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Potential use of PRI and active fluorescence for the diagnosis of physiological state of plants under ozone exposure and high atmospheric vapor pressure deficit

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Abstract

Assessing photosynthesis rates with remote sensing is important for tracking the physiological state of plants. The photochemical reflectance index (PRI) is a good estimator of short-term light-use efficiency (LUE) at the leaf scale but its responses to environmental factors are poorly understood. In this study, we assessed changes in the responses of PRI to ozone exposure and to an increase in atmospheric drought (separately and combined) in oak (*Quercus robur*) and holm oak (*Quercus ilex*) that were planted in climatic cells under controlled conditions. The aim was to evaluate the ability of PRI as a relevant indicator to assess the impact of abiotic factors on photosynthesis. Leaf-scale measurements of biochemical, physiological and spectral properties, including PRI in dim light on dark-adapted leaves (PRI₀), kinetic of PRI responses to PAR variations (photosynthetically active radiation), and leaf chlorophyll fluorescence parameters were performed. The results show that PRI₀ is a good proxy of the leaf chlorophyll content, and is correlated to chlorophyll fluorescence parameters on dark adapted leaves (F₀, Fₘ). The correction of PRI from the leaf chlorophyll content variations (PRIc) significantly improves correlations between PRI and NPQ (non-photochemical quenching). The variability of PARₜₐₙ (estimated PAR value at PRI saturation using PRI vs. PAR relationships) depends on ozone exposure and on the increase in atmospheric vapor pressure deficit. For *Quercus robur*, results highlight that PARₜₐₙ is linked to abiotic stress indicating that PRI may be used as a relevant indicator of abiotic factors limiting the photosynthesis. *Quercus ilex* did not show significant variability in PRI₀ and PARₜₐₙ, which suggest that it is a more drought resistant species than *Q. robur*.

Introduction

Photosynthesis is driven by the conversion of solar energy into chemical energy. However, the photosystem cannot use all the absorbed light, particularly under full-sun
exposure when the light energy flux exceeds the photosynthesis conversion rate. Cases of light energy that exceed the photosynthesis capacity are common and well known\textsuperscript{1,2}, and the surplus energy represents a danger to the plant and must be dissipated. The energy excess is rapidly dissipated as light via chlorophyll fluorescence or re-emitted as heat. Heat dissipation mechanisms are reversible and referred to as non-photochemical quenching (NPQ). The NPQ, the primary mechanism to dissipate excess excitation energy\textsuperscript{3,4}, requires the activation of the violaxanthin de-epoxidase enzyme (VDE) in the antenna complexes of the photosystem\textsuperscript{5-9}. This enzyme allows to redirect the energy usage from chlorophyll to xanthophyll cycle for the NPQ\textsuperscript{10,11}. The VDE converts violaxanthin into zeaxanthin via antheraxanthin and is activated by the proton gradient across the thylakoid membrane\textsuperscript{12}.

Environmental constraints limit the photosynthetic activity and potential growth of vegetation. Moreover, the increase in excess excitation energy can lead to the formation of reactive oxygen species\textsuperscript{13} (ROS). Most stress conditions involve similar plant responses that alleviate the effects of excess energy absorption. These response mechanisms primarily involve stomatal closure followed by photoinhibition mechanisms linked to NPQ. Photoinhibition processes cause a reduced yield of photosynthesis that decreases the plant's ability to use the light energy. Regardless of the stressor, if the stress persists, then the use of such mechanisms may cause severe damage to the plant and lead to a loss of plant vitality and productivity. Climate events such as water limitation represent stresses that can significantly affect plant productivity and cause leaf browning and early leaf loss\textsuperscript{14}. Chronic exposure to an air pollutant can also cause yield losses\textsuperscript{15-18}, which affects the photosynthetic capacity of photosystems. Ozone is considered the most important phytotoxic air pollutant, especially in the Mediterranean region\textsuperscript{19,20}. Ozone has a high oxidative capacity and induces ROS that can impair the light and dark reactions of photosynthesis\textsuperscript{21}. Like other plant stress factors, ozone
affects the photosynthetic performance and biochemical processes of plants, inducing interference with photosynthesis.

The effect of climate events and pollution on photosynthesis and on plants in general must be monitored for the early detection of damage. Two approaches are commonly implemented to monitor the effects of climate events on plants: the use of chlorophyll fluorescence and spectral properties. Fluorescence-based indicators of photosynthetic functioning at leaf scale can be estimated when the leaf is subjected to a strong pulse-amplitude-modulated (PAM) light used to excite chlorophyll fluorescence, and several parameters related to photosynthesis and other energy dissipation processes can be determined.\textsuperscript{22-24} Nevertheless, the use of active fluorescence is difficult to apply at ecosystem and regional scales.\textsuperscript{25}

