Relationship between photochemical reflectance index and leaf ecophysiological and biochemical parameters under two different water statuses: towards a rapid and efficient correction method using real-time measurements

G. Hmimina, E. Dufrène & K. Soudani

Laboratoire Ecologie Systématique et Evolution, CNRS, University of Paris–Sud, UMR8079, F-91405 Orsay, France

ABSTRACT

The use of the photochemical reflectance index (PRI) as a promising proxy of light use efficiency (LUE) has been extensively studied, and some issues have been identified, notably the sensitivity of PRI to leaf pigment composition and the variability in PRI response to LUE because of stress. In this study, we introduce a method that enables us to track the short-term PRI response to LUE changes because of photosynthetically active radiation (PAR) changes. The analysis of these short-term relationships between PRI and LUE throughout the growing season in two species (Quercus robur L. and Fagus sylvatica L.) under two different soil water statuses showed a clear change in PRI response to LUE, which is related to leaf pigment content. The use of an estimated or approximated PRI0, defined as the PRI of perfectly dark-adapted leaves, allowed us to separate the PRI variability due to leaf pigment content changes and the physiologically related PRI variability over both daily (PAR-related) and seasonal (soil water content-related) scales. The corrected PRI obtained by subtracting PRI0 from the PRI measurements showed a good correlation with the LUE over both of the species, soil water statuses and over the entire growing season.

Key-words: drought; light use efficiency (LUE).

INTRODUCTION

The terrestrial biosphere is one of the main components of the carbon cycle and is very sensitive to abiotic stresses. Climate change is expected to increase the frequency and intensity of drought events, and will have a considerable impact on carbon and water budgets (Sheffield & Wood 2008). Understanding carbon and water fluxes, which exhibit wide spatial and temporal variability (Falge et al. 2002; Le Quere et al. 2009), is of considerable importance and is a subject of increasing interest. The direct measurement of carbon and water fluxes between the biosphere and the atmosphere is currently possible only locally using the eddy covariance method. Over 500 tower sites from approximately 30 regional networks across five continents and covering the majority of terrestrial biomes, with different spatial densities and organized within the global network, FLUXNET is used to track the temporal dynamics of carbon and water fluxes at an intra-daily scale (Baldocchi et al. 2001). Nevertheless, the acquired data are still insufficient to accurately describe the functioning of the biosphere and the global carbon exchange because of the great diversity of ecosystems and the wide range of variability of ecosystem structure, physiological functioning and environmental abiotic and biotic factors.

Remote sensing is considered to be an alternative method of estimating carbon fluxes and stocks on large scales while allowing for the consideration of great diversity and spatial heterogeneity of terrestrial vegetation. Since 2000, the approach built around the moderate-resolution imaging spectroradiometer (MODIS) project has provided maps of gross primary production (GPP) and annual net primary production (NPP) across the globe with an 8 d time step (for GPP) and a spatial resolution of 1 km2. In this approach, MODIS daily imagery is used to derive the land cover, the fraction of absorbed photosynthetically active radiation (PAR), the leaf area index (LAI) and, based on the concept of light use efficiency (LUE) developed by Monteith (Monteith & Moss 1977), the estimates of the GPP at the global scale. Data are available from February 2000 to the present. LUE is highly variable and sensitive to abiotic stress factors and is notably one of the main factors of the variation of GPP in response to climatic events (Garbulsky et al. 2011). Currently, this variability is accounted for by considering a daily biome-specific maximum LUE value, which is then downscaled according to the modelled daily minimum temperature and vapour pressure deficit (VPD), as described by Running et al. (2000). MODIS GPP and NPP values are validated across different biomes by comparison with eddy covariance measurements (Heinsch et al. 2006; Coops et al. 2007), and the estimation of LUE is known to be a major source of uncertainty (Gebremichael & Barros 2006; Turner et al. 2006).

At the leaf scale, the most widely used technique to measure LUE is analysing chlorophyll fluorescence based on modulated fluorescence using the saturation pulse method (Maxwell & Johnson 2000). Fluorescence, photosynthesis (photochemistry) and heat dissipation are the three pathways of transformation of absorbed solar energy conducted by the
leaves. In natural conditions under actinic light, measurements of variable fluorescence (usually named $F_v$ or $F_v'$) and the maximum fluorescence under a saturating light pulse of a light-adapted leaf ($F_{m'}$) enable an accurate estimate of the proportion of photons used in the photosystem II (PSII) centres in chloroplast thylakoids according to the Genty parameter or the quantum yield of PSII photochemistry (Genty, Briantais & Baker 1998). Because the quantum yield of PSII is directly related to the quantum yield of CO$_2$ fixation in the absence of photorespiration (Baker 2008), this parameter provides an accurate estimation of the LUE.

