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PRI assessment of long-term changes in carotenoids/chlorophyll ratio and short-term changes in de-epoxidation state of the xanthophyll cycle

I. FILELLA*†, A. PORCAR-CASTELL‡, S. MUNNÉ-BOSCH§, J. BÄCK‡,
M. F. GARBULSKY†¶ and J. PEÑUELAS†
†Unitat d'Ecofisiologia CSIC-CEAB-CREAF, CREAF (Center for Ecological
Research and Forestry Applications), Universitat Autònoma de Barcelona,
Edifici C, 08193 Bellaterra, Spain
‡Department of Forest Ecology, Latokartanonkaari 7, PO Box 27, 00014
University of Helsinki, Finland
§Departament de Biologia Vegetal, Universitat de Barcelona,
Facultat de Biologia, Avinguda Diagonal 645, 08028 Barcelona, Spain
¶Faculty of Agronomy, University of Buenos Aires, Argentina

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The Photochemical Reflectance Index (PRI), an index based on leaf reflectance at 531 nm, has been found suitable for tracking variations in photosynthetic activity from leaf to ecosystem levels. This suitability has been attributed to PRI correlation with xanthophyll interconversion and photosynthetic radiation-use efficiency. However, other pigments and factors may be involved in such relationships. We studied the relationship between PRI and xanthophylls and other carotenoids in saplings of two widely distributed evergreen species (Scots pine and Holm oak) submitted to experimentally changing light conditions in a field experiment. PRI was strongly correlated with the de-epoxidation state of xanthophylls (DEPS, an expression of the relative concentration of the three xanthophyll cycle pigments), but also with carotenoids/chlorophyll ratio and β-carotene/chlorophyll ratio in both species. However, following momentary decreases in light due to clouds, PRI changed following the DEPS changes, while the carotenoids/chlorophyll ratio remained constant. The results show that PRI was able to reveal short-term changes in de-epoxidation state, i.e. the signal of xanthophyll interconversion, but it also tracked long-term changes in carotenoids/chlorophyll. Carotenoids other than xanthophylls, e.g. β-carotene, are also related to photoprotective processes, thus also making PRI effective as a measure of changes in photosynthetic light-use efficiency in response to stress on a long-term level.

1. Introduction

Several plant mechanisms are involved in short-term strategies to deal with a harmful excess of light. Among them, non-photochemical energy dissipation via the xanthophyll cycle is one of the most important mechanisms. In the xanthophyll cycle, violaxanthin is converted to zeaxanthin through the intermediate antheraxanthin in the presence of ascorbate and an acidic pH induced by the light-promoted proton pump (Demmig-Adams and Adams 1992), leading to an increase in energy dissipation as heat, which is

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*Corresponding author. Email: i.filella@creaf.uab.cat

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considered a mechanism of protection against photodamage (Demmig-Adams and Adams 2006). This process of dissipation of excess radiation by plants has been found to be related to increases in absorbance near 531 nm (Bilger and Björkman 1990, Demmig-Adams 1990). So the measure of changes in reflectance at this wavelength can give an indirect estimation of this dissipation of excess radiation and, therefore, of photosynthetic performance (Gamon *et al.* 1990). Gamon *et al.* (1993) reported that at the leaf level a reflectance signal at 531 nm was present during increased illumination in a wide range of species with different leaf morphology, different photosynthetic pathways and in different habitats. The Photochemical Reflectance Index (PRI), an index based on reflectance at 531 nm, has been found to be correlated with xanthophyll interconversion and photosynthetic radiation-use efficiency (CO_2 assimilation rate/incident Photosynthetic Photon Flux Density) at leaf (Peñuelas *et al.* 1995a, 1997, Gamon *et al.* 1997, Richardson and Berlyn 2002), proximal canopy (Gamon *et al.* 1992, Filella *et al.* 1996, Trotter *et al.* 2002) and ecosystem scale (Nichol *et al.* 2000, Peñuelas and Inoue 2000). Moreover, PRI has been found to be suitable for tracking both daily (Gamon *et al.* 1992, Peñuelas *et al.* 1994) and seasonal (Stylinski *et al.* 2002, Garbulsky *et al.* 2008) variations in photosynthetic activity. PRI is currently being tested at the ecosystem, region and biosphere scale for the estimation of the carbon balance (Rahman *et al.* 2001, Nichol *et al.* 2002, 2006, Garbulsky *et al.* 2008), which is one of the main concerns in the study of the ecology of global change (Ciais *et al.* 2005, Boisvenue and Running 2006, Schulze 2006).