The second approach is based on the use of spectral variations of reflectance and sun induced fluorescence of leaves and canopies. Increasing interest has been focused on the photochemical reflectance index (PRI) signal. The PRI, developed by Gamon et al.\textsuperscript{26}, is based on changes in reflectance at 531 nm: which are related to the conversion of violaxanthin to zeaxanthin (prior to heat dissipation), as well as a reference band at 570 nm. The PRI has been shown to be effective at tracking the light use efficiency (LUE) over a wide range of species under different conditions at the leaf and canopy scales.\textsuperscript{27,28} However, recent studies have shown that leaf pigment contents play a major role in the variability of the PRI, and it can mask the PRI vs. LUE relationship or blur its interpretation.\textsuperscript{29-31} Indeed, the PRI is a composite signal that responds to the physiological properties of leaves, which may be linked to photosynthetic functions, as well as the biochemical properties of leaves, such as chlorophyll content.\textsuperscript{28,29,32-34} In Hmimina et al.\textsuperscript{32,33}, Soudani et al.\textsuperscript{35} and Merlier et al.\textsuperscript{34}, the physiological and biochemical sources of PRI variability at the leaf and canopy scales were deconvoluted using PRI measurements at low radiation levels, which were determined immediately after leaf dark adaptation (PRI\textsubscript{0}) at the leaf scale\textsuperscript{32} or estimated from the PRI vs. PAR
(photosynthetically active radiation) relationship at the canopy scale. The PRI is strongly correlated to the leaf chlorophyll content at both the leaf and canopy scales. The corrected PRI, which was obtained by subtracting the pigment-related variability \( \text{PRI}_0 \) from the PRI measurement, was highly correlated with the LUE measurements that expressed the physiological variability of the PRI.

In this study, we further investigated the dependence of the PRI on the leaf biochemical and physiological properties under different abiotic conditions. Indeed, PRI responses to abiotic constraints are poorly understood and the effective scope of the PRI as an indicator of the responses of plant to abiotic constraints deserves to be evaluated carefully. In this work, we studied the responses of the PRI at leaf scale under abiotic constraints in controlled conditions. The main objectives were to 1) investigate the PRI responses to abiotic constraints induced separately and combined by ozone accumulation and atmospheric water deficit induced by decreasing of air humidity; 2) study the strength of the relationships between the parameters derived from the PRI variations and the factors linked to abiotic constraints; and 3) use these relationships to separate the constitutive components from the physiological components of PRI variability.

**Materials and methods**

**Experimental design, conditions and plant material.**

The experiment was conducted under controlled conditions using environmental cells in the Ecolab, which is available at the CEREEP-Ecotron (Research Centre in Experimental and Predictive Ecology, CNRS/ENS, Foljuif, France). The Ecolab is a modular autonomous structure consisting of three climate chambers (13 m³ volume each) that can be controlled independently for air humidity, temperature, light quality and quantity, rainfall and certain atmospheric gas concentrations, such as ozone and carbon dioxide.
Two-year-old saplings of oak (*Quercus robur* L.) and holm oak (*Quercus ilex* L.) were maintained outdoor, in garden pots, and then distributed into the 3 environmental cells, with 7 pots of each species in each cell. The pots were maintained 3 weeks in the environmental cells for their acclimation before the beginning of the experiment. During the experiment, which lasted 5 weeks, the first cell was used as a control and the other were used as treatments. The controlled parameters were air temperature, air humidity, light, water and the concentrations of CO$_2$, O$_2$ and O$_3$. In the three cells, natural light conditions were mimicked using a daily pattern maintained for 14 hours in the dark and 10 hours in the light using a lamp (model POWERSTAR HQI-BT 400 W/D PRO, OSRAM, Molsheim, France).

The temperature of the three cells was kept constant at 25°C during the diurnal period and at 17°C during the nocturnal period, the relative air humidity was maintained at 60% (except in the “air humidity” treatment, referred to as RH hereafter), the CO$_2$ concentration was 350 ppm, the O$_2$ content was 21% and the light was 500 µmol m$^{-2}$ s$^{-1}$ of PAR during light hours. In the control cell, the parameters remained stable throughout the experiment. In the “RH” treatment, air humidity was increased to 80% then decreased from 80% to 30% in intervals of 10% to provoke atmosphere-induced plant water constraint conditions. In the third cell, the “ozone” (referred to as “OZ” hereafter) treatment consisted of plants submitted to oxidative constraint caused by chronic elevated ozone exposure (maintained automatically at 100 ppb for 4 hours per day).

Throughout the experiment and for each cell, the biochemical, physiological and optical properties of the leaves were monitored. The biochemical properties were related to the leaf pigment contents, whereas the physiological properties were related to the chlorophyll fluorescence parameters.

During the experimental period, 10 leaves (kept on plant) of each species are sampled every day in two of the three cell, corresponding to 40 leafs sampled per day (see Fig. 2 for
the detail of treatments sampled each day). The leaf biochemical properties were estimated using the SPAD-502 meter (Konica-Minolta, Tokyo, Japan) and the DUALEX® meter (FORCE-A DUALEX meter, Orsay, France). Chlorophyll content was measured by the both instruments and, in addition, the amount of nitrogen and flavonoids are available using the DUALEX® meter.

After biochemical measurements, leaves were wrapped in aluminum foil to avoid illumination during 8 hours, before the fluorescence measurements. The leaf physiological and optical measurements were done on the subsequent day on the same leaves used for the biochemical measurements.