Satellite-based measurements of solar-induced chlorophyll fluorescence are technically and methodologically challenging mainly because the fluorescence intensity of chlorophyll is very weak, that is, approximately 1–2% of the absorbed radiation (Maxwell & Johnson 2000), and is strongly affected by atmospheric absorption. Recent works (Rascher et al. 2009; Guanter et al. 2012) showed that the retrieval of sun-induced chlorophyll variable fluorescence ($F_v$) might be feasible but remains challenging. Moreover, satellite-based $F_v$ measurements provide information about canopy photosynthetic activity, which depends on both LUE and other parameters, such as the LAI, chlorophyll concentration and light conditions, among other factors. Hence, $F_v$ needs to be standardized using a reference, such as $F_{m'}$.

Spectral vegetation indices based on reflected radiation, such as the enhanced vegetation index (EVI) and the normalized difference vegetation index (NDVI), are ineffective in the absence of severe water stress (Myneni, Los & Asrar 1995). These indices report changes in chlorophyll biomass, but are not able to track changes of photosynthetic efficiency over short timescales. Other spectral indices, particularly the photochemical reflectance index (PRI), which uses reflectance measured at 531 nm, have proven to be effective to track the LUE of different species and under different conditions at both the leaf and canopy scales. Indeed, a change in leaf reflectance at 531 nm related to the state of epoxidation of the violaxanthin-antheraxanthin-zeaxanthin cycle has been shown (Gamon et al. 1990). The epoxidation state of the xanthophyll cycle pigments is caused by excess light energy and allows the dissipation of this excess energy as heat (Yamamoto 1979; Schreiber & Bilger 1993; Pfündel & Bilger 1994). This mechanism responds to changes in absorbed PAR over short timescales of a few minutes and is slowly reversible in darkness (Jahns 1995; Hartel et al. 1996; Nilkens et al. 2010). At the leaf scale, the PRI has proven to be an accurate estimate of the quantum yield of PSII, as measured by fluorescence analysis, and LUE (Gamon et al. 1990; Gamon, Peñuelas & Field 1992; Peñuelas, Filella & Gamon 1995; Gamon, Serrano & Surfus 1997; Stylinski, Gamon & Oechel 2002). The first works of Gamon et al. opened a way for the assessment of the photosynthetic LUE from space.

Over the past 10 years, considerable effort has been made to evaluate the potential use of PRI as a proxy of LUE based on in situ and satellite-based measurements (Nichol et al. 2000; Asner et al. 2004; Drolet et al. 2005; Goerner, Reichstein & Rambal 2009; Peñuelas, Garbulsy & Filella 2011). At the canopy scale, the results are contrasting. The PRI versus LUE relationship was shown to be site dependent (Garbulsy et al. 2011) and exhibited variability over the seasonal scale (Soudani et al., unpublished data). To explain this variability in the PRI response to LUE, many studies have focused on the PRI sensitivity to the proportions of sunlit and shaded leaves in the canopy, which depend on the three-dimensional canopy structure and sun-view geometry (Barton & North 2001; Hall et al. 2008; Hilker et al. 2008, 2009, 2010). Recently, the PRI sensitivity to leaf pigment content (Moran et al. 2000; Gamon et al. 2001; Sims & Gamon 2002; Filella et al. 2004; Nakaji, Oguma & Fujinuma 2006), which was first shown to play a role in PRI response to LUE changes at seasonal scales (Garbulsy et al. 2011), was shown to introduce variability in PRI response to LUE at the leaf scale (Rahimzadeh-Bajgiran, Munehiro & Omasa 2012). Moreover, it was recently shown based on PRI kinetics following a dark-to-light transition (Gamon & Berry 2012) that PRI variability could be separated in two components: a facultative component linked to leaf physiological response to light and a constitutive component that was unrelated to the xanthophyll cycle.

Thus, PRI is a composite signal depending on the physical, chemical and physiological properties of leaves and canopies, and its variability is particularly difficult to interpret. A good understanding of this variability is necessary to judge the relevance of using PRI measurements as a proxy for LUE at the canopy scale, especially under different sun-view configurations and/or coarse temporal resolutions, which are extensively used based on satellite data (Drolet et al. 2005, 2008; Goerner et al. 2009; Moreno et al. 2012) or aircraft remote sensing (Zarco-Tejada et al. 2005; Suárez et al. 2008, 2010; Zarco-Tejada, González-Dugo & Berni 2012).

The use of the PRI as a proxy for LUE is not directly feasible and requires further study. The most challenging issue at hand is the deconvolution of the different sources of variability in PRI versus LUE relationships mentioned earlier. The development of methods to disentangle the seasonal variability due to changes in leaf pigment content from the variability due to climatic and edaphic constraints, which are related to the LUE, is particularly necessary.

In this study, we examine the temporal variability of the relations between PRI, fluorescence and the carbon assimilation at the leaf scale and throughout the season in two temperate deciduous tree species under two soil moisture treatments. More precisely, this study was designed with the following aims: (1) to assess PRI responses to PAR variations depending on the species and soil water status; (2) to assess the relationships between PRI and LUE; and (3) to attempt to disentangle the effects of seasonal variations of leaf biochemical properties on PRI versus LUE relationships.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Two hundred saplings of oak (*Quercus robur* L.) and beech (*Fagus sylvatica* L.) that were 3 years old were divided into two groups of 100 saplings each. For each species, the saplings were previously selected to have comparable sizes.
(approximately 45–60 cm for the oaks and 40–55 cm for the beeches). The saplings were planted in February 2011 in four planter boxes (50 individuals each), with two planter boxes for each species corresponding to the two soil water statuses. The planter boxes were 2.0 × 2.0 × 0.5 m each and were installed outside.

