



Chlorophyll fluorescence, photochemical reflective index and normalized difference vegetative index during plant senescence

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ABSTRACT

The relationship between the Photochemical Reflectance Index (PRI), Normalized Difference Vegetation Index (NDVI) and chlorophyll fluorescence along senescence was investigated in this work. Reflectance and radiance measurements were performed at canopy level in grass species presenting different photosynthetic metabolism: *Avena sativa* (C3) and *Setaria italica* (C4), at different stages of the natural senescence process. Sun induced-chlorophyll fluorescence at 760 nm (SIF₇₆₀) and the apparent fluorescence yield (SIF₇₆₀/a, with a = irradiance at time of measurement) were extracted from the radiance spectra of canopies using the Fraunhofer Line Discrimination-method. The photosynthetic parameters derived from Kautsky kinetics and pigment content were also calculated at leaf level.

Whilst stand level NDVI patterns were related to changes in the structure of canopies and not in pigment content, stand level PRI patterns suggested changes both in terms of canopy and of pigment content in leaves. Both SIF₇₆₀/a and Φ_{PSII} decreased progressively along senescence in both species. A strong increment in NPQ was evident in *A. sativa* while in *S. italica* NPQ values were lower. Our most important finding was that two chlorophyll fluorescence signals, Φ_{PSII} and SIF₇₆₀/a, correlated with the canopy PRI values in the two grasses assessed, even when tissues at different ontogenic stages were present. Even though significant changes occurred in the Total Chl/Car ratio along senescence in both studied species, significant correlations between PRI and chlorophyll fluorescence signals might indicate the usefulness of this reflectance index as a proxy of photosynthetic RUE, at least under the conditions of this study. The relationships between stand level PRI and the fluorescence estimators (Φ_{PSII} and SIF₇₆₀/a) were positive in both cases. Therefore, an increase in PRI values as in the fluorescence parameters would indicate higher RUE.

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1. Introduction

Net Primary Production (NPP), the rate of net C gains of vegetation, is a key functional attribute of ecosystems (McNaughton et al., 1989). During the last two decades, major improvements on NPP estimation have been made through the use of remotely sensed data (Running et al., 2000; Hilker et al., 2008). Remote sensing estimates of C gains rely on a simple model, proposed by Monteith, which states that C gains is the product of the absorbed photosyn-

thetically active radiation (APAR) by vegetation and the efficiency with which this radiation can be converted into biomass (Radiation Use Efficiency, RUE) (Monteith, 1972, 1977). APAR, in turn, results from the product of the incoming photosynthetically active radiation (PAR) and the fraction of PAR absorbed by green tissues (fPAR). Different studies showed a tight relationship between fPAR and spectral indices derived from the red and infrared reflectance of the surface (Sellers et al., 1992; Di Bella et al., 2004). The Normalized Difference Vegetation Index (NDVI) is the most widely used spectral index and the base of several primary production monitoring systems (Gilmanov et al., 2005; Grigera et al., 2007).

Perhaps, the major shortcoming in the use of Monteith model is the estimation of RUE (Hilker et al., 2008; Martínez et al., 2013). Field estimates are difficult to derive and data extrapolate

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tion in space and time has many uncertainties (Piñeiro et al., 2006; Oyarzabal et al., 2010). Different factors related to structural and functional differences among species and different types of stressors may modify the amount of C fixed per MJ of PAR absorbed at the canopy level (Schurr et al., 2006; Grace et al., 2007; Hilker et al., 2008). Ontogenic changes and senescence are also major controls of RUE (Hall et al., 1995).

Light energy absorbed by leaves that is not used in the process of photosynthesis must be discharged, to prevent damage in the photosynthetic apparatus (Demmig et al., 1987; Demmig-Adams and Adams, 1996). Therefore, another way of interpreting the photosynthetic RUE is as the ratio between the energy used in photochemical quenching and the energy dissipated by the two non-photochemical pathways: fluorescence emission and non radiative decay (heat loss).