A portable fluorometer with a leaf clip (PAM 2500, Walz, Effertlich, Germany) was used to measure the chlorophyll fluorescence. An optical fiber connected to a spectrometer (ASEQ, LR1-T spectrometer; 300-1000 nm; 0.6 nm spectral resolution; TEC module; 600 µm core VIS-IR fiber; Vancouver, Canada) that was directed towards the same area sampled by the PAM was used to obtain the spectral measurements (Fig. 1).

The measurements were performed according to the following order: first, in the dark, the aluminum foil was removed and the minimal fluorescence was measured; then a dim light (approximately 100 µmol m⁻² s⁻¹ of PAR on the leaf) was switched on using a LED panel (LED PLC230V303-BN, CONRAD, 20 W, 4000 K) and a series of spectra were measured for the same area used in the fluorescence measurement; and finally, a “reference” spectrum was measured over a spectralon panel at 98% under the same configuration used with the leaves.
Fig. 1: Experimental design. A leaf clip was used to acquire the fluorescence measurements as well as the spectral measurements, and the same field of view was used above a reference spectralon panel (a), above sampled leaf (b) and during the kinetics display on the leaf (c).

Subsequently, two distinct protocols were used. The first protocol involved half of the leaves (5 of each species and for each treatment), which were instantaneously removed after measurements at dim light and immersed in nitrogen liquid. The leaves were preserved at −80°C and used for pigment quantification.

The second protocol involved the other half of the leaves, which was used to investigate the PRI and chlorophyll fluorescence responses to PAR variations. A light kinetic of 6 different levels of PAR from 250 µmol m⁻² s⁻¹ to 2000 µmol m⁻² s⁻¹ was applied to these leaves for 6 minutes, to simulate increasing and decreasing levels of PAR. Only the red light of the PAM 2500 was used to avoid apparent reflectance variations caused by light in the wavelength range used for the PRI. Optical measurements were acquired every second during the light kinetic, and fluorescence measurements were acquired each minute on the same area of the leaf (Fig. 1).

After 2 weeks from the start of the experiment, 3 pots of each species were moved from one treatment to the other one to study the impact of successive constraints. Therefore, 6 pots (two species) from the “OZ” treatment cell were placed into the “RH” treatment cell
and 6 pots from the “RH” treatment cell were placed into the “OZ” treatment cell (Fig. 2).

![Temporal design of the cells sampled by day of year, for each treatment. RH indicates the atmospheric water constraint treatment (in blue), OZ indicates the ozone treatment (in red), RH_OZ indicates the plots in the OZ treatment after undergoing the RH treatment (red line on blue background) and OZ_RH indicates the plots in the RH treatment after undergoing the OZ treatment (blue line on red background).](image)

**Leaf pigment contents quantification**

To quantify the leaf pigment contents, 15 mg of leaf material was extracted with 10 ml of methanol and stirred for 90 minutes at 45°C. The samples were then centrifuged for 5 minutes at 4000 g based on the method described by Cerovic et al.\(^{37}\). The absorption spectra of the sample solutions were measured using a spectrophotometer (model HP 8453, Agilent UV-VIS, CA, USA). The total amount of chlorophyll (µg/cm², chlorophyll a and chlorophyll b) was calculated using the extinction coefficients for pure methanol given in Porra et al.\(^{38}\). The total amount of carotenoids and anthocyanin was calculated using Lichtenthaler\(^{39}\) and Cerovic et al.\(^{40}\) respectively.

**Data analysis**

At each step of the experiment, the effects of the applied constraints were monitored using their cumulative effects. For the “OZ” treatment, the AOT40 (accumulated ozone exposure over a threshold of 40 ppb) was calculated to determine the ozone impact on the leaves. The AOT40 (expressed in ppb) is the sum of the differences between the hourly ozone concentrations...
(greater than 40 ppb) and 40 ppb over a given period of time. In this study, the AOT40 was calculated daily. The threshold concentration of 40 ppb is commonly reported as the value at which significant damage can be observed on the vegetation41.

In the “RH” treatment, the effect of the air humidity was expressed in terms of air evaporative demand (EA). This demand is expressed in mm of water and calculated using the formula in the Penman evapotranspiration equation:

\[ EA = 0.26 \left[ e(Ta) - ea \right] \] (Eq.1)

where \( e(Ta) \) is the saturation vapor pressure (hPa) and \( ea \) the average water vapor pressure in the air (hPa).

In Eq. 1, the evaporative demand of the atmosphere is only dependent on the temperature and relative humidity and assumes no wind.

