RESEARCH PAPER

Estimating chlorophyll content and photochemical yield of photosystem II ($\Phi_{\text{PSII}}$) using solar-induced chlorophyll fluorescence measurements at different growing stages of attached leaves

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Abstract

This paper illustrates the possibility of measuring chlorophyll (Chl) content and Chl fluorescence parameters by the solar-induced Chl fluorescence (SIF) method using the Fraunhofer line depth (FLD) principle, and compares the results with the standard measurement methods. A high-spectral resolution HR2000+ and an ordinary USB4000 spectrometer were used to measure leaf reflectance under solar and artificial light, respectively, to estimate Chl fluorescence. Using leaves of Capsicum annuum cv. ‘Sven’ (paprika), the relationships between the Chl content and the steady-state Chl fluorescence near oxygen absorption bands of O$_2$B (686 nm) and O$_2$A (760 nm), measured under artificial and solar light at different growing stages of leaves, were evaluated. The Chl fluorescence yields of $\Phi F_{686\text{nm}}/\Phi F_{760\text{nm}}$ ratios obtained from both methods correlated well with the Chl content (steady-state solar light: $R^2 = 0.73$; artificial light: $R^2 = 0.94$). The SIF method was less accurate for Chl content estimation when Chl content was high. The steady-state solar-induced Chl fluorescence yield ratio correlated very well with the artificial-light-induced one ($R^2 = 0.84$). A new methodology is then presented to estimate photochemical yield of photosystem II ($\Phi_{\text{PSII}}$) from the SIF measurements, which was verified against the standard Chl fluorescence measurement method (pulse-amplitude modulated). The high coefficient of determination ($R^2 = 0.74$) between the $\Phi_{\text{PSII}}$ of the two methods shows that photosynthesis process parameters can be successfully estimated using the presented methodology.

Key words: Chlorophyll content; different leaf age; Fraunhofer line depth principle; photochemical yield of photosystem II ($\Phi_{\text{PSII}}$); solar-induced chlorophyll fluorescence.
**Introduction**

Chlorophyll (Chl) fluorescence has been studied as a useful probe for photosynthesis research (Govindjee, 1995; Papageorgiou and Govindjee, 2004; Buschmann, 2007; Baker, 2008; Omasa et al., 2009; van der Tol et al., 2009; Kalaji et al., 2012; Murchie and Lawson, 2013). It has been extensively applied for active phenotyping remote sensing of photosynthetic functioning, and for studying biotic and abiotic stress in plants (Omasa, 1998; Omasa et al., 1987, 2007; Lichtenthaler et al., 1992; Kolber et al., 1998; Kim et al., 2001; Omasa and Takayama, 2003; Schreiber, 2004; Moya et al., 2004; Konishi et al., 2009; Pieruschka et al., 2012, 2014).

However, the common active remote sensing method might be limited to short-distance use for individual leaves and small plants. Efforts have been made to expand the application of active Chl fluorescence measurement methods for long-distance, canopy-scale studies using the laser induced fluorescence transient (LIFT) method. The LIFT approach employs low-intensity pulses instead of a saturating pulse to measure the fluorescence transient, which is interpolated to a maximum fluorescence level using a fluorescence model. The technique is capable of measuring Chl fluorescence up to a distance of 50 m but there are still challenges for more long-distance remote sensing (Kolber et al., 2005; Pieruschka et al., 2012, 2014).

Passive remote sensing of steady-state solar-induced Chl fluorescence (SIF) has more recently become available (Meroni et al., 2009). The advantages of the SIF method are that the technique does not require an artificial excitation source and it can be used on air-borne and space-borne devices for canopy and larger-scale applications. However, this technique requires instruments with high spectral resolution and high accuracy to be able to differentiate the weak fluorescence signal from the strong background light reflection (Meroni and Colombo, 2006).

SIF measurement techniques have been developed for field and air-borne applications in recent years (Plasecky et al., 1975; Moya et al., 2004; Liu et al., 2005; Corp et al., 2006; Meroni et al., 2006, 2009; Rascher et al., 2008; Liu and Cheng, 2010; Zarco-Tejada et al., 2012). The development of space-borne SIF remote sensing of GOSAT satellite has provided the opportunity to assess global terrestrial carbon cycle and has attracted a considerable amount of attention within scientific communities (Frankenberg et al., 2011; Joiner et al., 2011; Porcar-Castel et al., 2014).