In the four planter boxes, the soil was identical and was composed of a mixture of two-thirds compost and one-third sand. The bottom of the planter box was permeable, and two drains were installed in each of the two planter boxes that were submitted to the drought treatment to facilitate drainage. With the aim of causing drought, the two planter boxes were also covered with clear plastic tarps only during rain events. The goal of this experiment was not to completely exclude rain, which may lead to a severe drought and therefore premature senescence and shedding of leaves, but to produce two contrasted conditions of soil moisture between the control and the treated plot. Therefore, the soil moisture in both the stands was controlled by watering.

**Measurements at the canopy and leaf scales and statistical analysis**

**Canopy scale and soil moisture measurements**

The four planter boxes were topped by an arch-shaped greenhouse structure with a top height of 4.5 m and made with galvanized steel pipes (Fig. 1). Optical fibres were placed on two cross bars of the greenhouse structure at approximately 2 m from the top tree canopy directly above the centre of each planter box. The fibres [numerical aperture 0.37, core diameter 200 μm, field of view (FOV) 43.4°; Thorlabs Inc., Newton, NJ, USA] pointed downward to collect the reflected radiation from the tree canopy in each planter box. The area covered by the FOV of each fibre was approximately 1.90 m in diameter. Two other optical fibres were used: one directed towards the sky and equipped with a cosine corrector that was mounted on top of the structure and used to measure the incident PAR, and the second fibre was installed at 7 cm from a Spectralon reference panel (Spectralon 99% reflectance, 25 × 25 cm; Labsphere Inc., North Sutton, NH, USA) and looking downward, collecting the reflected upwelling irradiance. The Spectralon reference panel was located next to the planter boxes and positioned at approximately 1.5 m height at the same level as the top of the tree canopy. All of the optical fibres were connected to input ports of an optical multiplexer (MPM-2000; Ocean Optics, Dunedin, FL, USA). The multiplexer had 16 input ports and 1 output port. The free input ports were blocked and used for dark noise measurements (instrumental noise). The output port was connected to a spectrometer [USB2000 + 350–1100 nm, 0.33 nm full width at half maximum (FWHM); Ocean Optics]. Each port was scanned sequentially by the

![Figure 1. Picture of the experimental set-up.](image)
multiplexer, allowing a temporal resolution of approximately 7 min between two successive acquisitions on the same planter box. After the installation and at their final locations, each fibre was calibrated for radiance measurements (w nm\(^{-1}\) m\(^{-2}\)) by measuring the spectrum at the output port of the multiplexer coming from a calibration lamp (HL-2000 CAL; Ocean Optics) connected at the end of the fibre.

In this study, the spectral data acquired above the vegetation were used to derive spectral indices as indicators of the temporal variation of canopy greenness (LAI) during the experiment. The reflected radiances from vegetation and from the Spectralon reference panel were used to calculate the reflectance. The NDVI, as an indicator of the canopy structure, was calculated based on the reflectance measured within a 25 nm wavelength band centred on 655 nm for the red and 800 nm for the near infrared using the following expression:

\[
NDVI = \frac{\rho_{655} - \rho_{800}}{\rho_{655} + \rho_{800}}
\]

The soil water content was monitored over the whole profile in the four planter boxes. An access tube was installed near the centre of each box, allowing the monitoring of the soil water content every 5 or 6 d over a 5 cm resolution profile using a PR2 soil moisture profile probe (Delta-T Devices, Cambridge, UK). The PR2 measurements were calibrated for volumetric humidity over 10,250 cm\(^3\) soil samples for each box (with a total of 60 samples).

**Leaf scale measurements**

At the leaf scale, measurements of fluorescence, photosynthesis and optical properties were begun when a sufficient contrast in terms of soil moisture was observed between the treated and the control plots, and during a long period of NDVI stability to minimize the effects of strong eventual temporal changes in the canopy structure and leaf pigment content. More precisely, the measurement campaigns took place from early July [date of year (DOY) 206] until late August (DOY 240).

For the planter boxes occupied by the oak saplings, data were acquired during four measurement campaigns (DOY 212, 215, 233 and 247). For the planter boxes occupied by the beech saplings, only two measurement campaigns were performed (DOY 213 and 234). The treatment and control plots were always sampled on the same day. From each plot, 10 leaves (five leaves from the top and five leaves from the bottom of the tree crowns) on five different trees were randomly selected. The same leaves were numbered and monitored throughout the experiment.