However, the xanthophyll cycle signal alone seems too weak to explain such successful estimation of photosynthetic performance in diverse conditions and at varying temporal and spatial scales. The PRI may also be assessing stronger pigment changes other than just those of the xanthophyll cycle. PRI has recently been reported to be related to the carotenoid/chlorophyll ratio in green leaves (Sims and Gamon 2002, Filella *et al.* 2004). Carotenoids such as β-carotene or lutein are also related to photoprotective processes (Frank and Brudvig 2004, Telfer 2005, Dall'Osto *et al.* 2006). PRI variation may thus be a combined function of both short-term (diurnal) changes in the levels of xanthophyll cycle pigment and long-term (seasonal) relative changes in the pools of carotenoid and chlorophylls over a time span of several weeks. This influence of carotenoids/chlorophyll ratio on PRI could help to account for the significant relationships between PRI and photosynthetic radiation-use efficiency.

We aimed to study the relationship of PRI with xanthophylls and their de-epoxidation state (DEPS – an expression of the relative concentrations of the three xanthophyll cycle pigments), and with other carotenoids in two widely distributed evergreen species (Scots pine/Boreal Regions, and Holm oak/Mediterranean Regions) in a field experiment where we modified the light environment of potted plants in order to induce changes in the pigment content of the thylakoid membrane. We also measured F_v/F_m to track concurrently the state of photosystems or photosynthetic capacity. We aimed to evaluate the relationship between changes in the different carotenoids and the PRI both at short-term (daily) and long-term (seasonal) time-scales, together with the physiological status of the plant.

2. Materials and methods

2.1 *Species and experimental setting*

We studied two species: Scots pine (*Pinus sylvestris* L.) and Holm oak (*Quercus ilex* L.). Forty potted four-year-old Scots pine saplings and thirty potted four-year-old

Holm oak saplings were purchased from Forestal Catalana S.A. (Barcelona) and transferred to the experimental site on 20 June 2006. On 21 June, the saplings were separated into two different light environments (50%–50%) (shade and full sunlight) for their long-term acclimation. The saplings under the shading structure received approximately 50% of the light intensity compared to the full sunlight conditions. During the summer, the saplings were regularly watered to avoid any water stress.

Approximately three months later, on the 18 September, ten pine saplings from the shaded conditions were transferred to full sunlight (Shade–Sun treatment), and ten saplings from full sunlight transferred to the shade (Sun–Shade treatment). The rest of the saplings in each light environment were left as controls (Shade and Sun treatments). The same process was followed with the Holm oak on the 26 September. However, the number of trees was smaller, with seven trees in the Sun and Shade controls, and eight trees in the transferring treatments (Sun–Shade and Shade–Sun).

Measurements of the different variables were taken 14, 21 and 28 days after the beginning of the treatments in *P. sylvestris* saplings, and 8, 15 and 29 days after the beginning of treatment in *Q. ilex* saplings.

2.2 *Environmental data*

Two light sensors (QUANTUM: Q 30258, Q 30259, LI-COR, USA), and two self-manufactured and calibrated thermistors were used to monitor photosynthetic flux density (PFD) and air temperature, respectively, in each of the light environments. A single measurement was recorded every 30 seconds with a data logger (Micrologger 21X, Campbell Scientific Ltd, England).

2.3 *Monitoring of photosynthetic capacity*

Maximum photochemical efficiency $(F_m\text{-}F_o)/F_m$ (or F_v/F_m) was monitored with a fluorescence system (PAM-2000, Walz, Germany). Minimum chlorophyll fluorescence intensity (F_o) was measured after a minimum of one hour of dark acclimation, and maximum chlorophyll fluorescence intensity (F_m) was obtained subsequently by supplying a light-saturating pulse to the leaf. Two current-year needle pairs (*P. sylvestris*), or one new and fully developed leaf (*Q. ilex*), were dark-acclimated using clips in Scots pine and Holm oak, respectively. Three measurements per tree were taken, and three trees from each of the four treatments (monitoring trees) were sampled every sampling day. Leaves were randomly selected from the seedling's foliage since we assumed that the light environment remained homogeneous inside the open seedling canopies. Measurements were usually performed around noon (11:30–12:30, local time).

2.4 *Monitoring of pigment contents*

Three to four current-year needle pairs (Scots pine) and two to three new and fully developed leaves (Holm oak) were randomly collected from the seedling's foliage and immediately frozen in liquid nitrogen each sampling day. Three trees per treatment, the same trees in which reflectance and fluorescence measurements were taken, were sampled. Pigment sampling was carried out around noon (12:00–13:00 local time). Samples were subsequently stored at –80°C until extraction.