The group of pigments called xanthophylls is involved in a specific biochemical process called xanthophyll cycle which has the ability to dissipate energy through the non-photochemical quenching (NPQ), consequently, avoiding photooxidative damage. This facultative mechanism is called photoprotection (Demmig et al., 1987; Demmig and Adams, 1996). Gamon and collaborators (1992) developed the Photochemical Reflectance Index (PRI), which was initially employed to estimate rapid changes in the relative levels of the pigments in the xanthophyll cycle, from the differences observed in the reflectance spectral signal around 531 nm (Gamon et al., 1992, 1997; Peñuelas et al., 1995). PRI compares the reflectance at 531 nm with a reference set at 570 nm. The first band not only gives evidence of rapid changes related to light energy distribution, carried out by the xanthophyll cycle (Gamon et al., 2015), but also belongs to a spectral region characterized by absorption of carotenoids and chlorophyll. The reference band, on the other hand, is only related to the chlorophyll absorption (Gamon et al., 1992). Nowadays, it is widely recognized that depending on the time scale in which the studies are developed, changes in PRI may show different plant functions. In a diurnal timescale, PRI variations assess changes in the pigments levels of the xanthophyll cycle (facultative response). On the other hand, in studies at large temporal or spatial scales, PRI is mostly influenced by the Chlorophylls/Carotenoids ratio (constitutive response) (Sims and Gamon, 2002; Filella et al., 2009; Gamon et al., 2015). In a very recent work Gamon et al., 2015 pointed out that both, the facultative and the constitutive response of PRI are strongly correlated with the photosynthetic activity over different timescales and by means of different mechanisms.

Several authors reported that PRI has strongly correlated with RUE both at leaf (Gamon et al., 1992, 1997; Peñuelas et al., 1995) and ecosystem levels (Trotter et al., 2002; Nichol et al., 2006; Grace et al., 2007). This is because changes at short temporal scale are closely related to fast redistribution of light energy associated with photosystem II (Gamon et al., 1997; Gamon et al., 2015). Furthermore, Filella and colleagues (2009), in a study where PRI was evaluated in both temporal scales, found a strong influence of Carotenoid/Chlorophyll ratio on PRI in the long-term. In this case, they still considered PRI as an effective reflectance index capable of measuring changes in photosynthetic RUE in response to stress. They argued that, in addition to xanthophylls, carotenoids as β -carotene or lutein are also related to photoprotective processes. For this reason they hold that PRI might still be useful for assessment of photosynthetic RUE to the extent that RUE is correlated with Carotenoid/Chlorophyll ratio (Sims and Gamon, 2002; Filella et al., 2009). In a comprehensive review of the existing literature on PRI, Garbulsky et al. concluded that this index is a reliable estimator of the ecophysiological variables related to photosynthetic efficiency at leaf and canopy levels in a wide variety of species, functional types and temporal scales (Garbulsky et al., 2011).

Some authors suggested that it is not possible to monitor a dynamic physiological process such as C gain using only the

reflectance of vegetation, and simultaneously emphasized the potential of chlorophyll fluorescence emission to estimate directly the photosynthetic RUE. At the same time, chlorophyll fluorescence provides very specific information about the vitality of plants and productivity (Cordon and Lagorio, 2007; Malenovský et al., 2009; Hmimina et al., 2013). Recent studies have shown that passive remote sensing techniques such as measurement of chlorophyll fluorescence induced by sunlight (sun induced fluorescence, SIF), which is modulated by the photosynthetic efficiency, should improve the knowledge of CO₂ assimilation processes (Pedrós et al., 2010; Moya et al., 2004; Louis et al., 2005; Damm et al., 2010). SIF techniques are suitable for studying the efficiency of photosynthetic processes at canopy level (Malenovský et al., 2009) and, hence, a promising alternative to quantify RUE directly (Damm et al., 2010).

Field canopies include not only a combination of structurally and functionally different species but also tissues at different ontogenic stages. Understanding the influence of species composition and ontogeny on RUE at canopy level is critical to improve C gain estimations from models based on remotely sensed data. Based on this observation, the aims of this work were:

- a to investigate the relationship between PRI and chlorophyll fluorescence since both would allow valuable estimates of RUE. This was assessed at two levels, leaf and stand levels, along the natural senescence process and in species with different photosynthetic metabolism.
- b to analyze if changes in the pigment pool (mainly in Chlorophyll/Carotenoid ratio) related to ontogeny, and/or changes in canopy structures are responsible for the behavior of NDVI, PRI and SIF. Given the nature of this study (we performed measurements weekly throughout the natural process of plants senescence) we focused on the constitutive mechanism of the PRI index. However, this index would still give us information on RUE, as we discussed above.
- c to assess the relationship between NDVI and PRI in these grasses. Together, they could be very useful to estimate NPP from remotely sensed data using Monteith's model (Gamon et al., 2001; Gamon et al., 2015).