The measurements were conducted on leaves still attached to the tree and previously wrapped in aluminium foil to keep them in the dark for fluorescence measurements. The leaves were dark adapted for 12 h. The measurements were performed on the same leaves, at first using a PAM-2000 fluorometer (Walz, Effeltrich, Germany) and then using the Li-Cor 6400 (Lincoln, NE, USA) for fluorescence and photosynthesis measurements on the other half of the leaf. The measurements of leaf optical properties were performed simultaneously with the PAM-2000 fluorescence measurements. The protocols are described in detail later:

For fluorescence measurements using the PAM-2000 and leaf optical properties, each leaf, still wrapped in aluminium foil, was clipped with a leaf clip holder 2030-B (Walz, Effeltrich, Germany). An optical fibre (50 μm, FOV 25°; Ocean Optics) fixed on the leaf clip holder and equipped with a collimating lens (to reduce the FOV of the optic fibre) and connected to a USB2000 spectrometer (350–1100 nm, 0.66 nm FWHM; Ocean Optics) was used to measure the leaf optical properties on the same portion of the leaf exposed to saturating light pulses generated by the PAM-2000.

The optical measurements started by measuring the radiance on a grey Spectralon panel (4% reflectance; Labsphere Inc.) clipped to the leaf and then the radiance reflected by the leaf immediately after removing the grey Spectralon and the aluminium foil. The aluminium foil was removed only from the first half of the leaf. The other half was kept dark adapted for Li-Cor measurements.

Immediately after the measurement of the leaf’s reflected radiance (approximately 1 or 2 s after), the fluorescence parameters \(F_0\) (dark-adapted initial minimum fluorescence) and \(F_m\) (maximum fluorescence measured during the first saturation pulse on dark-adapted leaves) were measured. After these two measurements, the \(F_s\) (stationary chlorophyll fluorescence level) and \(F_m'\) (maximum chlorophyll fluorescence in a light-adapted leaf) were measured continuously for 24 min at different increasing and decreasing light intensities emitted by a light-emitting diode (LED) array actinic lamp (0–472 μmol m\(^{-2}\) s\(^{-1}\)) to obtain light–response curves of the leaf under LED and natural light conditions. The quantum yield of PSII and its maximum value were determined as follows:

\[
\phi_{PSII} = \frac{F_{m'} - F_s}{F_m'}
\]
\[
\phi_{PSII_{max}} = \frac{F_m - F_0}{F_m}
\]

Simultaneously with the PAM-2000 measurements and throughout the sequence of increasing and decreasing light intensities, automatic measurements of the leaf optical spectrum were taken continuously at a very high temporal frequency (the maximum difference observed between two successive acquisitions of spectra was 0.5 s, and the mean difference was 0.13 s). At the end of the PAM-2000 measurements, another spectrum was measured on the Spectralon reference panel.

The spectra obtained from the leaves were then used to calculate PRI using the following formula:

\[
PRI = \frac{\rho_{570} - \rho_{531}}{\rho_{531} + \rho_{570}}
\]

where \(\rho_{531}\) and \(\rho_{570}\) represent the leaf reflectance integrated over a 10 nm wavelength band centred on 531 and 570 nm, respectively.
The first spectrum measured immediately after removing the aluminium foil and before switching on the PAM LEDs was also used to calculate the modified red-edge normalized difference index (mNDI\textsubscript{705}) as an indicator of leaf chlorophyll content based on the reflectance measured within a 25 nm wavelength band centred on 445, 705 and 750 nm, respectively.

\[
m\text{NDI}_{705} = \frac{\rho_{750} - \rho_{705}}{\rho_{750} + \rho_{705} - 2 \times \rho_{445}}
\]

where \(\rho_{445}, \rho_{705}\) and \(\rho_{750}\) represent the reflectance integrated over a 25 nm waveband centred on 445, 705 and 750 nm, respectively.

Note that only the PRI values computed from measurements taken 2 s after each PAM-2000 pulse were used to obtain a response curve of the PRI to increasing and decreasing PAR levels. This choice was made to avoid the contribution of the PAM pulses to the reflected radiation by the leaf. In addition, the spectrum of actinic light provided by the PAM-2000 LED sources and measured in this study peaked at 655 nm and ranged between 600 and 700 nm (FWHM 642–671 nm), and thus did not overlap the PRI wavelengths (531 and 570 nm).

After the PAM-2000 measurements, 6 of 10 leaves from each measurement campaign were kept for fluorescence and \(\text{CO}_2\) assimilation measurements with a Li-Cor 6400 with the 6400-40 leaf chamber fluorometer. \(\text{CO}_2\) assimilation measurements were used to estimate the LUE and the leaf total chlorophyll content. Reflectance and \(\text{CO}_2\) assimilation measurements were acquired simultaneously during a sequence of 30 min of an increasing and decreasing PAR (0–2000 \(\mu\text{mol m}^{-2} \text{s}^{-1}\)) after removing the aluminium foil covering the remaining portion of the leaf.