The extraction and HPLC analyses of the photosynthetic pigments chlorophyll-*a*, chlorophyll-*b*, neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and β-carotene were carried out as described by Munné-Bosch and Alegre (2000). In short, leaves were ground in liquid nitrogen and repeatedly extracted with ice-cold 85 (v/v) and 100% acetone using sonication. Pigments were separated on a Dupont non-endcapped Zorbax ODS-5 µm column (250×4.6 mm, 20%C, Scharlau, Barcelona, Spain) at 30°C at a flow rate of 1 ml min^{-1}. The solvents consisted of (A) acetonitrile/ methanol (85: 15, v/v) and (B) methanol/ethyl acetate (68: 32, v/v). The gradient used was: 0–14 min 100% A, 14–16 min decreasing to 0% A, 16–28 min 0% A, 28–30 min increasing to 100% A, and 30–38 min 100%A. Detection was carried out at 445 nm (Diode array detector, HP1100 Series, Agilent Technologies, Santa Clara, CA, USA). Compounds were identified by coelution with authentic standards, which were obtained from Fluka (Buchs, Switzerland).

De-epoxidation state (DEPS), was calculated as (zeaxanthin+0.5antheraxanthn)/ (violaxanthin+zeaxanthin+antheraxanthin) (Thayer and Bjorkman 1990).

2.5 *Reflectance measurements and indices*

Leaf-based reflectance was measured with a UniSpec Spectral Analysis System/ Reflectometer (PP Systems, Haverhill, MA, USA), operated with a palmtop PC. Single leaves and needles from five plants per treatment per date were individually sampled by clamping them into a leaf clip (adaxial side upwards) attached to a fibre-optic halogen light source and detector. Four scans per sample were integrated (integration time 10 ms). Reflectance measurements were preceded by a dark scan, and were compared with reflectance from a Spectralon (Labsphere Inc., North Sutton, NH, USA) white standard to obtain percent reflectance. From the reflectance data, PRI was calculated as $(R_{531} - R_{570})/(R_{531} + R_{570})$ (Gamon *et al.* 1992, Peñuelas *et al.* 1995a).

2.6 *Data treatment and statistical analyses*

Repeated measures analyses of variance (RM-ANOVA), with pigment ratios, DEPS, F_v/F_m and PRI as dependent variables and irradiance treatment as the independent factor, and regression analyses between PRI and the other variables were conducted. Statistical differences between treatments in each season were also analysed with a Student *t*-test. For all these analyses the program package STATISTICA v. 6.0 for Windows (StatSoft, Inc. Tulsa, Oklahoma) was used.

3. Results

3.1 *Xanthophylls and other carotenoids*

The de-epoxidation state of the xanthophyll cycle pigments was higher in sun treatments than in shade treatments, both in *P. sylvestris* and *Q. ilex* ($p < 0.005$ for *P. sylvestris* and $p < 0.001$ for *Q. ilex*), and decreased to reach the values of the shade treatments when the weather was cloudy, or even when leaf collection coincided with momentary cloudy conditions (figure 1). The total xanthophyll pool relative to chlorophyll-*a*+*b* content in *Q. ilex* tended to be higher in the full sunlight treatments compared to the shaded ones, and increased significantly in the treatment Shade–Sun one month after transfer from shade to sun conditions ($p < 0.005$) (figure 1). However, in *P. sylvestris*, the pool of xanthophylls in relation to chlorophyll-*a*+*b* did not change between the treatments or during the experiment. Only in *Q. ilex*, the xanthophyll pool increased in the saplings transferred from shade to full sunlight (figure 1), in line with the hypothetical acclimation to the new light conditions.

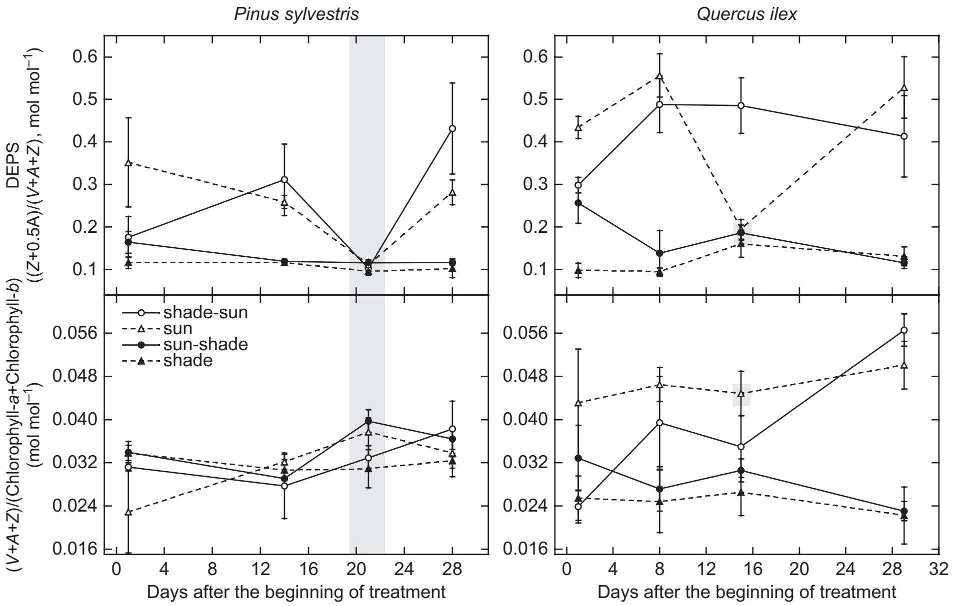

Figure 1. Time courses of de-epoxidation state (DEPS) and V+A+Z (violaxanthin+anthera xanthin+zeaxanthin)/chlorophyll-*a*+*b* after the beginning of treatment in *Pinus sylvestris* and *Quercus ilex*. The shaded area indicates cloudy conditions while sampling. Error bars are SE ($n = 3$).