Current trends of research in the SIF field demonstrate the need for further evaluations of this technique. There are intrinsic differences between the basis of active Chl fluorescence measurement methods based on pulse-amplitude modulated (PAM) measurement and that of the SIF; thus, consequent challenges are currently encountered. Further, leaf-level experimental work is needed to characterize relationships between SIF- and PAM-measured Chl fluorescence (Porcar-Castel et al., 2014). The steady-state Chl fluorescence has been shown to be partially re-absorbed by Chl (Lichtenthaler and Rinderle, 1988; Buschmann, 2007), which necessitates the evaluation of Chl fluorescence at various life stages of the plants when Chl content is variable. In addition, interest in relationships between steady-state SIF and the photosynthesis reaction at the physiology level, as well as growth primary production and the enhanced vegetation index (Frankenberg et al., 2011; Joiner et al., 2011), which are related to biomass and Chl content, is growing (Van der Tol et al., 2014).

To progress our understanding of the potential to detect SIF signals from vegetation reflectance to study photosynthesis, the main objective of this paper is to evaluate the performance of the SIF measurement method throughout the lifespan of leaves with varying Chl content through comparison with the standard Chl measurement method. This is carried out via an estimation of Chl content and photochemical yield of photosystem II (ΦPSII). Not many studies have examined the correlation between both measurement methods of Chl fluorescence on the same sample. Moya et al. (2004) found high correlation between both fluorescence measurements; however, the experiments were limited to a small range of Chl content (350–450 mg m⁻²). As far as could be determined, there is currently no report available to estimate and compare large-range Chl content or ΦPSII obtained from the two methods. In the present paper, the focus has, therefore, been on the relationships between the steady-state Chl fluorescence and Chl content near the oxygen absorption bands of O₂B (686 nm) and O₂A (760 nm), measured under artificial light and solar light at different growing stages of leaves. A new technique to estimate ΦPSII from the SIF method is also presented.

**Materials and methods**

**Plant material and growth conditions**

Paprika (Capsicum annuum cv. ‘Sven’) plants were grown in an environmentally controlled growth chamber for 10–18 weeks. The plants were illuminated for 12h each day with fluorescent lights and halogen lamps at a photosynthetic photon flux (PPF) of 400 μmol m⁻² s⁻¹. The growth chamber air temperature was 25.0°C during the day and 20.0°C at night with relative humidity of ~70%. Plants were watered daily with a nutrient solution (1:1000 dilution of HYPONeX). Fully expanded mature and aging leaves of different growth stages were used in the experiments.

**Chl fluorescence measurement under artificial light**

Comparable veinless sites of attached leaves were used to measure the steady-state artificial-light-induced Chl fluorescence. The fluorescence spectra of leaves were measured at an interval of 500 ms (integration time) with a USB4000 spectrometer (Ocean Optics, USA) under a halogen light (Sumita Optical Glass, Inc., LS-100F) through short-pass (Optical Coatings Japan, wavelength λ < 600 nm) and heat-absorbing filters in a dark room. The USB4000 spectrometer was set to the 350–850 nm range with 3648 channels, grating of 600 lines mm⁻¹.
and a 50 μm slit. It was connected to a 3 m-long fibre cosine corrector-diffuser. The instrument has 1.5–2.3 nm full width at half maximum (FWHM). The attached leaf was set at a 45° angle to the optical light beam and the fibre optic diffuser was set at 5 cm distance from the leaf surface. The fibre-optic diffuser was equipped with a long-pass filter (Optical Coatings Japan, λ > 640 nm) to block the reflected light from the leaf. The LS-1-CAL calibrated tungsten halogen NIST-traceable light source (Ocean Optics, USA) was used to calibrate the spectrometer. The leaf area was illuminated for 20 min (PPF: 150 μmol m⁻² s⁻¹), and then the steady-state Chl fluorescence was measured. Black wool paper was set at the back of the leaf to remove the reflection of light transmitted through the leaf. Chl fluorescence spectra were recorded to computer via a USB port using SpectraSuite operating software.