Spectral measurements were performed below the LED panel, and the PRI and PRI\(_0\) were calculated as described below:

\[ PRI = \left( \frac{R_{531} - R_{570}}{R_{531} + R_{570}} \right) \] (Eq. 2)

The PRI responses to PAR variations were analyzed using a non-linear regression based on the following expression given in Merlier et al.34:

\[ PRI(t) = PRI_0 + \Delta PRI \times erf\left( \frac{PAR(t)}{PAR_{sat}} \right) \] (Eq.3)

with:

\[ erf\left( \frac{PAR(t)}{PAR_{sat}} \right) = \frac{2}{\sqrt{\pi}} \int_0^{\frac{PAR(t)}{PAR_{sat}}} \exp^{-x^2} dx \]

where \( PAR_{sat} \) and \( \Delta PRI \) are parameters estimated using the mean square minimization and erf is the error function (also called the Gauss error function).
At the leaf scale, the chlorophyll content was also estimated using the mNDI<sub>705</sub> (modified red-edge Normalized Difference Index<sup>42</sup>) as shown in previous studies<sup>32</sup>.

\[
mNDI_{705} = \frac{R_{715} - R_{705}}{R_{715} + R_{705} - 2 \times R_{445}}
\]  
(Eq.4)

R<sub>445</sub>, R<sub>531</sub>, R<sub>570</sub>, R<sub>705</sub> and R<sub>715</sub> are reflectances integrated over a 5 nm waveband centered on 445 nm, 531 nm, 570 nm, 705 nm and 715 nm, respectively. The signal at 750 nm that is usually used for the mNDI<sub>705</sub> is noisy due to the actinic light; thus, it was replaced by the signal at 715 nm.

The fluorescence data were used to derive different parameters. The Y(II) is the effective quantum yield of PSII<sup>43</sup> which expresses the photosynthesis rate.

\[
Y(II) = \frac{Fm' - F}{Fm'}
\]  
(Eq.5)

Fm' is the maximum fluorescence levels induced by saturating light pulses: which temporarily close all PS II reaction centers and F corresponds to the momentary fluorescence yield of an illuminated sample.

The Y(NPQ) is the fraction of energy dissipated via the regulated NPQ linked to heat dissipation and xanthophyll cycle<sup>44</sup>.

\[
Y(NPQ) = \frac{F}{Fm'} - \frac{F}{Fm}
\]  
(Eq.6)

Fm is the maximum fluorescence level elicited by a strong light pulse: which closes all PS II reaction centers on a dark adapted leaf.

Y(NO) is the fraction of energy passively dissipated in the form of heat and fluorescence, and it corresponds to NPQ because of photo-inactivation of PSII and constitutive thermal dissipation independent of the xanthophyll cycle<sup>44</sup>. Y(NO) may also be involved in the PRI
when the predominant mode of excess energy dissipation does not involve the xanthophyll cycle\textsuperscript{45}.

\[
Y(NO) = \frac{F}{Fm} \quad \text{(Eq.7)}
\]

Statistical analyses were performed in MATLAB. Analyses of the correlations between the fluorescence parameters, optical indices and pigment contents were determined using regressions between these variables. The relationships between PRI responses to PAR variations (Eq.3) were fitted using a non-linear optimization algorithm (interior-point algorithm). The relationships between parameters (PRI\textsubscript{0} and PAR\textsubscript{sat}) and explanatory variables (AOT40 and EA) were investigated using multiple regression analysis and slopes were compared between treatments and species.

We note that, in our experimental design, the trees were maintained under these constraining conditions to provoke significant changes in the biochemical and ecophysiological properties of the leaves and then study the responses of PRI to these changes. From statistical point of view, the aim is not to study the variability of leaf responses differences between treatments or within the same treatment, but to understand and quantify the nature and strength of the links between PRI and biochemical and functional characteristics of the sampled leaves. The goal of statistical analysis described above is to give insights to these issues.

**Results**

The chlorophyll contents were measured using direct measurements with the DUALEX and SPAD meters and after methanol extraction according to the spectrophotometric assay. Measurements of the chlorophyll content with DUALEX meter were validated in previous works\textsuperscript{37,46} and in this study using chlorophyll content measurements obtained by spectrophotometry and SPAD (data not shown).
The relationship between mNDI$_{705}$ (Eq.4) and leaf chlorophyll content using DUALEX is shown in Fig. 3. This relationship is highly significant ($R^2=0.8$, $p<0.001$) and validates the use of mNDI$_{705}$ to estimate the leaf chlorophyll content. This relationship is highlighted for both species, *Q. robur* ($R^2=0.42$, $p<0.001$) and *Q. ilex* ($R^2=0.40$, $p<0.001$). Relationships were established among the physiological properties of the dark-adapted leaves obtained by fluorescence measurements (Fo and Fm) and optical measurements in dim light (PRI$_0$). For both species, the PRI$_0$ values were correlated with the measured leaf chlorophyll contents (Fig. 4.a and Table 1; $R^2=0.56$, $p<0.001$). The strength of these relationships decreased when the species were considered independently ($R^2=0.22$, $p<0.001$ for both species), due to low intra-specific chlorophyll variability. The mNDI$_{705}$ was a good estimator of PRI$_0$ when the two species are grouped together ($R^2=0.61$, $p<0.001$), and the strength of the relationship decreased when the species were separated, with a better correlation for *Q. robur* ($R^2=0.32$, $p<0.001$) than for *Q. ilex* ($R^2=0.23$, $p<0.001$). The Fv/Fm ((Fm-Fo)/Fm) value, which is the quantum yield of the open centers of PSII, is correlated to PRI$_0$ value for both species ($R^2=0.35$ $p<0.001$) (Table 1). The correlation between “Fm-Fo” and PRI$_0$ (Fig. 4.b and Table 1) was highly significant when the two species were pooled together ($R^2=0.61$, $p<0.001$) and for *Q. robur* independently ($R^2=0.52$ $p<0.001$), although the correlation was not as strong for *Q. ilex* alone ($R^2=0.1$, $p<0.001$).
The PAR$_{sat}$ varied significantly between days for $Q.\ robur$ ($p<0.05$) but not for $Q.\ ilex$, and it was not correlated with any of the variables listed in Table 1 (mNDI$_{705}$, Fo, Fm, Fm-Fo, Fv/Fm) when species are considered independently. The relationships became weakly significant when both species were pooled.