In both species, saplings brought to full sunlight conditions (Shade–Sun) experienced an increase in the relative proportion of carotenoids to chlorophyll-*a*+*b*, compared to the respective controls in the shade. In *P. sylvestris*, levels in sun treatments were significantly higher than in the shade ones after 20 days of treatment ($p < 0.005$). *Q. ilex* saplings presented a higher proportion of carotenoids relative to chlorophyll-*a*+*b* than *P. sylvestris* in all treatments (figure 2) and in *Q. ilex*, the carotenoids relative to chlorophyll-*a*+*b* in the Shade–Sun treatment plants had increased significantly after one month ($p < 0.001$). Furthermore, β-carotene/chlorophyll-*a*+*b* increased through the experiment in the control sun treatment in *P. sylvestris* and in the Shade–Sun treatment in *Q. ilex*. In *P. sylvestris* at all sampling dates and in *Q. ilex* at the end of the experiment the shade saplings presented the lowest values (figure 2). Lutein content was twice higher than β-carotene levels in *P. sylvestris*, while it presented levels similar to β-carotene in *Q. ilex*. We did not observe any difference in the lutein pattern between treatments (data not shown).

3.2 F_v/F_m and PRI

The maximum photochemical efficiency of photosystem II, $F_\mathrm{v}/F_\mathrm{m}$, decreased slightly in the *Q. ilex* saplings transferred to full light (Shade–Sun) but was almost totally recovered after two weeks. In contrast, the saplings transferred from full sunlight to shade (Sun–Shade) presented initially signs of stress, as indicated by the lower $F_\mathrm{v}/F_\mathrm{m}$ levels, but recovered rapidly reaching $F_\mathrm{v}/F_\mathrm{m}$ levels found in the non-stressed shade control (shade). In *P. sylvestris*, the saplings transferred to full light (Shade–Sun) presented lower $F_\mathrm{v}/F_\mathrm{m}$ values than the other treatments and did not recover during the

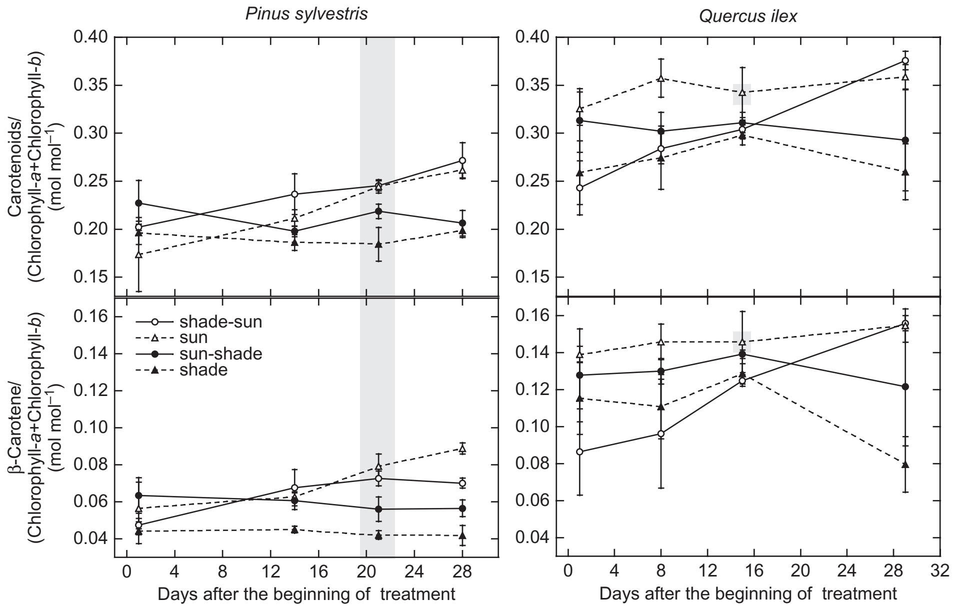

Figure 2. Time courses of carotenoids/chlorophyll-$a+b$ and β-carotene/chlorophyll-$a+b$ after the beginning of treatment in *Pinus sylvestris* and *Quercus ilex*. The shaded area indicates cloudy conditions while sampling. Error bars are SE ($n = 3$).

treatment. On the cloudy day, which followed a three-day cloudy period, all treatments showed an increase in F_v/F_m. Overall, the fluorescence data indicate that the most stressed leaves were those of the *P. sylvestris* saplings transferred from shade to sun conditions (Shade–Sun) (figure 3).