**Spectral radiant intensity measurement for chlorophyll content estimation under solar light using the FLD method**

After the above-mentioned artificial-light-induced measurements, the spectral radiant intensity of the same leaf area was measured at an interval of 200 ms (integration time) under solar light using a 0.035 nm FWHM HR2000+ spectrometer (Ocean Optics, USA) in the 680–770 nm range with 2048 channels. The spectrometer was built with grating H11 of 1800 lines/mm, a 5 μm slit, an L2 detector collection lens, an OF1-G590 long-pass filter, and a set of high-reflectivity AgPlus mirrors model SAG+UPGD-HR. The spectrometer was connected to a 2 m-long fibre optic, bundled 600 μm optical fibre up, with a CC-3 VIS-NIR cosine corrector-diffuser for spectral radiant intensity measurement. The attached leaf was set vertically to incident solar light after sufficient light adaptation. The fibre-optic diffuser was set at 5 cm distance from the leaf surface, and it was held at 45° to light to reduce specular reflection from the leaf surface. An 18% Gray Card (Kodak, USA) set at the same angle and position as the leaf was used as the non-fluorescent reference standard. Both the spectra of the leaf and the non-fluorescent reference were recorded to computer via a USB port using OPWave+ operating software (Ocean Optics, USA). Black wool paper was set at the back of the leaf to remove the reflection of light transmitted through the leaf. During the experiments, a LI-COR LI-250 light meter was used to measure the PPF (800–1800 μmol m⁻² s⁻¹).

**Spectral radiant intensity measurement for F_{PSII} estimation under solar light**

The attached leaf was set vertically to incident solar light using a JUNIOR-B leaf clip (Walz). The fibre-optic diffuser of the HR2000+ spectrometer was set at 5 mm distance from the leaf surface, and it was held at 45° to solar light. During the experiment, the spectral leaf radiant intensity was continuously measured at an interval of 200 ms (integration time) under solar light using the spectrometer after sufficient light adaptation. The saturation light pulse of about 5000 μmol m⁻² s⁻¹ from a red laser (KaLaser: 660 nm, 200 mW) was illuminated for 1 s at 60° to the leaf from a distance of 40 cm and at about a 30 mm diameter footprint. For the verification, the photochemical yield of photosystem II (F_{PSII}) was measured at a 1 mm distance from the leaf by JUNIOR PAM (about 10000 μmol m⁻² s⁻¹ blue LED saturation pulse for 0.6 s and measuring beam of 5 Hz) to give F_{PSII,PAM}. The spectral radiant intensity data were recorded by the above-mentioned computer system. The set of sequential experiments included (i) measurement of solar light radiant intensity reflected from a non-fluorescent reference (90% White Card), (ii) measurement of solar light radiant intensity from the attached leaf, (iii) measurement of saturation laser pulse radiant intensity from the leaf, (iv) measurement of F_{PSII,PAM} of the leaf using JUNIOR PAM, (v) measurement of solar light radiant intensity from the leaf, (vi) measurement of the second laser pulse radiant intensity from the leaf, and (vii) measurement of solar light radiant intensity reflected from the non-fluorescent reference at each interval of 20 s in (ii) to (vi) and about 60 s in (i) and (vii) under solar light. A data set with very little change in solar light intensity during the experiment was used for the calculation of F_{PSII} estimated by the Fraunhofer line depth principle (FLD), designated F_{PSII,FLD}. The solar light intensity in the experiment was 200–1600 μmol m⁻² s⁻¹.

**Solar-induced Chl fluorescence estimation using FLD**

It is difficult to quantify the SIF from the ordinary spectral leaf radiant intensity because the signal is obscured by the reflected light. However, the leaf radiant intensity in Fraunhofer lines increases the ratio of Chl fluorescence emission to reflected light in the leaf radiant intensity as compared with other wavelengths, hence the fluorescence emission can be quantified by the FLD (Lichtenthaler et al., 1992; Moya et al., 2004).

The FLD method was originally proposed by Plascecyk and Gabriel (1975), then successfully used by Meroni et al. (2006; 2008a) and Rascher et al. (2009) at the leaf level, and at a canopy level by Moya et al. (2004), Meroni et al. (2008b), and Daumard et al. (2012). At the ground level, two oxygen absorption bands of terrestrial atmosphere at 760 nm (O₂ A) and 686 nm (O₂ B) are usually used to estimate the steady-state SIF because the absorption bands include Chl fluorescence emission spectra.