Fig. 3: Relationship between mNDI$_{705}$ and chlorophyll content (mg/m$^2$) for both species ($Q.\ robur$ in blue and $Q.\ ilex$ in green).

Fig. 4: (a) Relationship between the PRI$_0$ and mNDI$_{705}$ and (b) relationship between the PRI$_0$ and (Fm-Fo) for both species ($Q.\ robur$ in blue and $Q.\ ilex$ in green).
Table 1: Summary of the regression for mNDI\textsubscript{705}, Fo, Fm and Fm-Fo on PRI\textsubscript{0} and PAR\textsubscript{sat} for the entire dataset ($R^2$; *, ** and *** indicate significant effects at $p<0.05$, $p<0.01$ and $p<0.001$, respectively).

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<th>Chlorophyll content (mg/m\textsuperscript{2})</th>
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<th>Fm</th>
<th>Fm-Fo</th>
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The PRI<sub>0</sub> vs. total chlorophyll relationship had a better relationship than PRI vs. total chlorophyll relationship (R<sup>2</sup>=0.35 p<0.001 vs. R<sup>2</sup>=0.04).

Fig. 5: Dynamics of the (a) chlorophyll content (mg/m²) and (b) PRI<sub>0</sub> for the entire experiment. RH represents the atmospheric water constraint treatment and OZ represents the ozone treatment; crosses in blue for Q. robur, and green for Q. ilex, red represents the combined treatments.
(atmospheric water deficit treatment followed by ozone treatment and vice-versa), vertical black lines represent standard deviation. The linear regressions are represented in black lines. The grey lines represent linear regressions for combined treatments (red measurements).

The temporal dynamics of carotenoids (mg/g) and the ratio between chlorophyll a and chlorophyll b (Chla/Chlb) for the entire experiment are represented in Fig. 6. The total amount of carotenoids showed an increasing trend in RH treatment for *Q. robur*, with the increase of VPD. The ratio of chlorophyll a and chlorophyll b showed a decreasing trend in OZ treatment for both species. There is a strong relationship between total chlorophyll content and total carotenoids ($R^2$=0.70) and anthocyanins ($R^2$=0.55) that makes interpretation of the PRI vs. chlorophyll content relationship difficult to deconvolute in terms of the pigments actually involved.

**Fig. 6:** Temporal dynamics of the carotenoids (mg/g, cross symbol and left y-axis) and the ratio between chlorophyll a and chlorophyll b (Chla/Chlb, point symbol and right y-axis) for the entire experiment. RH represents the atmospheric water constraint treatment and OZ represents the ozone treatment. Symbols in red represent the combined treatments (atmospheric water deficit treatment followed by ozone treatment and vice-versa). The linear regressions are represented in solid lines for
carotenoids and dotted for Chla/Chlb. The linear regressions in grey lines represent combined treatments (red measurements).

The effects of ozone and atmospheric water deficit on the parameters determined from Eq. 3, which summarize the PRI vs. PAR responses under different constraint conditions, were assessed using multiple linear models. To determine the effects of ozone, the AOT40 was used as a predictor of changes in PRI₀ and PAR_sat in the OZ and RH_OZ treatments. To determine the effects of atmospheric water deficits, the air evaporative demand (Ea – Eq.1) was used as the explanatory variable in all the treatments (control, RH and OZ). The RH data were measured within the three cells as described in section 2, and the results of these analyses are in Table 2.

The AOT40 explains the variability of PRI₀ and PAR_sat in a nuanced manner depending on the species. In Q. robur, the AOT40 explained 28% of the variance of PRI₀, and this relationship was significant in the two treatments OZ and RH_OZ, whereas in Q. ilex, the relationship was only significant in the treatment RH_OZ.

An increased amount of cumulative vapor deficit pressure induced PRI₀ variations in both species. For Q. robur, this factor was significant in all the treatments except OZ_RH, whereas for Q. ilex, this factor was significant for all the treatments except OZ and OZ_RH (Table 2).