2. Materials and methods

2.1. Plant material and experimental conditions

Plants of *Avena sativa* and *Setaria italica* were cultivated from seeds in pots of 30 cm of diameter. The pots were kept under normal field conditions, exposed to sun and watered daily. A sufficient amount of nutrients was supplied in order to avoid a potential deficiency.

Measurements began when the first panicle became evident. All measurements were carried out on sunny days, between 11 a.m. and 2 p.m. The average temperature of those days was 23 °C (temperature varied between 18 and 27 °C) and the average Photosynthetic Photon Flux Density (PPFD) was about 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at noon. Measurements were performed at leaf (reflectance, Kautsky kinetics parameters and pigments contents) and stand levels (reflectance and radiance).

Leaf-level measurements (reflectance, Kautsky kinetic parameters and pigment contents) were extended to other species. For these measurements leaves were chosen at different stages of senescence within the same individual. The plants used were *Paspalum dilatatum* (C4), *Dactylis glomerata* (C3), and *Brassica napus L* (C3). All of them were in excellent state of nutrition as they were supplemented with Hoagland nutrient solution (Hoagland and Arnon, 1950). Leaves of various colors (red, yellow and green)

from two species of deciduous trees were also collected at the campus of the College of Agronomy (UBA): *Liquidambar styraciflua* and *Rhus sylvestris*. These leaves were kept in water until measurements, which were performed within two hours after collection.

The first fully developed leaf was always used in leaf-level measurements of *Avena sativa*, *Setaria italica*, *Paspalum dilatatum*, *Dactylis glomerata* and *Brassica napus* L. In the case of *Liquidambar styraciflua* and *Rhus sylvestris*, we specifically used leaves with different pigment content and, for this reason, the selected leaves were chosen according to their color.

2.2. Pigment contents and RGB values

The contents of chlorophyll a, chlorophyll-b and carotenoids were measured in the same leaves whose reflectance and photo-synthetic parameters were determined (see sections 2.3 and 2.4). Leaves were washed with distilled water and dried with paper towels. Approximately 0.4 g of each leaf was weighed and the plant material was placed in a mortar containing liquid nitrogen to facilitate grinding. The pigments were then extracted from the formed powder by adding 2 ml of a solution consisting of 80% acetone and 20% of TRIS buffer (final concentration 1% w/v, adjusted to pH = 8 with HCl), previously cooled with ice. The extract was centrifuged at 12,000g for 3 min. A defined amount of the supernatant (1 ml) which was diluted to 3.0 ml was taken. The absorbance of the solution was determined as a function of wavelength using a Shimadzu Spectrophotometer UV 160A. Lastly, the concentration of pigments was calculated from the measured absorbance according to Sims and Gamon (2002). The reported value for each date is an average of 10 measurements. We finally expressed the contents of carotenoids and chlorophylls in leaves as nmol/cm² of leaf, having previously determined the specific leaf area.

From the reflectance spectrum it was possible to calculate the tristimulus values XYZ. For this conversion the functions defined by the CIE standard observer were used. In this case, the spectral distribution of D65 illuminant was employed. Then, it was possible to transform XYZ values, whose components are in the nominal range (0, 1), into RGB values according to the equations proposed by Bruce Lindbloom. The values of R, G and B were calculated in the range of 0–255 in arbitrary units. The Spectral Calculator, developed by Bruce Lindbloom (<http://www.brucelindbloom.com>), was used for this purpose. RGB values reported are the average of 10 leaves.

2.3. Radiance and reflectance measurements

The leaf-level reflectance spectra were recorded with a portable spectroradiometer ASD FieldSpec Pro FR equipped with plant probe and leaf-clip accessories which allowed obtaining the spectrum of the first fully developed leaf, without cutting the plants. On each date 10 measurements were taken at leaf level. The use of the leaf-clip avoided the entrance of ambient light. Grasses, whose leaves did not cover the entire sampling area, were grouped (two or three, always the first fully developed) to cover the entire measuring area but with special care to avoid overlapping (Daughtry et al., 1989).