**Leaf biochemical measurements**

During the entire experiment and throughout the growing season, a total of 45 oak and 25 beech non-dark-adapted leaves were sampled in each planter box. Before the sampling, their reflectance spectra were measured. Immediately after sampling, the leaves were frozen in liquid nitrogen, lyophilized, weighed and ground. Fifteen micrograms of the resulting powder from each leaf was dissolved in 10 mL 90% acetone at 60 °C during 1 h. Then, the absorbance spectrum of the solution was measured using an Agilent 8453 UV-VIS spectrophotometer (Santa Clara, CA, USA) to determine the total chlorophyll concentration of the leaf. Reflectance and leaf chlorophyll concentration measurements were used to establish a calibration relationship between the mNDI\textsubscript{705} and leaf total chlorophyll content.

**Data analysis**

The spectra were processed using MATLAB 7.0 (MathWorks, Natick, MA, USA). The reflectance spectra obtained using radiances measured from the leaves and the Spectralon reference panel were smoothed using a robust loess local regression. The spectra were then used to derive the spectral indices (NDVI, mNDI\textsubscript{705} and PRI) described earlier.

The spectral, fluorescence and \(\text{CO}_2\) assimilation data were then analysed to describe the temporal and treatment-related variability and to assess relationships between the PRI, PAR, fluorescence and LUE at the leaf scale. The temporal and treatment-related variability was described based on summary statistics and a Kruskal–Wallis non-parametric analysis of variance. Relationships between the PRI, PAR, fluorescence and LUE were described using regression analysis, and their robustness was assessed using the resulting \(R^2\) values.

**RESULTS**

**General characterization of the soil water status, temporal patterns of canopy structure and leaf chlorophyll content in the experimental plots**

The soil moisture and canopy structure dynamics during the measurement campaigns are shown in Fig. 2a,b.

As stated in the Materials and Methods, the four plots were regularly watered to produce a difference in the soil water content between the control and treated groups. Intense hydric stress was avoided to prevent strong changes in the structural and biochemical canopy characteristics. Nevertheless, the soil moisture time courses (Fig. 2a) showed different hydric statuses between the plots. For the oak, the soil moisture varied between 13 and 19% for the control plot, and between 6 and 15% for the treated plot. Throughout the duration of the experiment, the average difference in soil moisture in the control and treated oak plots was approximately 7%. For beech, the soil moisture varied between 24 and 31% for the control plot, and between 15 and 19% for the treated plot. During the entire experiment, the average absolute difference in soil moisture between the control and the treated beech plots was approximately 10%. These absolute differences did not lead to perceptible changes of the canopies structural properties. As shown in Fig. 2b, the NDVI time courses did not show any significant variation in canopy structure between the control and the treated plots. Finally, between the two species, the lower soil moisture reached in the treated oak plot can be explained by a higher canopy LAI, as suggested by the level and length of the stable NDVI region during the measurement campaigns.

At the leaf scale, temporal patterns of chlorophyll content were investigated based on mNDI\textsubscript{705} measurements calibrated against direct measurements, as described earlier. A robust linear calibration relationship \([R^2 = 0.95; \text{root mean square error} (\text{RMSE}) = 0.4]\) was obtained, with a slope of 17.5 mg g\(^{-1}\) and an intercept of \(-9.8\) mg g\(^{-1}\) (Fig. 3a). No significant differences in leaf chlorophyll content were observed between the treatments during the measurement campaigns (Fig. 3b,c).

**Dynamics of ecophysiological responses and PRI**

As noted earlier, ecophysiological responses of the four plots were investigated during the NDVI plateau period.
Photosynthetic functioning at the leaf scale was assessed through measurements of CO₂ and water exchange (Fig. 4) and through parameters of chlorophyll fluorescence measurements under different levels of imposed PAR (Fig. 5).

Summary statistics of the leaf stomatal conductance and LUE are illustrated in Fig. 4. As described in the Materials and Methods, the leaf stomatal conductance was determined based on the Li-Cor measurements made in the same conditions and over the same PAR range for every leaf. For oak, Fig. 4a does not show any significant difference between the control and the treated plots at the beginning of the experiment. However, significant differences can be observed on
DOY 233 and 247 ($P < 0.001$ and $P < 0.0015$, respectively). Significant differences between the beech control and treated plots were also observed at the end of the experiment, as shown in Fig. 4b ($P < 0.003$).

Figure 4c,d shows the temporal variability of the LUE. No significant differences between the control and the treated plots were observed for oak (Fig. 4c). Nevertheless, during the entire period of measurements, the average LUE was always lower in the treated plot. In the beech plots (Fig. 4d), LUE was significantly lower during the end of the experiment ($P < 0.027$).

Summary statistics of the maximum fluorescence yield from PAM measurements are illustrated in Fig. 5. The results
confirm the conclusions derived based on the LUE measurements. The maximum fluorescence yield was measured on dark-adapted leaves and consequently was not PAR dependent. A decrease in maximum fluorescence yield is observed, as well as the significant difference between the control and the treated plots for oak at the end of the experiment, and for beech (Fig. 5a,b).