Slopes of regressions between the two parameters (PRI₀ and PAR_sat) and the two predictors (EA and AOT40) are estimated for each species and treatments (Table 2). The relationship between the PRI₀ and both the predictors (EA and AOT40) was different between the two species, with Q. ilex being less impacted by the abiotic constraint. However, the slopes of the relationships between PAR_sat vs. EA and PAR_sat vs. AOT40 remained constant between species and between treatments. Therefore, the PAR_sat responses to constraints led to a unique relationship.
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|                      |         |    |    |       |       |             |              |             |       |
| **EA (mm)**          |         |    |    |       |       |             |              |             |       |
| *Q. robur*           |         |    |    |       |       |             |              |             |       |
| $PRI_o$              |         |    |    |       |       |             |              |             |       |
| **-6.32e-05**        | ***     | *  |     | -2.99e-04|   ***  | 0.24        | ***          | 282         | 0.0203|
| (5.48e-05)           |         |    |    |       |       |             |              |             |       |
| $PAR_{sat}$          |         |    |    |       |       |             |              |             |       |
| **-4.522**           | **      | *  |     | -8.2673|       | 0.23        |              | 83          | 0.461 |
| (2.2153)             |         |    |    |       |       |             |              |             |       |
| *Q. ilex*            |         |    |    |       |       |             |              |             |       |
| $PRI_o$              |         |    |    |       |       |             |              |             |       |
| **7.75e-05**         | *       |     | *** | 4.06e-05|     | 0.12        | ***          | 296         | 0.0122|
| (3.38e-05)           |         |    |    |       |       |             |              |             |       |
| $PAR_{sat}$          |         |    |    |       |       |             |              |             |       |
| ns                   | ns      |     | *** |       |       |             |              |             |       |
| **Both**             |         |    |    |       |       |             |              |             |       |
| $PRI_o$              |         |    |    |       |       |             |              |             |       |
| **-2.54e-04**        | ***     | *** |     | 4.57e-05|     | 0.05        | ***          | 578         | 0.024 |
| (5.58e-05)           |         |    |    |       |       |             |              |             |       |
| $PAR_{sat}$          |         |    |    |       |       |             |              |             |       |
| ns                   | ns      |     | *** |       |       |             |              |             |       |
| *species*            |         |    |    |       |       |             |              |             |       |
| $PAR_{sat}$          |         |    |    |       |       |             |              |             |       |
| **-6.0771**          | ***     | *** |     | 1.4209|       | 0.15        | ***          | 142         | 0.416 |
| (1.7991)             |         |    |    |       |       |             |              |             |       |

Table 2: Relationships of the AOT40 (ppb) and EA (mm) with the PRI_o and PAR_sat for Q. robur and Q. ilex in the 5 treatments; The significance of the predictors is *, ** and *** , which indicates significant effects at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, estimates of regression slopes and standard error. Control represents the control treatment, RH represents atmospheric water deficit treatment, OZ represents the ozone treatment, RH_OZ represents the plots in OZ treatment after undergoing RH treatment and OZ_RH represents the plots in RH treatment after undergoing OZ treatment. Sample size: number of observations (leaves). RMSE: root mean square error.
To obtain a corrected PRI that is not sensitive to the structural and biochemical properties of leaves, the PRI₀ measurements were subtracted from the PRI measured at different intensities of PAR. The results of this deconvolution procedure are illustrated in Fig. 7, which shows the relationships between the fluorescence quantum yields and PRI before and after correction (PRI_c). Quantum yield of photochemistry Y(II) shows a relationship with the PRI, mainly due to chlorophyll content, although it disappears when PRI₀ is subtracted from PRI (PRI_c). Low values of Y(II) are explained by low actinic light applied during the measurements of PAM fluorescence. The NPQ shows a significant correlation with the PRI (R²=0.12, p<0.001) for the two species together. This relationship increases and becomes more consistent with PRI_c (R²=0.32, p<0.001), highlighting the ability of PRI to track the xanthophyll cycle linked to NPQ. The goodness of the fit of the relationship with Y(NO) does not change with the use of PRI or PRI_c (R²=0.32, p<0.001 for both).
Fig. 7: Relationships between the quantum yield of photochemistry $Y(\text{II})$, active non-photochemical quenching $Y(\text{NPQ})$, passive non-photochemical quenching $Y(\text{NO})$, PRI and PRIc (PRI corrected). Blue points are Q. robur species and green points are Q. ilex species.

Discussion

The use of PRI as a proxy of photosynthesis activity requires a better understanding of its variability to distinguish between the variability linked to changes in the content of constitutive (slow) pigments, and other phenological leaf properties independent of the leaf physiological responses to stress, from the variability linked to photosynthetic activity and physiological functioning. Phenological effects may blur the physiological responses of PRI linked to LUE. For species with high chlorophyll content variability, such as deciduous species, or during exposure to severe constraints, the PRI signal interpretation may lead to a misunderstanding of the PRI vs. LUE relationship, which was previously highlighted in Nichol et al., Meroni et al. and Hmimina et al. Thus, taking account of the phenological component is required to correct the PRI.
Different approaches have been developed to differentiate PRI variability unrelated to LUE to better interpret the PRI signal linked to photosynthesis activity\textsuperscript{27-29}. Wu et al.\textsuperscript{19} defined a structurally related signal in PRI as a function of the leaf area index (LAI) to improve accuracy in the estimation of LUE. Soudani et al.\textsuperscript{35}, Hmimina et al.\textsuperscript{32-33} and Merlier et al.\textsuperscript{34} used the PRI\textsubscript{0}, which corresponds to PRI values at very low light estimated from PRI vs. PAR relationships, to correct the PRI and assess its variability related to changes in the xanthophyll cycle. This approach assumes that PRI variability, as LUE variability, is principally linked to PAR at short-term temporal scales, intra-daily or over a few days when variations of leaf chlorophyll content are not significant, implying the importance of studying their variability simultaneously. In this study, leaf-scale measurements of biochemical and spectral properties, PRI responses to PAR variations and leaf chlorophyll fluorescence parameters were used to distinguish between phenological and physiological variability of the PRI under different constraint conditions, ozone application and atmospheric water deficit.