Fig. 1 shows the FLD method. In Fig. 1A, B, the solid line is the non-fluorescent reference radiant intensity spectra and the dashed line is the leaf radiant intensity spectra measured in O₂ A and O₂ B bands. Mₐ and M₈ are the mean values of radiant intensity (digital numbers, which is not calibrated to exact radiance) from the non-fluorescent reference in band a (border of the well, A: W = 685.93 to 686.32 nm; B: W = 758.76 to 759.17 nm; W: the wavelength width) and in band b (bottom of the well, A: W = 686.54 to 686.93 nm; B: W = 760.24 to 760.64 nm), and Mᵦ and Mᵦ are the mean values of radiant intensity from the leaf in band c (the same W as a) and in band d (the same W as b), respectively. Following Plascecyk and Gabriel (1975) and Moya et al. (2004), the reflectance coefficient (R) and the solar-induced fluorescence intensity (Fs) were derived from the measured values of Mₐ, Mₐ, Mᵦ, and Mᵦ as follows:
Chl fluorescence yield ratio

The Chl fluorescence yield (Φf, the ratio of fluorescence photons to absorbed photons) can more accurately represent the relationship between the Chl fluorescence signal magnitude and light intensity than Chl fluorescence intensity (Govindjee, 1995). The solar-induced and artificial-light-induced Chl fluorescence yields near the 686 nm and 760 nm bands were approximately calculated by

\[
\Phi_f = F / (PPF \times 0.84)
\]

where \( F \) is the fluorescence intensity (relative units), PPF is the photosynthetic photon flux (μmol m\(^{-2}\) s\(^{-1}\)), and 0.84 is the leaf absorption coefficient. The solar-induced (Φs) and artificial-light-induced (Φc) Chl fluorescence yield ratios of ΦF686.7/ΦF760.4 and ΦF686.4/ΦF760.5 were calculated and their relationships at different Chl content levels compared. The ratio of [ΦF686.7/ΦF760.4][ΦF686.4/ΦF760.5] was then calculated to assess the steady-state SIF at different leaf-growing stages. The wavelength width (W in Fig. 1) to minimize the mean absolute error of SIF estimation was 0.4 nm.

ΦPSII.FLD estimation from solar-induced and red laser saturation pulse-induced Chl fluorescence yields

From a set of temporal changes in the above-mentioned spectral intensity measurement of (1) to (7), the changes in the SIF yield (ΦF760.4) and laser saturation pulse-induced yield (ΦFm’760.4) of the O2A band were calculated by the FLD method. ΦPSII.FLD was calculated by

\[
\Phi_{PSII. FLD} = (\Phi Fm' - \Phi Fs) / \Phi Fm'
\]

where ΦFs is a constant value after median filtering to data measured during the 10 s just before the saturation laser pulse for noise reduction, and ΦFm’ is the mean value of the highest three data points during the saturation laser pulse.

Leaf spectral absorptance

Chl fluorescence spectra largely overlap with Chl absorption, and the red fluorescence re-absorbed by Chl itself is larger than the far-red fluorescence (Govindjee, 1995; Gitelson et al. 1998). To investigate the re-absorption in Chl fluorescence spectra, the reflectance (R) and transmittance (T) of the leaf were measured using a spectrophotometer equipped with an integrating sphere (V570, JASCO, Japan) in a range of 600–800 nm and a spectral resolution of 2 nm. The reflectance spectra were measured against barium sulphate as a reference standard; black wool paper was set at the back of the leaf to remove the re-reflection of light transmitted through the leaf. Leaf spectral absorptance was calculated as 1-T-R (Buschmann et al., 1994; Gitelson et al., 1998, 2003).

Chlorophyll content

A 1.5 cm-diameter leaf disc was cut from the marked point of the leaf using a standard leaf punch. After weighing, the leaf disc was mashed in 96% ethanol solution using a mortar and a pestle. The pigments were completely separated in sedimentation tubes by a centrifugal separator set for 5 min at 2000 rpm. The absorption spectrum of the top layer solution in the tube was measured by a V570 spectrophotometer (JASCO, Japan) at 0.5 nm intervals between 600 nm and 800 nm. Total Chl content was calculated from the absorption spectra using equations reported by Wintermans and de Mots (1965).