PRI values of *P. sylvestris* saplings were significantly lower in the sun treatments (Sun, Shade–Sun) ($p < 0.01$) and showed a recovery on the cloudy day (figure 3), mirroring the DEPS behaviour (figure 1). In *Q. ilex*, although there was a trend towards lower PRI values in sun saplings, the difference was significant only at the end of the experiment. There was an increase in the PRI value in the Sun and Shade–Sun pine saplings on the twenty-first day of treatment and in the Sun *Q. ilex* saplings on the fifteenth day of treatment, coinciding with the observed decrease in DEPS caused by the cloudy conditions. Carotenoids/chlorophyll-$a+b$ in light treatments did not follow the changes in PRI, DEPS and F_v/F_m on the cloudy day (figures 2 and 3).

3.3 *Relationship of PRI with fluorescence and pigments*

PRI was inversely correlated with DEPS in both species ($p < 0.01$) (figure 4). The correlation was, however, driven by the strong changes depending on sun and shade conditions. PRI was also inversely correlated with carotenoids/chlorophyll-$a+b$ and β-carotene/chlorophyll-$a+b$ in both *P. sylvestris* and *Q. ilex* (figure 4), and directly correlated with F_v/F_m in *P. sylvestris* (data not shown, $R^2 = 0.5$, $p = 0.01$). When focusing on the Sun and Shade–Sun saplings of *P. sylvestris* or on those of *Q. ilex* which responded to the changes in radiation caused by clouds, PRI was still correlated with the DEPS ($p < 0.02$ for *P. sylvestris*), while it was not correlated with carotenoids/chlorophyll-$a+b$ (figure 5). When separating data from sun and shade

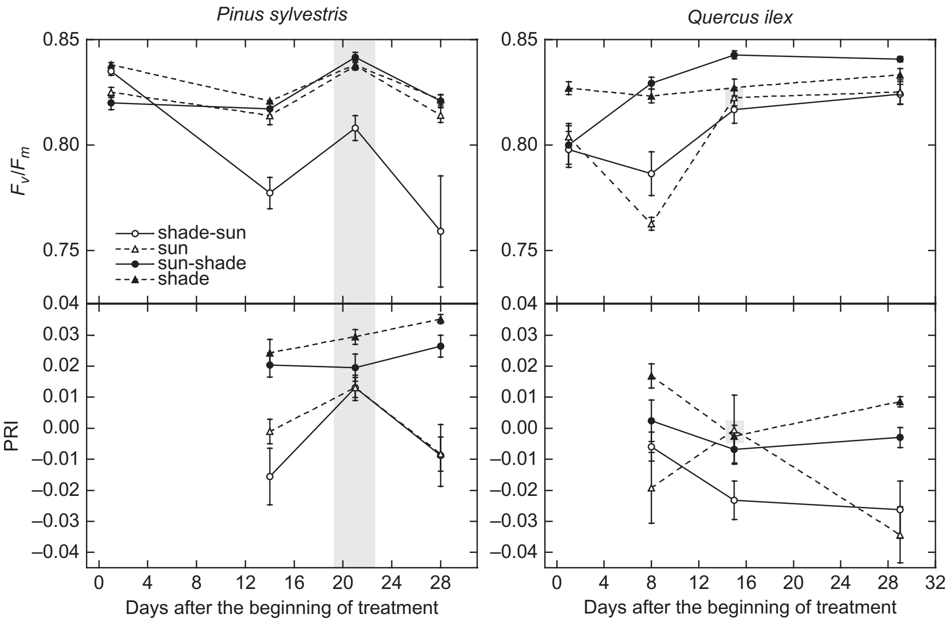

Figure 3. Time courses of F_v/F_m (maximum photochemical efficiency of photosystem II) and PRI (Photochemical Reflectance Index) after the beginning of treatment in *Pinus sylvestris* and *Quercus ilex*. The shaded area indicates cloudy conditions while sampling. Error bars are SE ($n = 9$ for F_v/F_m, and $n = 3$ for PRI).

conditions, i.e. either with high or low DEPS, carotenoids/chlorophyll-*a*+*b* remained correlated with PRI (figure 6).

