient CO_2 concentration on respiration, R_n was measured in a set of leaves similar to those used in the previous experiments. A total of seven leaves was considered. Leaf temperature was kept at 20°C, and R_n was estimated first at an ambient CO_2 concentration of 350 μL L^{-1} after a stabilization period of 45 min. Then ambient CO_2 concentration in the cuvette was changed to 35 μL L^{-1}, and the respiration rate was estimated after allowing the leaf to stabilize for 1 h.

RESULTS

R_d

Figures 1 and 2 show an example of the results obtained by both methods with a leaf of *L. fragans*. In the case of the Kok method (Fig. 1), there was a change in slope of the straight line at approximately 5 μmol photons m^{-2} s^{-1}. The intercept of the regression line (open symbols) with the ordinate represents R_d[a]. That value was 1.73 nmol CO_2 g^{-1} s^{-1} (arrow in the figure) for the leaf considered in the example.

Figure 2 shows the results obtained with the same leaf using the Laisk method. The regression lines of **A** over c_i at different PPFDs intercepted at one point, having the coordinates Γ_* and R_d, where R_d equals 2.87 nmol CO_2 g^{-1} s^{-1} (arrow in the figure).

In the two species the average values of R_d[a] by the Kok method were lower (about 28%, $P < 0.05$, Student's t test) than those corrected following the method of Kirschbaum and Farquhar (1987) (Table I). The mean values of R_d for *H. arbutifolia* obtained by the methods of Kok (after correcting for the c_i effect) and Laisk were 2.27 and 3.06 nmol CO_2 g^{-1} s^{-1}, respectively, and for *L. fragans* they were 2.49 and 3.79 nmol CO_2 g^{-1} s^{-1}, respectively (Table I).

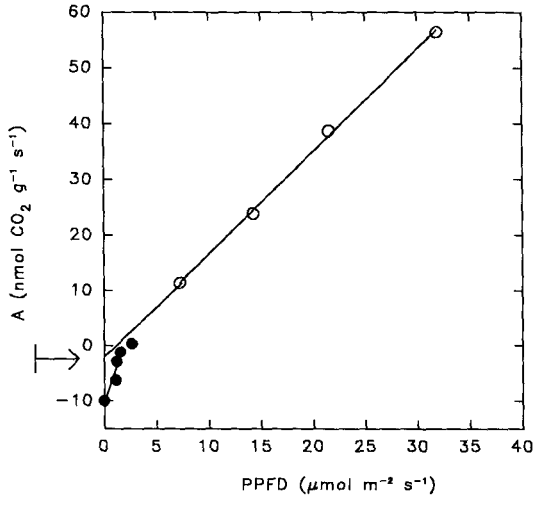

Figure 1. **A** at limiting PPFDs of incident PAR (method of Kok) for a leaf of *L. fragans*. Lines represent linear regressions before (○) and after (●) the inflection point of the light curve. The arrow indicates the value of R_d[a].

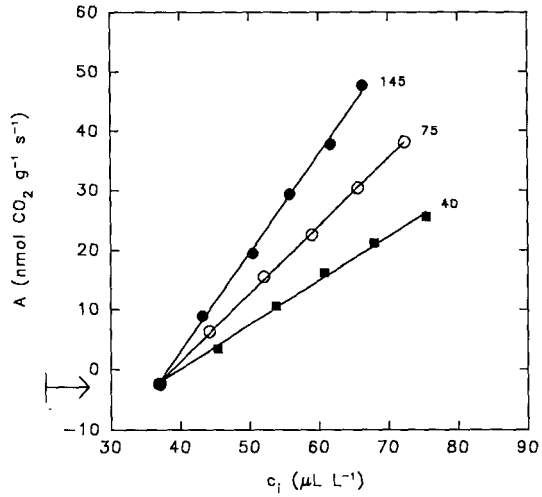

Figure 2. **A** as a function of c_i (μL L^{-1}) (Laisk method) for the same leaf as in Figure 1. Numbers in the figure indicate the incident PPFD under which measurements were made (μmol photons m^{-2} s^{-1}). Lines represent linear regressions at these PPFDs. The arrow indicates the value of R_d for that particular leaf.