Results

Figure 2A shows the steady-state Chl fluorescence emission spectrum of three leaves with different Chl content taken under the artificial illumination light (λ < 600 nm). Two maxima appeared in the red region around 686 nm (F686) and far-red region around 740 nm (F740). Near the red region, Chl fluorescence increase sharply with the reduction of Chl content, but near the far-red region, it increased with the increase of Chl content. The Chl absorbance near 686 nm greatly increased with increasing Chl content, whereas there was little difference near 760 nm (Fig. 2B).

Figure 3 shows relationships between leaf Chl content and steady-state Chl fluorescence yields of ΦF686.4 and ΦF760.5 under artificial light. The Chl fluorescence yields of ΦF686.4 decreased with an increase in Chl content (Fig. 3A), whereas those of ΦF760.5 increased (Fig. 3B), although the yields greatly varied at low Chl content below about 200 mg.
m$^{-2}$. At higher Chl content with more than about 400 mg m$^{-2}$, the changes in $\Phi_{F686.4}$ and $\Phi_{F760.5}$ become small.

Figure 4 shows the relationships between leaf Chl content and steady-state solar-induced Chl fluorescence yields of $\Phi_{F686.7}$ and $\Phi_{F760.4}$. The Chl fluorescence yields of $\Phi_{F686.7}$ decreased with an increase in Chl content (Fig. 4A), whereas the Chl fluorescence yields of $\Phi_{F760.4}$ increased, although the yields varied largely, especially in $\Phi_{F760.4}$ (Fig. 4B). Comparing Figs. 3 and 4, the solar-induced Chl fluorescence yield variation was larger than that induced by artificial light, probably because of a weak Chl fluorescence signal, lack of the sensitivity of the spectrometer, and solar fluctuation during the measurement.

Figure 5 shows the relationships between leaf Chl content and steady-state Chl fluorescence yield ratios calculated from Figs. 3 and 4. Under artificial light, the ratio of $\Phi_{F686.4}/\Phi_{F760.5}$ showed a very clearly inverse curvilinear relationship with the Chl content of leaves (Fig. 5A; $R^2 = 0.94$). A similar highly inverse curvilinear relationship was also obtained under solar light (Fig. 5B; $R^2 = 0.73$) regardless of the large variation shown in Fig. 4. The lower coefficient of determination value obtained for the solar light-induced yield ratio is caused by wider variation at all Chl content values. A comparison of root mean square error (RMSE) values as indicated on the figure shows a much larger RMSE for the SIF method. However, when RMSEs are calculated for Chl content values below 400 mg m$^{-2}$, the RMSEs of the two methods become closer and amount to a maximum of 40 mg m$^{-2}$ for the SIF method, indicating that the SIF method might be less accurate for higher Chl content (above 400 mg m$^{-2}$) estimation.

Figure 6 shows a linear correlation between artificial-light-induced Chl fluorescence yield ratio ($\Phi_{F686.4}/\Phi_{F760.5}$) and the solar-induced one ($\Phi_{F686.7}/\Phi_{F760.4}$) obtained at the optimal W (W = 0.4 nm). The regression line was very close to the $y = x$ line.

Figure 7 shows an example of Chl fluorescence measurement using the FLD method under solar light and saturation pulse for estimating $\Phi_{PSII}$ of the attached leaf. The temporal changes in radiant intensity measurement of (ii) to (vi)
Figure 8 shows a linear correlation between \( \Phi_{\text{PSII, PAM}} \) (measured by JUNIOR PAM) and \( \Phi_{\text{PSII, FLD}} \) (estimated by FLD method) at different growing stages of the attached leaves. The regression line was approximated by \( y = x \).