4. Discussion

PRI was strongly inversely correlated with DEPS, carotenoids/chlorophyll-*a*+*b* ratio and β-carotene/chlorophyll-*a*+*b* ratio (figure 4). However, when considering only Sun treatment saplings, responding to momentary decreases in light because of clouds, PRI changed following the changes in DEPS, while the carotenoids/chlorophyll-*a*+*b* ratio remained constant (figure 5). When considering separately the data obtained in sun or shade conditions, i.e. with high or low DEPS, PRI still followed the carotenoids/chlorophyll-*a*+*b* ratio. PRI was thus able to assess rapid interconversions between xanthophyll cycle pigments, occurring on a time-scale of minutes, in addition to changes in carotenoid and chlorophyll contents occurring over periods of days to weeks. PRI was designed to follow the changes in the levels of xanthophylls comparing the reflectance at 531 nm, where xanthophylls absorb, with reflectance at 570 nm as a reference. However, since both carotenoids and chlorophylls still absorb in the 530 nm spectral region, and chlorophyll only slightly absorbs at 570 nm, PRI also measures the relative proportion between these two pigment groups.

Sims and Gamon (2002) suggested that PRI variation over the diurnal cycle will be a function primarily of xanthophyll cycle changes, while PRI variation over weeks or months may be a combined function of the xanthophyll cycle and changes in the pools of carotenoids and chlorophylls (Sims and Gamon 2002, Stylinski *et al.* 2002). We found here that, in effect, PRI was affected by the pool of carotenoids relative to

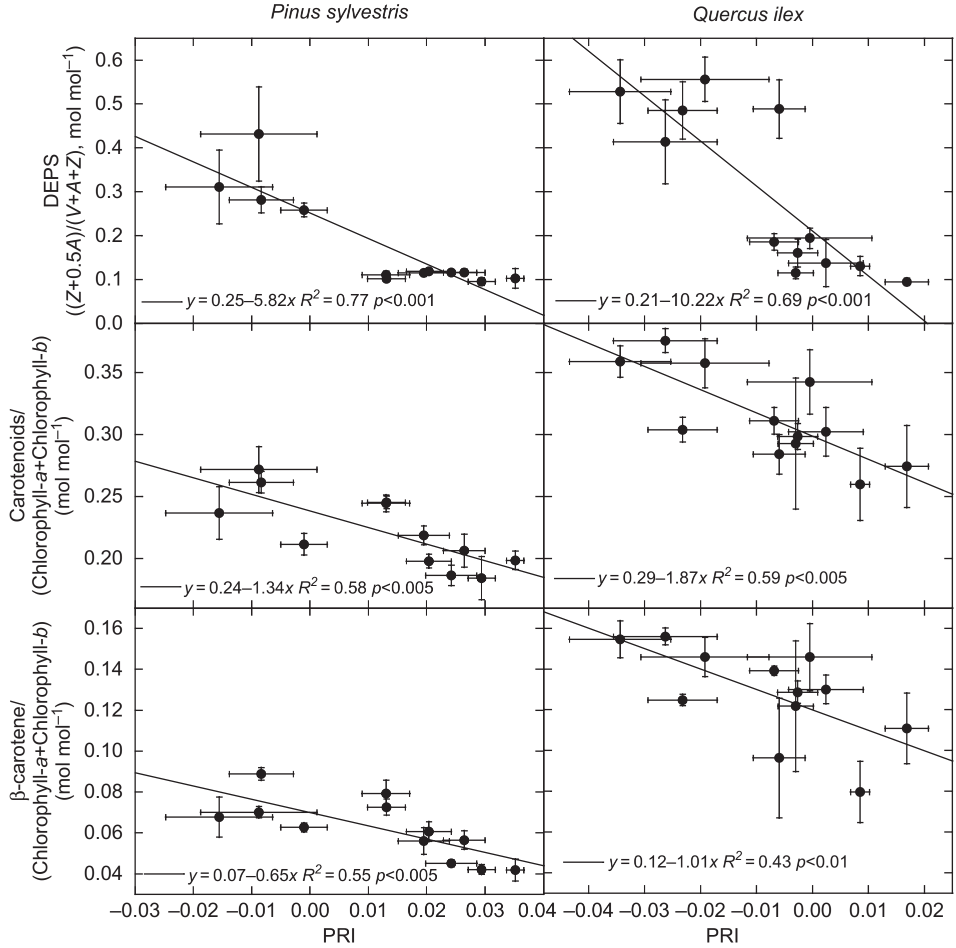

Figure 4. Relationship between PRI (Photochemical Reflectance Index) and DEPS (de-epoxidation state), carotenoids/chlorophyll-*a*+*b* and β-carotene/chlorophyll-*a* + *b* in *Pinus sylvestris* and *Quercus ilex*. Error bars are SE (n = 3).

chlorophylls and was able to discriminate the signal of xanthophyll interconversion over the higher absorption of carotenoids.