Within each species, and comparing individual leaves, the values of R_d obtained by the Laisk method were always higher (about 52%, $P < 0.01$, Student's t test) than those obtained by the Kok method. In contrast, for each particular method, there were no significant differences between the mean values of R_d of the two species studied (Student's t test). Finally, it should be noted that the values of Γ_* were similar in the leaves of the two species (Table I).

Effect of Light Intensity on R_d

Figure 3 shows the relationship between the measured values of R_d at different PPFDs. The relationship was linear ($r = -0.84$, $P < 0.05$) and was defined by the equation:

$$R_d = 2.3521 - 3.9568 \times 10^{-3} \times I \qquad (3)$$

where I is the PPFD.

This equation can be used for predicting R_d at different PPFDs, and it shows that the value of R_d decreases from 2.05 nmol CO_2 g^{-1} s^{-1} at 75 μmol photons m^{-2} s^{-1} (the average PPFD used in the Laisk experiments) to values near zero at PPFD of approximately 500 μmol photons m^{-2} s^{-1}.

The average PPFD values used in the Kok and the Laisk experiments in this study were 20 and 75 μmol photons m^{-2} s^{-1}, respectively. For those values, Equation 3 predicts the values of R_d of 2.27 and 2.05 nmol CO_2 g^{-1} s^{-1}, respectively, for the leaf of *H. arbutifolia*. These values are quite close (difference less than 10%).

Table I. *Mean values of R_d (methods of Kok and Laisk), R_n, and Γ_* in the two species*

In the case of the Kok method, R_d^a is before any correction for c_i and R_d is after this correction. See the text for explanation.

	R_d			R_n	Γ_*
	Kok		Laisk		
	R_d^a	R_d			
	nmol CO_2 g^{-1} s^{-1}				μL L^{-1} CO_2
H. arbutifolia					
Mean	1.63	2.27	3.06	4.40	35.7
SD (n = 4)	±0.68	±0.67	±0.74	±1.28	±0.8
L. fragans					
Mean	1.80	2.49	3.79	6.25	35.3
SD (n = 5)	±0.44	±0.76	±0.78	±2.09	±1.7

R_n

Table I shows the values of R_n in the leaves of the two species in a normal CO_2 atmosphere (350 μL L^{-1}). No significant differences were detected (Student's t test) between the mean values of R_n of the two species studied. The mean values of R_n in *H. arbutifolia* (4.40 nmol CO_2 g^{-1} s^{-1}) and *L. fragans* (6.25 nmol CO_2 g^{-1} s^{-1}) were higher than the values of R_d. There were significant differences between R_d obtained by the Kok method and R_n ($P < 0.05$, Student's t test).

Figure 4 shows the relationship between the measured values of R_n at ambient concentrations of CO_2 of 35 and 350 μL L^{-1}. The relationship was linear ($r = 0.95$, $P < 0.01$) and was defined by the equation:

$$R_n = -0.17724 + 0.69639 \times R_{n\,35} \quad (4)$$

where R_n and $R_{n\,35}$ were estimated at ambient CO_2 concentrations of 350 and 35 μL L^{-1}, respectively.

This equation indicated that R_n at 350 μL L^{-1} CO_2 was lower (approximately 30%) than that at 35 μL L^{-1} CO_2.

DISCUSSION

In spite of the differences between the leaves of the two species, i.e. chemical composition, specific leaf area, leaf duration (Merino et al., 1984), the mean values of R_d for these two species were similar (Table I). However, there were strong, consistent differences depending on the method used to determine R_d. The method of Kok, used more commonly to separate respiration in light from that in darkness (Kok, 1948; Sharp et al., 1984; Kirschbaum and Farquhar, 1987), is more direct than that of Laisk.