**Discussion**

Active Chl fluorescence analysis such as the PAM method to assess photosynthesis activities is the most common method employed for laboratory-scale photosynthesis studies and can provide invaluable information on the factors affecting the photosynthesis process. However, this method has limitations for large-size plants or at canopy level. For this reason, the passive remote sensing of photosynthesis can be more feasibly carried out either through SIF studies or the photochemical reflectance index (PRI) measurement introduced by Gaman et al. (1990). Although PRI has been widely used for photosynthesis measurement, recent studies have shown that PRI use may be limited by its sensitivity to plant pigment variation at various life stages of the leaves (Rahimzadeh-Bajgiran et al., 2012).

A few studies have been recently performed on the solar-induced Chl fluorescence at both laboratory scale and canopy level for plant photosynthesis measurement (Moya et al., 2004; Meroni et al., 2006; 2009; Rascher et al., 2008). Under solar light, the steady-state Chl fluorescence cannot be measured directly, because the photon emission from the canopy is less than 1% of what is emitted by the sun (Liu et al., 2005). Hence, the SIF measurement using Fraunhofer line spectra such as the FLD principle has been proposed as the only way to remotely sense Chl fluorescence in natural environments. As outlined by others (Malenovsky et al., 2009; Porcar-Castel et al., 2014; Van der Tol et al., 2014), however, there is a need for further studies relating the steady-state SIF measurements to photosynthetic performance of plants.

The result presented here show that the steady-state Chl fluorescence emission spectrum in the red peak near 686 nm strongly overlaps with the maximum of the leaf absorption spectrum near 680 nm (Fig. 2). At the red region near \( \text{O}_2\text{B} \) (686 nm), the steady-state Chl fluorescence yield decreased sharply with the increase of Chl content regardless of the type of the light source-artificial or solar (Figs. 3A and 4A). This is speculated to be due to the fact that a large part of the red Chl fluorescence is re-absorbed by the Chl pigment before it can be measured. At the far-red region near \( \text{O}_2\text{B} \) (760 nm), because of the sharp decrease of the absorption of the leaf, the Chl fluorescence yield rose with Chl content increase (Figs. 3B and 4B). The phenomenon that Chl fluorescence is strongly re-absorbed by Chl pigment near the red region more than the far-red region has previously been reported (Lichtenthaler et al., 1986; Gitelson et al., 1998; Buschmann, 2007). The present results obtained by the FLD principle using \( \text{O}_2\text{A} \) and \( \text{O}_2\text{B} \) bands followed the same phenomenon.

Under solar light, the Chl fluorescence signal largely overlapped with the high-reflection signal of incident solar light and the re-absorption bands of pigments, and it is easily affected by the instrument conditions, the intensity of exciting light, and the angle of detection. The measurement of \( \text{O}_2\text{A} \) mentioned in Materials and methods, Spectral radiant intensity measurement for \( \Phi_{\text{PSII}} \) estimation under solar light, are shown in Fig. 7A. The radiant intensity increased with the saturation pulses of 660 nm laser (SL_{RL}) and blue LED (SL_{PAM}) of JUNIOR PAM. Fig. 7B, C shows temporal changes in Chl fluorescence intensity (F760.4) calculated by the FLD method and those in Chl fluorescence yield (\( \Phi F760.4 \)) calculated by equation (3). The solid line in Fig. 7C is the sunlight fluorescence yield (\( \Phi F \)) without noise after median filtering during 10 s before the SL_{RL} illumination for estimating \( \Phi_{\text{PSII}} \). The saturation laser-induced fluorescence yield (\( \Phi Fm' \)) under solar light was larger than the yield induced by SL_{PAM}. This result might have been caused by the difference in beam footprint sizes between the HR2000+ spectrometer and JUNIOR PAM.
and O₂B reflectance under solar light is very difficult and one should be careful to provide identical illumination to the sample and the reference, because the depth of oxygen absorption bands may change easily (Zarco-Tejada et al., 2003; Moya et al., 2004; Meroni et al., 2006; Campbell et al., 2008). In this study, the reflectance of the standard reflectance panel was measured immediately after the measurement of the sample reflectance, but changes in the oxygen absorption bands leading to measurement errors are inherently inevitable. Also, under the unstable solar light intensity, the instability of photosynthetic states results in a change in the percentage of Chl fluorescence emission and Chl re-absorption capacity (Buschmann, 2007).