As shown in Fig. 3, the mNDI\textsubscript{705} was highly correlated to chlorophyll content (R\textsuperscript{2}=0.8 \(p<0.001\)), which was measured using two field-portable instruments (DUALEX and SPAD) and by the standard laboratory-based spectrophotometric method. The mNDI\textsubscript{705}, as an optical measurement that was made directly on the studied area of each leaf, was chosen and used to estimate the leaf chlorophyll content hereafter. The PRI\textsubscript{0} measurements were linked to the structural and biochemical properties of the leaves and explained by the chlorophyll content, which is indicated by the strong relationship between the mNDI\textsubscript{705} and PRI\textsubscript{0} (Fig. 4.a) for both species (R\textsuperscript{2}=0.61, \(p<0.001\), Table 1). Similar results were previously reported in Hmimina et al.\textsuperscript{32} and at the canopy scale in Hmimina et al.\textsuperscript{33} and Merlier et al.\textsuperscript{34}. The relationship between PRI\textsubscript{0} and chlorophyll content was significant in each treatment and for the two species during the entire experiment regardless of the phenological and physiological cycle. The relationship between PRI\textsubscript{0} and chlorophyll content may imply other pigments, particularly total carotenoid
pigment, but being correlated with each other, it is difficult to determine precisely their respective contributions (Fig. 5 & 6). These results highlight the relevant use of PRI₀ as an optical proxy of constitutive (slow) pigment pools and more particularly chlorophyll content.

The PRI₀ was linked to the minimal fluorescence yield (Fo), which is the state when all reaction centers are in an active “open” state (R²=0.34, p<0.01 for both species together, Table 1). The Fo, the initial fluorescence of dark adapted leaf, is principally an emission of antenna chlorophyll-a pigments⁵⁰ that can be mobilized for photosynthesis and it was related to the chlorophyll content measurements of Q. Robur. The maximal fluorescence of the dark adapted leaf, Fm, appears when all of the reaction centers are in an inactive “close” form, and this value was also linked to the chlorophyll content and with the PRI₀ (R²=0.61, p<0.05 for both species together). “Fm-Fo” was the more relevant fluorescence parameter correlated to PRI₀ (R²=0.61, p<0.001 for Q. robur and Q. ilex pooled; R²=0.52, p<0.001 for Q. robur; R²=0.1, p<0.001 for Q. ilex). These results highlight the relevant use of PRI₀ as a proxy for the leaf fluorescence on dark adapted leaves.

The impacts of ozone accumulation and atmospheric water deficit induced a decreasing trend on chlorophyll-a to chlorophyll-b ratio, as previously reported under water-limiting conditions (Jaleel et al. 2009). Moreover, the amount of total carotenoid showed an increasing trend, suggesting the establishment of protecting photochemical processes⁵¹.

The impact of these constraints on the PRI₀, as a proxy of leaf pigment content, at the leaf scale were investigated using multiple regression analysis. Ozone accumulation was expressed as AOT40, and atmospheric water deficit was expressed as EA. The PRI₀ was explained by EA and AOT40 and was used to assess changes in the chlorophyll pigment content caused by the treatments for the two species (Table 2). The slopes of the regressions between PRI₀ vs. EA or PRI₀ vs. AOT40 were significantly different between the species and between the treatments,
highlighting the different biochemical responses of each species to constraint conditions. According to Karlsson et al.\textsuperscript{52}, \textit{Q. ilex} is particularly resistant to abiotic constraints, especially to ozone exposure, because of its larger leaf thickness, which is an important factor influencing the development of leaf injuries. However, after exposure to drought constraint, the addition of oxidative constraint induced by ozone caused additional constraint to the plant, especially on PRI\textsubscript{0}. The PRI\textsubscript{0} of \textit{Q. ilex} significantly varied when the atmospheric water deficit increased and then ozone injection occurred (Fig. 5 and Table 2). As reported in previous studies, water constraint before ozone constraint may result in a lower physiological response of the plant to ozone exposure\textsuperscript{53,54} compared to ozone exposure without water constraint. This result may be explained by defense mechanisms induced by drought constraint, which can limit the absorption of ozone and its impact on leaves\textsuperscript{55}. These effects were not observed for plants undergoing atmospheric water deficit after the ozone exposure (Table 2), but this result does not allow us to clearly conclude due to the possible insufficient ozone exposure. In our experiment, for this successive constraint (OZ\_RH), the application of an atmospheric water deficit occurred after that the ozone exposure had reached an AOT40 of 1300 ppb, which may be insufficient to cause additional effects on leaf properties.