It must be pointed out that, in spite of the laborious procedure of the Laisk method, the values of Γ_* are practically identical in all leaves measured. This is as expected, since the value of Γ_* is dependent mainly on the kinetic characteristics of Rubisco, which should be similar in leaves of the same plant population and possibly in leaves of species of similar ecology (Jordan and Ogren, 1981). In fact, the average values

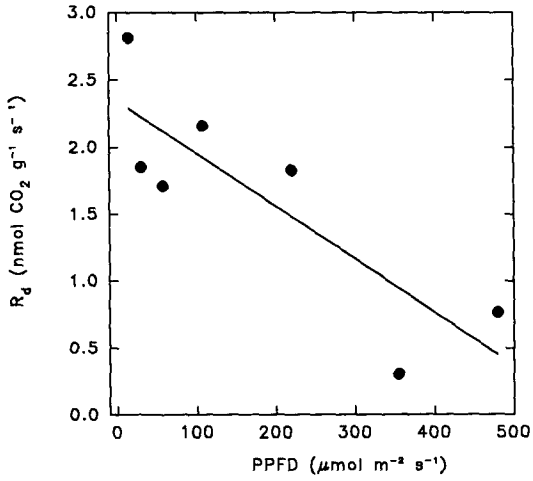

Figure 3. Relationship between R_d and incident PPFD in *H. arbutifolia*. The line represents the regression $y = 2.3521 - 3.9568 \times 10^{-3} \times x$ ($r = 0.84$, $P < 0.05$).

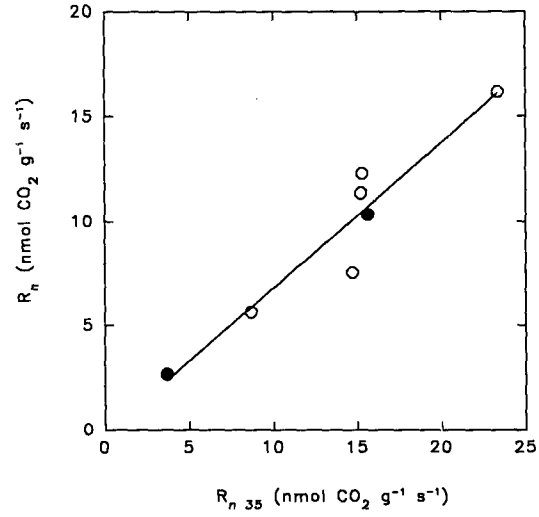

Figure 4. Relationship between R_n measured at 350 and that at 35 μL L^{-1} CO_2 concentration in air (R_n and $R_{n\,35}$, respectively) in *H. arbutifolia* (●) and *L. fragans* (○) leaves. The line represents the regression $R_n = -0.17724 + 0.69639 \times Rn_{35}$ ($r = 0.95$, $P < 0.01$).

of Γ_* found for the two species considered (35.7 and 35.3 μL L^{-1} CO_2) are similar to the average value (35.5 μL L^{-1} CO_2) found by Brooks and Farquhar (1985) for leaves of *Spinacia oleracea* in experiments conducted at the same temperature (20°C) as in the present study.

All of the above underline the high uniformity in the specificity of Rubisco and, in addition, indicate a high consistency in the results yielded by the Laisk method. The agreement between the published values of Γ_* and those found in the present study suggests the existence of no significant errors in the application of the method.

The question is raised as to the reason for the higher R_d values obtained by the Laisk method (Table I). The difference observed between the average values of R_d obtained by the methods of Kok and Laisk (Table I) may not be explained as a consequence of the different average light intensity during the experiments (20 and 75 μmol photons m^{-2} s^{-1}, respectively), since the values of R_d predicted for these light intensities using Equation 3 were very close (2.27 and 2.05 nmol CO_2 g^{-1} s^{-1}, a difference of less than 10%).