Relations between the PRI and leaf ecophysiological and biochemical parameters

Relations between the PRI and ecophysiological responses

Simultaneous reflectance spectra and fluorescence measurements are illustrated in Figs 6a,b and 7. Typical reflectance spectra measured at the leaf scale under different levels of incident radiation coming from PAM LEDs and from ambient light conditions are illustrated in Fig. 6a. PAM LED radiation was applied in increasing and decreasing sequences. The general shape of the reflectance spectrum was similar to that typically measured above the leaves. In the visible spectrum, reflectance peaked in green, and as low in the blue and red regions. Then, reflectance increased sharply at the red edge with an inflection point around 720 nm and reached its maximum in the near infrared. The peaks observed around 655 nm were due to the increased actinic red light that came from the PAM LEDs. As noted earlier, the emission spectrum of the PAM LEDs did not overlap with the wavelengths used in PRI. Measurements of this spectrum (data not shown) showed that the emission spectrum was limited between approximately 611 and 687 nm. In addition, the peak located at approximately 760 nm was largely due to the sun-induced chlorophyll fluorescence in the Fraunhofer line of the oxygen absorption.

Figure 6a (inset) shows a significant temporal variability of the reflectance in the region surrounding the peak at 555 nm due to increasing and decreasing variations of PAR. This variability was asymmetrical and more important in the left side than in the right side.

The corresponding sequence of imposed PAR and the resulting typical PRI and fluorescence kinetics are illustrated in Fig. 6b. The PRI and fluorescence yield were both negatively correlated with the PAR, and their variation was not completely reversible.

The PAR-dependent variability of the reflectance calculated for every wavelength as the relative difference between the reflectance at time \( t \) and reflectance at the beginning of the increasing sequence of PAR (time zero) is illustrated in Fig. 7. First, over the entire kinetic path from the beginning of the increasing sequence of PAR to the end of the decreasing sequence, the reflectance level remained lower than the level of reflectance measured at time zero. This result indicates a hysteresis phenomenon, meaning that the curve of the decrease of reflectance produced by the increase of PAR does not overlap the curve of the increase of reflectance produced by the decrease of PAR. This figure also shows an important variability depending on the wavelength. The maximum variation was found in two spectral bands centred on 525 and 540 nm. The variation observed in the 525 nm band was not completely reversed with decreasing PAR (the \( R_{525} \) nm at the end of the decreasing sequence remained lower than the \( R_{525} \) nm at time zero). On the contrary,
the variation observed around the 540 nm spectral band appeared with low PAR values as soon as the leaf was exposed and was quickly reversed under increasing red light.

Based on measurements of PRI and fluorescence from all the leaves under increasing and decreasing sequences of PAR according to the protocol described earlier, the relationships between PAR, fluorescence yield, LUE and PRI are shown in Fig. 8.

As shown in Fig. 8, the relationships between PRI and PAR, fluorescence and LUE at the leaf scale were very scattered because of the strong variability of the intercepts.

Investigating causes of the variability of the relations between PRI and fluorescence yield

As noted earlier, the PRI, as well as the intercept of the linear regressions of PRI versus PAR (Fig. 8), hereafter called regression-based PRI0 (the hypothetical PRI at PAR near 0), exhibited a strong variability between the leaves. Figure 9 shows summary statistics of the PRI and PRI0 for the oak and beech during the different measurement campaigns.

Note that regression-based PRI0 values were estimated from PRI versus PAR relationships established from measurements acquired on dark-adapted leaves. The comparison of the regression-based PRI0 estimates to the PRI values measured on the same leaves immediately after the removal of the aluminium foil that covered each leaf is shown in Fig. 10a. Significant correlations between the offsets and the dark-adapted PRI values were obtained (R² = 0.84, P < 0.001, RMSE = 0.006). Moreover, the measured and estimated dark-adapted PRI0 were positively correlated (R² = 0.83, P < 0.001, RMSE = 0.33), with the leaf chlorophyll content as estimated using the calibrated mNDI705 (Fig. 10b).

A simple procedure for disentangling the dependencies of PRI on the variability of leaf chlorophyll content between leaves and during the season may consist of subtracting the corresponding PRI0 values from the PRI measurements achieved during the different campaigns. The relationships between the corrected PRI and fluorescence yield from PAM, and between the corrected PRI and LUE from Li-Cor data are shown in Fig. 11a,b. Whereas the correlation between the PRI and fluorescence yield and LUE was poor (R² = 0.09, P < 0.001, RMSE = 0.039 and R² = 0.09, P < 0.001,
RMSE = 0.038, respectively), the obtained corrected PRI versus LUE over 260 measurements made on 16 oak leaves and 8 beech leaves (4 leaves per sampling date) was strong ($R^2 = 0.72$, $P < 0.001$, RMSE = 0.0042 and $R^2 = 0.93$, $P < 0.001$, RMSE = 0.0016, respectively) and linear, accounting for both inter-leaf and seasonal variability.

