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Short communication

Calibration matters: On the procedure of using the chlorophyll fluorescence method to estimate mesophyll conductance

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ABSTRACT

Estimates of mesophyll conductance (g_m), when calculated from chlorophyll fluorescence, are uncertain, especially when the photosystem II (PSII) operating efficiency is measured from the traditional single saturation pulse methodology. The multiphase flash method has recently been recommended to replace the single saturation pulse method, allowing a more reliable estimation of g_m. Also, many researchers still directly use the PSII operating efficiency to derive linear electron transport rate J (that is required to estimate g_m), without appropriate calibration using measurements under non-photorespiratory conditions. Here we demonstrate for tomato and rice that (i) using the multiphase flash method did not yield realistic estimates of g_m if no calibration was conducted; and (ii) using the single saturation pulse method still gave reasonable estimates of g_m when calibration based on the non-photorespiratory measurements was properly conducted. Therefore, conducting calibration based on data under non-photorespiratory conditions was indispensable for a reliable estimation of g_m, regardless whether the multiphase flash or the single saturation pulse method was used for measuring the PSII operating efficiency. Other issues related to the procedure of using the chlorophyll fluorescence method to estimate g_m were discussed.

1. Introduction

Mesophyll conductance (g_m) for CO_2 transfer from intercellular airspaces to carboxylating sites of Rubisco in chloroplasts has received growing attention in studying leaf photosynthesis of C_3 plants. This parameter has mainly been estimated using either the carbon isotope discrimination method (Evans et al., 1994) or the chlorophyll fluorescence method (Harley et al., 1992), although other methods have also been suggested (see reviews of Warren 2006; Flexas et al., 2008; Pons et al., 2009). The chlorophyll fluorescence method has been widely used because the technique has become routinely available in many laboratories with the advent of portable integrated fluorometer and gas exchange systems like LI-COR.

The chlorophyll fluorescence method to estimate g_m relies on an equation of the model of Farquhar et al. (1980) for the electron transport limited net rate of leaf photosynthesis (A):

$$A = \frac{C_c - \Gamma_*}{C_c + 2\Gamma_*}\frac{J}{4} - R_d \quad (1)$$

where C_c is the CO_2 level at carboxylating sites of Rubisco in chloroplasts, J is the rate of linear electron transport supporting the Calvin cycle and photorespiration, and Γ_* is the C_c-based CO_2 compensation point in the absence of day respiration (R_d), i.e. the respiratory CO_2 release in the light. Data for variable A in the equation can be measured from the gas exchange system, and variable J can be derived from the simultaneously measured photosystem II (PSII) electron transport efficiency from the integrated fluorometer ($\Delta F/F_m'$, where F_m' is the maximum fluorescence yield during a saturating pulse of light, and ΔF is the difference between F_m' and the steady-state fluorescence yield F_s, Genty et al., 1989); so, combining Eq. (1) with the equation for diffusion of CO_2 from intercellular airspaces (C_i) to chloroplast stroma: $A = g_m(C_i - C_c)$, one can solve g_m as (Harley et al., 1992):

$$g_m = \frac{A}{C_i - \frac{\Gamma_*[J/4 + 2(A + R_d)]}{J/4 - (A + R_d)}} \quad (2)$$

Values of g_m can be calculated using Eq. (2) for each C_i or incoming irradiance (I_{inc}) level, at which gas exchange and chlorophyll fluorescence data are simultaneously collected, and the average value is often considered as g_m for the range of C_i or I_{inc} involved. However, as generally recognised, the calculated g_m values from Eq. (2) are very sensitive to random measurement errors in A, C_i and $\Delta F/F_m'$ (Harley et al., 1992). Yin and Struik (2009) suggested several methods to minimise this sensitivity, and the best one is to use the equation that is obtained from solving for A from Eq. (2):

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$$A = 0.5\left\{J/4 - R_d + g_m(C_i + 2\Gamma_*) - \sqrt{\begin{array}{l}[J/4 - R_d + g_m(C_i + 2\Gamma_*)]^2 \\ - 4g_m[(C_i - \Gamma_*)J/4 - R_d(C_i + 2\Gamma_*)]\end{array}}\right\} \quad (3)$$

By fitting Eq. (3) to data obtained using a range of C_i or I_{inc}, g_m within that range of C_i or I_{inc} can be estimated, which is considerably less sensitive to measurement errors than the average value of g_m calculated using Eq. (2) for individual C_i or I_{inc} (Yin and Struik 2009).

Obviously, the accuracy of data on J has a strong influence on the estimation of g_m, regardless whether Eq. (2) or (3) is used. Values of J are calculated routinely as (Baker, 2008):

$$J = \beta\rho_2 I_{Inc}(\Delta F/F_m') \quad (4)$$

where β is absorptance by leaf photosynthetic pigments, and ρ_2 is the proportion of absorbed irradiance that is partitioned to PSII. Real values of β and ρ_2 are hard to measure and β is often approximated to total leaf absorptance as measured by a spectroradiometer and integrating sphere, assuming the absorptance by non-photosynthetic pigments is negligible. If not measured at all, 0.84 (or 0.85) and 0.5 have frequently been assumed as the default values of β and ρ_2, respectively, for healthy leaves to estimate g_m (e.g. Li et al., 2009; Adachi et al., 2013) and this assumption appears to be continuously made in the literature (He et al., 2017). However, even if these represent the real values for leaves or total leaf absorptance represents the absorptance by photosynthetic pigments, Eq. (4) may be criticised because it ignores the any occurrence of alternative electron sinks. Furthermore, it is possible that the chlorophyll fluorescence-based $\Delta F/F_m'$ does not accurately represent the true PSII electron transport efficiency of the whole leaf (Φ_2), or in other words, ξ, the ratio of Φ_2 to ($\Delta F/F_m'$), may not be equal to 1. In the presence of alternative sinks, it is the total electron flux passing PSII, J_2, that is equal to $\beta\rho_2 I_{inc}\Delta F/F_m'$, and J_2 and the linear electron flux J differ as (Yin et al., 2009):

$$J = \left(1 - \frac{f_{pseudo}}{1 - f_{cyc}}\right)J_2 \quad (5)$$

where f_{cyc} and f_{pseudo} are fractions of total electron flux passing PSI that is used as cyclic and pseudocyclic electron transport, respectively. Here, f_{pseudo} refers to the fraction allocated to all other noncyclic electron sinks than the Calvin cycle and photorespiration (like nitrite reduction, the Mehler reaction, malate export, and so on).