In practice, the Chl fluorescence intensity ratio of F685/F730, F690/F735, or F690/F740 is used to assess the steady-state artificial-light-induced Chl fluorescence signals and determine the Chl content in a non-destructive way (Agati et al., 1995; Buschmann, 2007; Campbell et al., 2008). Gitelson et al. (1998) pointed out that ≥90% of the variation in the fluorescence ratio F690/F735 is exclusively dominated by Chl content. In contrast, at the ground level, two oxygen absorption bands of terrestrial atmosphere at 686 nm (O₂B) and 760 nm (O₂A) are usually used to estimate steady-state SIF because the position of the absorption bands are very close to the position of the Chl fluorescence spectrum peaks (Moya et al., 2004; Meroni et al., 2009). Here, the Chl fluorescence yield ratio of O₂A and O₂B was used to estimate the change of steady-state Chl fluorescence with Chl content and was considered to better express the relationship between the Chl fluorescence and illumination light.

The steady-state artificial-light-induced Chl fluorescence yield ratio of ΦF₆₈₆.₄/ΦF₇₆₀.₅ and the steady-state SIF yield ratio of ΦF₆₈₆.₇/ΦF₇₆₀.₄ showed very clear inverse curvilinear correlations with Chl content with R² values of 0.94 and 0.73, respectively (Fig. 5). Moya et al. (1992) mentioned that a strong inverse relationship existed between fluorescence quantum yield and Chl content. Owing to the strong re-absorption influence of Chl pigment for Chl fluorescence near the 686 nm band, the Chl fluorescence yield ratios of ΦF₆₈₆.₄/ΦF₇₆₀.₅ and ΦF₆₈₆.₇/ΦF₇₆₀.₄ decreased with the increase of Chl content. Similar trends and shapes of the curves can be observed in Fig. 5A, B. However, the coefficients of regression lines are different. This is solely related to the lower accuracy of either measurement method to estimate Chl content at higher Chl content values. Up to a Chl content of about 300 mg m⁻², the coefficients of the power law functions are very similar with close R² values (y = 223.2x⁻⁰.₈₅₁, R² = 0.80 for Fig. 5A; y = 241.₄x⁻⁰.₈₆₇, R² = 0.79 for Fig. 5B). Once data pertaining to higher Chl content are included, the curves start to divert from each other, causing considerable differences in coefficients. This shows that both the active and passive methods used here have the same limitation at higher Chl content: under solar light, when the Chl content is very high (more than 400 mg m⁻²), the value of ΦF₆₈₆.₇/ΦF₇₆₀.₄ becomes disperse; it appears that sometimes the Chl fluorescence yield ratio is difficult to estimate because of the too-low fluorescence intensity due to the high re-absorption of Chl pigment (Fig. 5B).

At the band width of 0.4 nm, the steady-state Chl fluorescence yield ratios of the two methods were very well correlated with an R² of 0.84 (Fig. 6). The work by Moya et al. (2004) reported a high correlation between active and passive fluorescence measurements (R² = 0.99) (not the ratio, just absolute values), but this was obtained over a limited range of Chl content, eliminating the effects of re-absorption.

The saturation pulse method has been proposed as a powerful tool for assessing photosynthetic parameters (Genty et al., 1989; Bilger and Björkman, 1990; Schreiber 2004) and many useful Chl fluorescence parameters, such as ΦPSII and non-photochemical quenching, have been developed and used as indices of photosynthetic activity under solar and artificial (actic) lights. The value obtained by multiplying the PPF value of actinic light by the ΦPSII value is used as an indicator of photosynthetic electron transport rate and CO₂ fixation rate (Baker and Oxborough, 2004; Schreiber, 2004).