**DISCUSSION**

Previous studies (Gamon *et al.* 1990, 1992) showed a clear response of PRI to the xanthophyll cycle epoxidation state and the LUE. However, other studies obtained much more contrasting results over the season at the leaf (Rahimzadeh-Bajgiran *et al.* 2012) and canopy scales (Grace *et al.* 2007; Garbulsky *et al.* 2011). Indeed, at the seasonal scale, a relationship between PRI and LUE may not always be observed (Gamon *et al.* 2001; Filella *et al.* 2004; Nakaji *et al.* 2006; Rahimzadeh-Bajgiran *et al.* 2012). Hereafter, we summarize the reasons that may explain the apparent loss of relationship between PRI and LUE, that is:

1. Insufficient LUE variability (e.g. due to abiotic and biotic stress conditions).
2. Dependency of the PRI on other factors (e.g. leaf biochemical composition, LAI, three-dimensional canopy structure and sun-view geometry) regardless of LUE.
3. Loss of PRI response to LUE changes (e.g. xanthophyll cycle inhibition or saturation and zeaxanthin-independent quenching).

At the leaf scale, most of the previous works focused on the steady-state response of PRI under fixed PAR, after stabilization (Gamon *et al.* 1990, 1992, 1997; Penuelas *et al.* 1997; Rahimzadeh-Bajgiran *et al.* 2012), rather than the dynamic response of PRI to PAR variability. Under natural conditions, the steady-state response of PRI may not be reached because of high PAR variability over very short timescales. Therefore, steady-state PRI measurements may not be similar to PRI responses under natural conditions, as suggested by Rascher & Nedbal (2006). On the other hand, some studies focused on the PRI kinetic under fixed PAR and after dark–light transition (Peñuelas *et al.* 1995; Gamon & Surfus 1999; Gamon & Berry 2012). In Gamon & Berry (2012), it was shown that two components of PRI variability could be distinguished: a constitutive component depending on leaf pigment content, and a facultative component, varying at short timescale because of the xanthophylls cycle. The constitutive component could be isolated using the first PRI measurement over dark-adapted leaves.

In the present study, PRI kinetics after dark–light transition were coupled with PRI light curve measurements under semi-controlled PAR variability. This enabled us to track the quantitative response of PRI to PAR variation under constant leaf pigment composition. Our results showed the same responses of reflectance at 525 and 540 nm as those reported by Gamon *et al.* (1997). The band centred on 525 nm that respond to the whole range of PAR may be linked to the xanthophylls cycle, whereas the band centred on 540 nm may be due to the reflectance variation due to light-scattering changes due to dark–light transition, as observed in Gamon *et al.* (1997) around 545 nm. These reflectance changes were clearly associated with the PRI changes related to the imposed sequences of PAR and to the resulting quantum yield changes (Figs 6 & 7). The protocol adopted in this study allowed us to avoid considering the possible causes of the deterioration of the relationship of PRI versus LUE that are described above. The measurements were made at very high temporal resolution under an imposed variability of PAR over leaves for which the biochemical composition remained unchanged during the measurements.

Our results were obtained based on measurements achieved over the entire growing season from two species (*Q. robur* and *F. sylvatica*) and under different soil water
contents. The soil water content differential triggered a significant decrease in leaf conductance in the treated groups of both species (Fig. 4a,b) as well as a decrease in LUE (Fig. 4c,d), and a decrease in maximum quantum yield (Fig. 5a,b). In contrast, the leaf biochemical and canopy structural properties of the plots were not significantly different, as illustrated by the canopy NDVI dynamics and leaf chlorophyll content (Figs 2 & 3). The PRI seasonal variability (Fig. 9a,b) exhibits both difference between treatment, in accordance with the soil water content measurements, and a seasonal trend comparable with the one observed in leaf chlorophyll content (Figs 2 & 3). At the leaf scale and over the entire season, the correlations between PRI and quantum yield, and between PRI and LUE were significantly higher.

Figure 9. (a, b) Leaf photochemical reflectance index (PRI) measured on oak (a) and beech (b) leaves in the control and in the treated plots. The top of each bar is the mean PRI of the leaf group, and the whiskers indicate the estimated error (95% confidence interval) around the mean. The control groups are presented in blue, and the treated groups are presented in red. (c, d) Leaf PRI0 measured on oak (c) and beech (d) leaves in the control and in the treated plots. The top of each bar is the mean PRI0 of the leaf group, and the whiskers indicate the estimated error (95% confidence interval) around the mean. The control group is presented in blue, and the treated group is presented in red (oak: n = 10 for each bar; beech: n = 10 for each bar).
than those reported in the review of Garbulsky et al. (2011). [The mean $R^2$ was 0.78 and 0.73 between PRI versus quantum yield and PRI versus LUE, respectively, in this study, and values of 0.5 and 0.25, respectively, were reported by Garbulsky et al. (2011.)] These values are comparable with those obtained on an intra-daily scale (Gammon et al. 1992; Penuelas et al. 1997; Guo & Trotter 2004). Moreover, these relations, shown in Fig. 8, were stable over the entire

Figure 10. (a) PRI$_0$, estimated as the intercept of photochemical reflectance index (PRI) photosynthetically active radiation (PAR) regressions versus PRI$_0$ measured immediately after dark adaptation. Symbols: blue and red symbols for the control and the treated plots, respectively, with circles for oak and squares for beech. (b) Measured PRI$_0$, versus the estimated total leaf chlorophyll content of leaves (mg g$^{-1}$). Symbols: blue and red symbols for the control and treated plots, respectively.