Plants have developed both short-term and long-term strategies to deal with excess light. At the daily and seasonal time-scale (weeks–months), several acclimation processes take place in leaves to adjust to the patterns of light and temperature. The long-term strategies are related mainly to changes in amount and composition of antenna pigments and increases in the levels of ROS (Reactive Oxygen Species) scavenging enzyme-systems (Telfer 2005). In evergreen species, such as Scots pine and Holm oak, the biochemical composition of the thylakoid membrane undergoes seasonal changes to adjust the energy absorption capacity to the prevailing energy demand of the plant (Öquist and Huner 2003, Ensminger *et al.* 2004). Any stress, such as drought, growth cessation or extreme temperatures may induce down-regulation of the photosynthetic capacity and, then, absorbed light may be in excess, thus requiring biochemical acclimation of the pigment components in the photosynthetic membrane

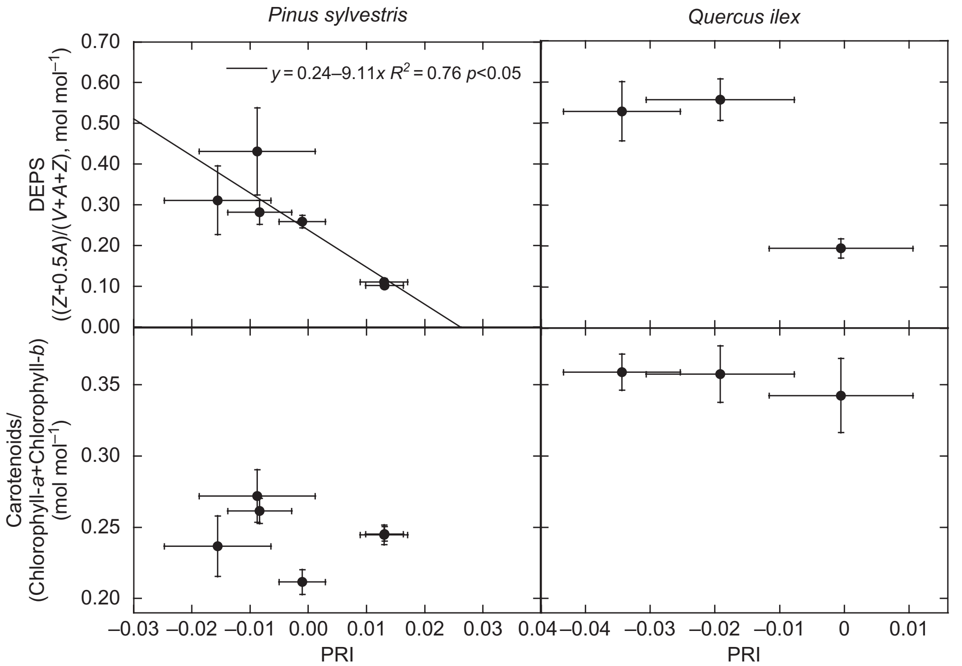

Figure 5. Relationship between PRI (Photochemical Reflectance Index) and DEPS (de-epoxidation state) and carotenoids/chlorophyll-*a*+*b* considering only treatments affected by changes in radiance produced by cloudy conditions, i.e. Sun and Shade–Sun treatments plants for *Pinus sylvestris*, and Sun treatment plants for *Quercus ilex*. Error bars are SE (n = 3).

(Huner *et al.* 1998). The light treatments caused different effects in the concentrations of photoprotective pigments in the two species. The proportion of xanthophyll to chlorophyll reflects the relative protection level that xanthophylls confer to the photosystem. Total xanthophyll pool relative to chlorophyll in *Q. ilex* was higher in the full sunlight treatments compared to the shaded ones, consistent with an acclimation to higher light environments (Demmig Adams 1998) and, thus, *Q. ilex* sun-exposed saplings reached much higher protection levels than shaded saplings (figure 1) in accordance with the literature (Demmig Adams 1998). However, *P. sylvestris* exhibited an already high pool of xanthophylls that did not change during the experiment or between treatments. The carotenoid composition of *P. sylvestris* has been found to vary seasonally with a twofold increase in lutein and the carotenoids of the xanthophyll cycle during winter, allowing *P. sylvestris* to maintain a large stock of chlorophyll in a photoprotected state, and a rapid recovery of photosynthesis in spring (Ottander *et al.* 1995, Ensminger *et al.* 2004, Porcar-Castell *et al.* 2005).