The high values of R_d obtained by the method of Laisk for the two species (Table I) may be related to the sensitivity of respiratory processes to the low concentration of CO_2 used for this experimental procedure. In fact, the results showed an obvious depressing effect of an increase in the ambient CO_2 concentration on R_n (Fig. 4). Such a depressing effect has been reported in other studies (Gifford et al., 1985; Reuveni and Gale, 1985; Amthor et al., 1992) and has been explained by CO_2 acting as an inhibitor of certain enzymes (e.g. succinate dehydrogenase; Shaish et al., 1989). However, other authors (Kirschbaum and Farquhar, 1987) did not find any response of R_d to an ambient CO_2 concentration increase.

Unfortunately, the experimental conditions required for the Laisk method do not allow direct exploration of the effect of CO_2 concentration on the value of R_d. Although the mechanism of the action of CO_2 on the respiration rate is not well known, there is no reason to suppose that the responses of R_n and R_d to CO_2 concentration will be different. In principle, the respiratory processes in the light do not seem essentially different from those taking place in darkness, since the physiological pathways are the same and the main difference appears to be related to the degree of activity of the enzymes involved (Graham, 1980). The similarity of the responses of R_d and R_n to a series of environmental factors (Brooks and Farquhar, 1985) agrees with this supposition.

If this is the case, the relationship between R_d estimated at ambient CO_2 concentrations of 35 and 350 μL L^{-1} should be similar to that for R_n at these same concentrations (Fig. 4) and expressed by Equation 4. Values of 1.96 and 2.46 nmol CO_2 g^{-1} s^{-1} are obtained for *H. arbutifolia* and *L. fragans*, respectively, using this equation to estimate the mean values of R_d (by the Laisk method) expected at an ambient CO_2 concentration of 350 μL L^{-1}. These new values for the Laisk method are closer to the values of R_d obtained by the Kok method in these two species (2.27 and 2.49 nmol CO_2 g^{-1} s^{-1}, respectively; Table I), making the differences between methods nonsignificant.

These results show that the R_d values in the two species are significantly lower ($P < 0.05$) than the R_n values (Table I). This indicates that dark respiration is partially inhibited in the light in the leaves of the two species. The average value for the degree of inhibition of dark respiration by light for the two species is approximately 55% (54% by the Kok method and 56% by the Laisk method). Results of other authors (Sharp et al., 1984; Brooks and Farquhar, 1985; Kirschbaum and Farquhar, 1987) show that the degree of inhibition in different species of agricultural or silvicultural interest ranges between 17 and 66%.

The nature of the inhibition may be due to the inhibiting effect of the light on the respiratory enzymes mediated by different cofactors, such as NADPH and ATP (Graham, 1980; McCashing et al., 1988). Although the ATP and reducing power generated in the photosynthetic process may follow many different pathways (Gardeström and Wigge, 1988; Krömer and Heldt, 1991), the utilization of ATP and NADPH produced directly from photosynthesis may decrease the requirements for ATP and NADH having catabolic origin and thus decrease respiration rates, resulting in a significant increase in growth efficiency (Raven, 1976).

The results show that during the day the average daytime rates of foliar respiration (R_d) in woody species can be at least 55% lower than the rates during the night (R_n) and even lower during a sunny day. This should be taken into account in the modeling of carbon balance.

In summary, the methods for estimating R_d considered in the present work are dependent on distinct factors; therefore, the results are not directly comparable when using distinct approaches. However, by correcting both **A** for c_i (Kok method) and R_d for the atmospheric CO_2 concentration (Laisk method), the agreement of the results by both methods should be quite acceptable.

The difficulty in detecting the change of slope in the light curves at low light intensities (Kok method) and the length of these experiments (usually a minimum of 5 h per leaf, in contrast to an average of 2 h in the case of the Laisk method) makes the latter more appropriate for cases in which the number of determinations is high and the R_n dependence on ambient CO_2 concentration is known.

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