Figure 11. (a) Corrected photochemical reflectance index (PRI) (PRI minus PRI$_0$ for each measurement series) versus fluorescence yield. Symbols: blue and red symbols for the control and the treated plots, respectively, with circles for oak and squares for beech. (b) Corrected PRI versus light use efficiency. Symbols: blue and red symbols for the control and treated plots, respectively, with circles for oak and squares for beech.
season for both species and both treatments. On the other hand, these relations exhibit a strong variability in intercepts between leaves and over the season.

In light of the results discussed earlier, we can conclude that we did not observe any loss of the robustness of the PRI response to PAR and to LUE over the growing season, but this PRI response changed depending on other factors unrelated to leaf physiological responses to PAR variability. This change is clearly shown in Fig. 8. This figure also shows that the PRI variability between leaves was much higher than the variability induced by PAR. To explain this variability, we defined PRI0 as the PRI of a completely dark-adapted leaves [analogous to the ground (F0) fluorescence of a dark-adapted leaf]. We used two different methods to determine the PRI0.

The first method consisted of directly approximating PRI0 as the PRI measured immediately (less than 100 ms on average) after the removal of the aluminium foil used for the leaf dark adaptation, as done previously in Gamon & Surnas (1999) and in Gamon & Berry (2012). We noticed that the estimated and measured PRI0 were highly correlated (Fig. 10a), meaning that the PRI0 measured directly after leaf dark adaptation can be used to track the PRI variability that is unrelated to leaf physiological responses.

The results show that PRI0 was highly linearly correlated with the leaf chlorophyll content (Fig. 10b), in accordance with Gamon & Berry (2012). Therefore, there was a strong influence of leaf pigment content on PRI, as shown by Filella et al. (2004) and Garbulsky et al. (2011). After subtracting the PRI0 from PRI measurements, the relations between the corrected PRI (PRI – PRI0) and LUE significantly improved over the entire growing season for both species and both treatments, as shown in Fig. 11a, b, respectively. The uncorrected PRI versus LUE relationship is shown in Fig. 8c.

Based on these results and as reported in Gamon & Berry (2012), the PRI variability can be separated into two components:

1. A constitutive component is mainly due to the leaf biochemical composition, which exhibited a seasonal pattern and a strong inter-leaf variability. This component was captured using the PRI0.
2. A physiological component due to LUE variability, mainly explained by the PAR and soil water status. This component was recovered as the corrected PRI.

The use of high-temporal resolution measurements on dark-adapted leaves under controlled PAR conditions, which allowed us to disentangle the PRI variability correlated to LUE from the effects of pigmentation changes, was clearly not suitable to correct the PRI acquired over vegetation canopies at large spatial and temporal scales. This constitutive PRI variability related to leaf pigment composition may therefore make it difficult to use the PRI as a proxy of the ecosystem LUE. Nevertheless, we think that the PRI0 at the canopy scale could be obtained following a variety of approaches, including:

1. Using PRI measurements acquired under low light after sunrise to minimize the contribution of the xanthophyll cycle to the measured signal. However, precautions should be taken because, as shown by Gamon et al. (1992) and confirmed in this study (Fig. 7), the PRI shows an exponential decline as soon as the leaves are exposed to light.
2. Using high-temporal resolution PRI measurements to obtain a PRI versus PAR regression, and to estimate PRI0 as the intercept of this regression. In this study, the PRI0 estimated using this approach was strongly correlated with the measured PRI0 (Fig. 10a). However, the intercept estimation depends on the quality of the PRI versus PAR relationship, which may not be conserved at larger scales because of high spatial heterogeneity or when photosynthesis is limited by other factors than the PAR (Soudani et al., unpublished data).
3. As suggested by Rahimzadeh-Bajgiran et al. (2012), combining PRI with optical indices sensitive to leaf pigmentation, such as the mNDI705, which was shown to be well correlated with the PRI0 in this study. Nevertheless, special caution should be taken when using optical indices to correct the PRI because it is unclear whether there is a single relationship over different species, scales, acquisition conditions and plant physiological statuses.

**CONCLUSION**

In this study, measurements at a high-temporal resolution of PRI on dark-adapted leaves in controlled PAR conditions showed strong relationships between the PRI, quantum yield and LUE at the leaf scale, but these relationships were strongly impacted by the leaf pigment content. These impacts may account for most of the PRI variability measured over coarse temporal and spatial scales. This effect may significantly hamper the use of PRI as a proxy of canopy LUE. Moreover, we showed that this PRI variability could be corrected using PRI measurements in low light or immediately after leaf dark adaptation, or the estimation of dark-adapted PRI based on light curve analysis. The new correction procedure allowed for the disentanglement of the effects of seasonal variations in leaf pigment content on the relation between the PRI and the LUE. Nevertheless, this correction method needs to be assessed at the leaf scale over a wide range of species under much more constraining conditions. At the canopy scale, the application of such a procedure using satellite data might be possible but must be tested.

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