It is hard to accurately measure or estimate leaf-specific values of individual parameters β, ρ_2, f_{cyc} and f_{pseudo}, and ξ. To collectively account for them, a common protocol is to establish a calibration curve although only β and ρ_2 are most commonly pointed out explicitly (Valentini et al., 1995; Gilbert et al., 2012; Martins et al., 2013; Bellasio et al., 2016; Singh and Reddy 2016). The calibration involves to conduct simultaneous gas exchange and chlorophyll fluorescence measurements under non-photorespiratory conditions (e.g. using combined low O_2 and high CO_2), typically at several levels of I_{inc} yet within the range where A is electron transport limited. The obtained parameters of calibration, typically through linear regression, are then used to calculate J under either non-photorespiratory or photorespiratory conditions. To enhance the calibration accuracy, measurements for both non-photorespiratory and photorespiratory conditions are advised to be made on the same leaf spots.

Most calibration procedures implicitly assume that parameter ξ stays constant across levels of I_{inc}. Two factors are known to contribute to the difference between Φ_2 and $\Delta F/F_m'$. First, chlorophyll fluorescence measurements may not sample chloroplast populations representative of the whole leaf that gas exchange data reflect (Evans 2009). Second, estimation of F_m' by traditional single saturation pulse methodology is prone to underestimation error, which arises because complete reduction of the primary quinone acceptor in PSII may be hindered by rapid turnover of the PSII acceptor pools, even when using very high single saturation pulse intensities (Earl and Ennahli 2004). Neither factor will guarantee that the ratio of Φ_2 to ($\Delta F/F_m'$) would stay constant across levels of I_{inc}.

To improve the measuring accuracy of chlorophyll fluorescence-based PSII electron transport efficiency, Loriaux et al. (2013) reported a method, referred to as the multiphase flash method, being capable of rapidly (within 1 s) describing the irradiance dependence of F_m' and estimating F_m' at infinite irradiance. They showed that the multiphase flash method can generate more accurate and consistent estimates of g_m than the single saturation pulse method. However, they used Eq. (4) to derive the values of J for both the single saturation pulse method and the multiphase flash method. This generates two questions. First, to what extent can the single saturation pulse method still give a reasonable estimate of g_m if an appropriate calibration procedure is applied? Secondly, can the multiphase flash method also yield an erroneous estimate of g_m in the absence of calibration?

The objective of the present communication is to address these unknowns by comparing the estimated g_m based on the multiphase flash vs single saturation pulse methods, either with or without a calibration procedure. To this end, we collected combined gas exchange and chlorophyll fluorescence data for two contrasting C_3 species, i.e. tomato and rice.

2. Materials and methods

2.1. Culturing plant material

An experiment was carried out in a glasshouse at Wageningen University, using tomato (*Solanum lycopersicum*) cv. “MoneyMaker” and rice (*Oryza sativa*) cv. “IR64” in four replicates.

Tomato plants were grown in a nursery bed and seedlings were transplanted after seven days in 10 L pots containing potting soil. The initial nitrogen content in the soil was 0.66 g per pot. On a weekly basis a standard tomato nutrient solution was applied. In seven applications a total of 0.85 g N, 0.39 g P_2O_5 and 1.60 g K_2O was added per pot. The glasshouse temperature was 24 ± 3 °C during the day (for 16 h) and 18 °C during the night, the relative humidity was 40–60% and the photoperiod was kept at 14 h d^{-1}. All measurements were carried out in the seventh week after sowing (April 2015), using distal leaflets of the compound leaves that were just fully expanded, typically leaf 8 counted from below.

Rice plants were grown in small pots and seedlings were transplanted after 14 days in 7-L pots containing sandy soil. The initial nitrogen content in the soil was 0.40 g per pot. With mixing granulate fertiliser through the soil 0.50 g N, 0.50 g P_2O_5 and 0.49 g K_2O was added per pot. The rice plants were grown under submerged conditions. The glasshouse temperature was 28 ± 2 °C during the day (for 12 h) and 23 °C during the night, the relative humidity was 40–80% and the photoperiod was kept at 12 h d^{-1}. All measurements were carried out in the eighth week after sowing (May 2015) on leaves that were just fully expanded, typically leaf 10 counted from below.

About 60% of the photosynthetically active incident radiation on the greenhouse was transmitted to the plant level. During daytime supplemental light from 600 W HPS Hortilux Schréder lamps (Monster, South Holland, The Netherlands) was switched on automatically when the photon flux of the solar incident radiation dropped below 340 μmol m^{-2} s^{-1} and was switched off when it exceeded 570 μmol m^{-2} s^{-1} in the tomato greenhouse. In the rice greenhouse these thresholds were 910 μmol m^{-2} s^{-1} and 1140 μmol m^{-2} s^{-1}, respectively.

2.2. Measurements

We used the LI-COR-6400XT open gas exchange system with an integrated fluorescence chamber head enclosing 2-cm^2 areas. Fully