For \textit{Q. robur}, the PAR\textsubscript{sat} significantly varied under constraint conditions and was correlated to the AOT40 and EA variability (Table 2). For \textit{Q. ilex}, the PAR\textsubscript{sat} did not vary significantly and was not correlated to the EA and AOT40, probably due to the relative resistance of this species to these climatic constraints. The slopes of the relationships between PAR\textsubscript{sat} vs. EA and PAR\textsubscript{sat} vs. AOT40 were not significantly different between treatments in \textit{Q. robur}. Thus, the PAR\textsubscript{sat} may be considered as a physiological component of PRI variability because its variations are independent of chlorophyll content variability, linked to the PRI\textsubscript{0}, but are correlated with abiotic constraints affecting photosynthesis, as previously reported in
Merlier et al.\textsuperscript{34}, which show that PAR\textsubscript{sat} values depend on soil water content, when chlorophyll content does not limit photosynthesis.

As shown in Fig. 7, the PRI is negatively linked to the quantum yield of photosystem, Y(II), and this relationship is lost when the PRI is corrected after the subtraction of the PRI\textsubscript{c}. As shown in previous studies, Y(II) is mainly under the control of chlorophyll content. This result can be explained by the importance of the phenological component in the PRI that is linked to the chlorophyll content\textsuperscript{33,34,49}, as opposed to the physiological component of PRI, especially in our conditions, wherein the leaves were maintained in low light due to Ecolab cell conditions. Leaves could be considered as shaded leaves, which could explains why photosynthesis and PRI saturate quickly under increasing PAR, leading to low Y(II) and low PRI variability. Subtracting the phenological component (PRI\textsubscript{c}) from PRI values leads to a decrease of the strength of the relationship between PRI\textsubscript{c} and Y(II). Nevertheless, this negative relationship between PRI and Y(II) appears to be an overlapping of several linear relationships controlled by biochemical properties (Fig. 7), as also shown in Hmimina et al.\textsuperscript{33}.

The Y(NPQ) values, the quantum yield of NPQ linked to the xanthophyll cycle, has a better relationship with the PRI\textsubscript{c} than with the PRI. These results highlight the effectiveness of the PRI to track NPQ and confirm the link between PRI and the xanthophyll cycle, especially when the phenological component, which is xanthophyll independent, is subtracted. The use of this correction did not modify the link between the PRI and Y(NO).

Finally, many studies have shown that the PRI could be used to detect variations of chlorophyll content before visible symptoms occurs\textsuperscript{56-58}, however, the use of PRI to track LUE is more erratic and not as well understood. The high sensitivity of the PRI in dim light on dark adapted leaves (PRI\textsubscript{c}) to structural and biochemical properties suggests that the relationship between the PRI and carbon assimilation of the canopy (LUE or GPP\textsuperscript{35,59,61}), which have been observed in many previous studies and established over long time scale, may mainly be
explained by canopy structural and biochemical variations and not directly caused by the effects of abiotic factors on photosynthetic functioning. The common methods of using the PRI, which do not exploit the close relationship between the PRI and PAR, may strongly limit the assessment of the physiological component of PRI. The approach developed here using the kinetics of PRI vs. PAR relationship to obtain the PRI$_0$ and PAR$_{sat}$ parameters allows for the separation of PRI's physiological and biochemical components. The physiological component of the PRI is highlighted by the relationships between PAR$_{sat}$ and abiotic factors (atmospheric water deficit and ozone in this study) and the biochemical component (PRI$_0$) is a proxy of the chlorophyll content.

**Conclusions**

The present study investigated the ability of the optical index PRI to inform on plant functioning under abiotic constraints. The responses of the PRI, which is linked to xanthophyll cycle, to abiotic constraint conditions (atmospheric water deficit and ozone accumulation) are studied for two species (*Q. robur* and *Q. ilex*) in climatic cells. Measurements of the optical and fluorescence parameters were simultaneously coupled, and the PRI responses to PAR variations were characterized under contrasting plant constraint conditions. Measurements of the PRI$_0$, PRI in dim light on dark adapted leaves, and PAR$_{sat}$, the value of PAR at PRI saturation, were determined in order to access the biochemical and physiological sources of PRI variability.

The induced constraints led to different responses between the studied species. *Q. ilex* appears more resistant to ozone accumulation and to atmospheric water deficit constraint while *Q. robur* is more sensitive to both factors, and significant variations in leaf biochemical and physiological characteristics were observed for this species in all treatments.

The PRI$_0$ is correlated with the leaf chlorophyll content whatever the species or the constraint applied and varied depending on the species. PRI$_0$ can therefore be used as a proxy of chlorophyll content and represents the PRI component that depends on pigment content.
rather than the plant physiological functioning. This dependency of PRI to chlorophyll content blurs its use as a proxy of photosynthesis activity *stricto sensu*. The use of the PRI₀ to correct the PRI allows for the assessment of the PRI’s physiological variability. This correction increased the strength of the relationship between PRI₀ and Y(NPQ), which is linked to the xanthophyll cycle. It is recommended to correct PRI values using PRI₀ measured at dim light on dark adapted leaves (or estimated by PRI vs. PAR relationships analysis).

The PARₙₐₜ varied, and decreased when constraints were applied on plants. The relationships between the PARₙₐₜ and the factors describing the abiotic constraints were independent from species or constraint. These results highlight the ability of PARₙₐₜ to track the physiological component of the PRI and suggest that it should be used as a potential indicator of the limiting factor affecting photosynthesis activity.

**Acknowledgments**

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