The PAM Chl fluorometer is the standard instrument for the measurement of the Chl fluorescence parameters using the saturation pulse method. Therefore, the Chl fluorescence parameter ΦPSII,FLD estimated using the FLD method under solar light and saturation pulse was verified with a PAM Chl fluorometer (JUNIOR PAM) according to the sequence shown in Fig. 7. Although the Chl fluorescence signal estimated by the FLD method was very noisy, the stable ΦF₇₆₀.₄ and ΦFm’₇₆₀.₄ in equation (4) for calculating ΦPSII,FLD were obtained as a constant value by the median filtering from data measured during the 10 s just before the saturation laser pulse (SLPAM) and averaging of the highest three data points during the saturation laser pulse. To assure the reliability in ΦPSII,FLD estimation, the small change in solar intensity was confirmed during a sequence from (i) to (vii) in the measurement mentioned in the Materials and methods. As a result, the ΦF₇₆₀.₄ and ΦFm’₇₆₀.₄ values were stable during double measurements by the saturation laser pulse. Though the JUNIOR PAM blue LED saturation pulse (SLPAM) during 0.6 s was illuminated on the leaf at about 10000 μmol m⁻² s⁻¹, the Chl fluorescence yield estimated by the FLD method hardly changed (Fig. 7C). This could be owing to the measured error of SLPAM (Fig. 7A), caused by the difference in beam footprint size between the HR2000+ spectrometer and JUNIOR PAM.

The ΦPSII,FLD and ΦPSII,PAM exhibited a good correlation with R² of 0.74 and RMSE of 0.08, regardless of different leaf ages and solar intensities of 200–1600 μmol m⁻² s⁻¹ (Fig. 8). The regression line was almost y = x. This result is therefore introduced as a newly developed ΦPSII measurement technique using the FLD method. Potential sources of error may include noise in the spectrometer, the solar fluctuation during the measurement, differences in the angle of the uneven leaf surface and non-fluorescent reference, the accuracy of light intensity measurement on the leaf, etc. These should be taken into consideration when trying to develop specific instruments designed based on the findings presented here.

Should the remote sensing of ΦPSII for whole-plant and canopy scale studies using the SIF method be desired, a high-power laser or LED for the saturation pulse would be needed. This is a limitation of the saturation pulse method,
but using the special sequence of laser illumination employing low-intensity pulses as described in Kolber et al. (1998), it will be possible to interpolate to a maximum fluorescence level. Therefore, there is the possibility for remote sensing of Chl fluorescence parameters such as ΦPSII without the use of accurate pulse-synchronized and modulated fluorimetric techniques such as PAM and LIFT using the SIF method and the illumination technique together.

Based on what was discussed above, the SIF measurement method could be used for both the Chl content estimation and photosynthesis parameter measurement (ΦPSII) needed for phenotyping studies of plants and remote sensing at different scales. In general, the SIF (FLD) method needs a high-spectral resolution and a high-sensitivity low-noise spectrometer to perform well. With more developments in instrument design and capability expected, the SIF method could potentially work well and perform more effectively should more research be focused on the development of spectrometers. For instance, if a cooled high-spectral resolution spectrometer with high sensitivity and low noise is used, more stable and low-noise data could be obtained.

**Conclusion**

This paper illustrates the possibility of measuring Chl content and Chl fluorescence parameters using SIF measurement (FLD method) and the saturation pulse method via a red laser at different life stages of plant leaves. The results were compared with those obtained through the standard Chl fluorescence measurement method. A high-resolution HR2000+ spectrometer was used to measure leaf reflectance under solar light using the FLD method to estimate the solar-induced Chl fluorescence. A USB4000 spectrometer measured the steady-state artificially induced Chl fluorescence spectrum in laboratory conditions.

The steady-state SIF yield ratio of O₂A and O₂B showed high correlation with Chl content but the relationship was weaker when comparing with results obtained using artificial light. Both methods proved to be less accurate at higher Chl content owing to the strong re-absorption influence of Chl pigment for Chl fluorescence, with the SIF method being less accurate than the artificial-light-induced method. The steady-state SIF yield ratio and the steady-state artificial-light-induced Chl fluorescence yield ratio were very well correlated (R² = 0.84), indicating that Chl content can be estimated from the proposed SIF measurement method.

A methodology was presented to estimate ΦPSII from the SIF measurements, and verified against the active Chl fluorescence method (PAM). The high coefficient of determination found between ΦPSII of the two methods shows that photosynthesis process parameters can be successfully estimated using the presented methodology. This can be the basis for future developments in remote sensing technologies for the estimation of both Chl content and Chl fluorescence parameters for passive phenotyping.

To scale-up the measurement techniques using SIF, measures should also be taken to remove the effect of noise, such as high background radiation, terrestrial atmosphere influence, and instrument measurement errors. Further studies are also recommended to evaluate these relationships beyond single leaf studies toward heterogeneous canopies.

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