The influence of all these changes in carotenoids/chlorophyll ratio on PRI can have important implications for the use of PRI as an estimator of photosynthetic radiation-use efficiency. However, considering that not only xanthophylls but also other carotenoids, such as β-carotene or lutein, are related to photoprotective processes (Frank and Brudvig 2004, Telfer 2005, Dall'Osto *et al.* 2006), we still regard PRI as an effective reflectance index capable of measuring changes in photosynthetic light-use efficiency in response to stress. It may provide an integrated remote sensing assessment of photoprotective and accessory pigments and, therefore, of photosynthetic

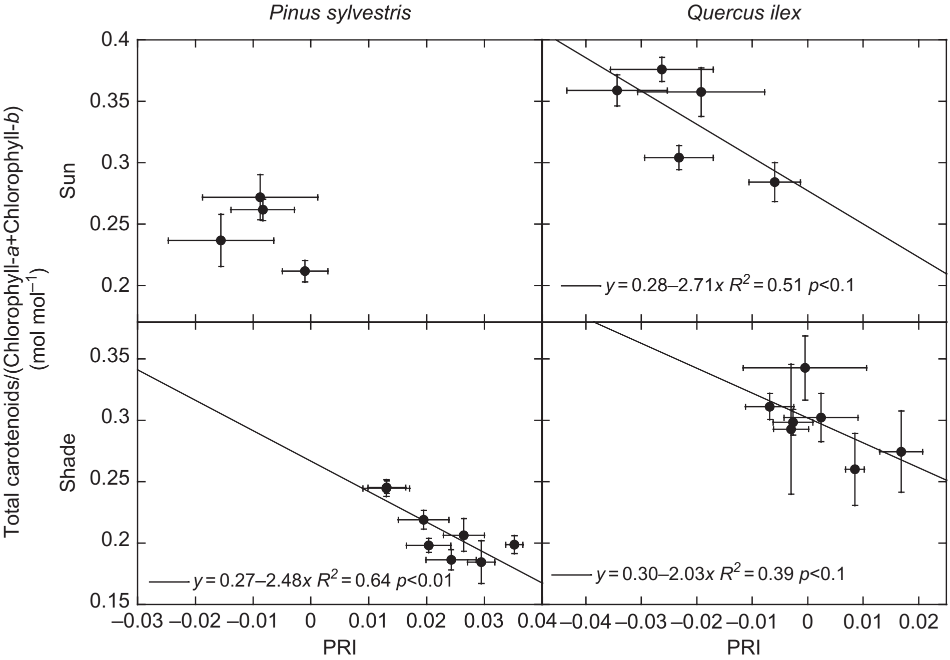

Figure 6. Relationship between PRI (Photochemical Reflectance Index) and carotenoids/chlorophyll-*a*+*b* in *Pinus sylvestris* and *Quercus ilex,* separating values for sun and shade conditions, corresponding to two classes without significant differences in DEPS. Error bars are SE ($n = 3$).

radiation-use efficiency to the extent that this is correlated with carotenoid/chlorophyll ratio. Stress usually results in an increase of this carotenoid/chlorophyll ratio and decreased photosynthetic light-use efficiency. Chlorophyll decreases more rapidly than carotenoids when plants are under stress or during leaf senescence (Gitelson and Merzlyak 1994, Peñuelas *et al.* 1995b). During senescence, however, with chlorophyll degradation and leaf yellowing, the carotenoids to chlorophyll ratio is more variable and the relationship between PRI and xanthophyll pigment cycle can break down. In fact, an inconsistent relationship between PRI and the epoxidation state of xanthophylls and radiation-use efficiency has been reported in senescent leaves (Gamon *et al.* 2001, Nakaji *et al.* 2006). In conclusion, over the long term (weeks), PRI tracked the variations in the carotenoids/chlorophyll-*a*+*b* ratio and, over the short term (minutes), the changes in the de-epoxidation state. We also found that PRI is able to discriminate the signal of xanthophyll interconversion above the signal of the absorption of carotenoids. These results reinforce the value of PRI as a remote sensing reflectance index able to estimate changes in photochemical efficiency linked to changes in carotenoids/chlorophyll-*a*+*b* in the long term, at a scale of days to weeks, in addition to changes in photochemical efficiency linked to short-term (minutes to hours) changes in the xanthophyll cycle. The efficiency with which light energy is converted varies significantly between plants, season and ecosystems. Tracking short-term changes in photosynthetic light regulation at photosystem with PRI provides a means of assessing this efficiency. In Mediterranean forest, for example, seasonal variation in radiation interception is very low and carbon absorption has important seasonal fluctuations with periods of very low photosynthesis (Garbulsky *et al.* 2008). Due to the negligible changes in structure and leaf area index (LAI) and, thus, in radiation interception, it is of great importance in this forest to have accurate measurements of short-term changes in radiation-use efficiency. With PRI it is now possible to improve assessments from airborne sensors of the carbon uptake in many of the world's ecosystems (Rahman *et al.* 2001, Nichol *et al.* 2002, 2006). However, PRI has been shown to be sensitive to LAI (Barton and North 2001) and soil background (Barton and North 2001, Filella *et al.* 2004) and incapable of detecting short-term changes in DEPS when cover or LAI are low. Simultaneous estimation of LAI can be helpful in those cases

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