We also used the portable hyperspectral spectroradiometer ASD FieldSpec Pro FR to measure both radiance and reflectance spectra at canopy level. The spectral range of this equipment varied between 350 nm to 2500 nm; its spectral resolution was 3 nm in the range 350–1000 nm and 10 nm in the 1000–2500 nm region of the electromagnetic spectrum, while the sampling interval was 1.4 nm and 2 nm for each zone, respectively. The full-width half maximum (FWHM) was 5.5 nm. The field of view of the sensor was 23°. During sampling we moved over the canopy made up with pots (5 × 5 pots) to cover the whole area (2.25 m²) and 10 spectra were

recorded each time. From the reflectance spectra we calculated PRI as:

$$PRI = (R_{531} - R_{570}) / (R_{531} + R_{570}) \quad (1)$$

where R₅₃₁ is the reflectance at 531 nm and R₅₇₀ reflectance at 570 nm. The Normalized Difference Vegetation Index (NDVI) was calculated as:

$$NDVI = (R_{774} - R_{677}) / (R_{774} + R_{677}) \quad (2)$$

in this case, R₆₇₇ is the reflectance at 677 nm and R₇₄₄ reflectance at 774 nm.

The sun-induced fluorescence (SIF, hereafter) of the canopy was derived from its radiance spectrum (L) and the incident irradiance (E) at the so-called Fraunhofer lines. We used the Fraunhofer Line Discrimination (FLD) method to obtain the magnitude of the fluorescence for O₂-A (Moya et al., 2004; Damm et al., 2010; Liu and Cheng, 2010). It was deduced by comparing the measured signal within the absorption band (*in*) with the signal measured outside it (*out*), which contains the solar irradiance background (Meroni et al., 2009). Incident solar irradiance (E) and the radiance of vegetation cover (L) on the bottom band (*in*) and on the shoulder of the band (*out*) were measured and sun induced fluorescence (SIF) was obtained according to (3):

$$SIF = [E(\lambda_{out}) \times L(\lambda_{in}) - L(\lambda_{out}) \times E(\lambda_{in})] / [E(\lambda_{out}) - E(\lambda_{in})] \quad (3)$$

Moreover, the value used within the absorption band (*in*) was set at 760 nm while the signal measured outside the absorption band (*out*) was an average value between 750 and 755 nm.

SIF has the same physical units as the radiance, W m⁻² nm⁻¹ sr⁻¹. The incident solar irradiance (E) was determined using a Spectralon panel (Labsphere, Inc.) of 51 × 51 cm.

The apparent fluorescence yield (SIF/a), that is, the ratio between the SIF and the irradiance reaching the canopy at the time of measurement (a) in the edge of the observed Fraunhofer line (O₂-A in this case), is a widely used parameter (Liu and Cheng, 2010). SIF₇₆₀/a becomes a dimensionless number that reflects the apparent fluorescence yield regardless of the level of light reaching the coverage, while SIF signal depends linearly on the illumination intensity of PAR as demonstrated by Liu and Cheng in 2010. We preferred to use SIF₇₆₀/a to eliminate the influence of PAR level.

An additional set of experiments was conducted in order to evaluate the fluorescence results obtained at stand level. In this case, a 3 × 4-pot (diameter 30 cm) canopy was designed to simulate a small canopy with plants of *Setaria italica* and *Cichorium intybus*. Radiance spectrum of the canopy (L) and the incident irradiance (E) obtained using the Spectralon panel were recorded. Then, the original pots of *S. italica* or *C. intybus* were replaced by an increasing number of pots containing completely dried plant material (wheat straw). Canopy PRI values were also calculated for each assembly of pots. In the first assembly we only replaced one pot with dry material, subsequently; we replaced 2, 4, 6, 8 and finally 10 pots with dried plant material (remaining only 2 pots with green plants from the original 12). On each assembly, 3 pots were arranged randomly and 3 radiance and irradiance spectra were recorded each time.

2.4. Kautsky kinetics parameters

Photochemical parameters were obtained with a modulated fluorometer Hansatech FMS1 on leaves still attached to the plants. In all cases, plants were dark-adapted for 20 min prior to measurements. To take into account the natural variability of leaves, at least 10 measurements on different leaves were performed and

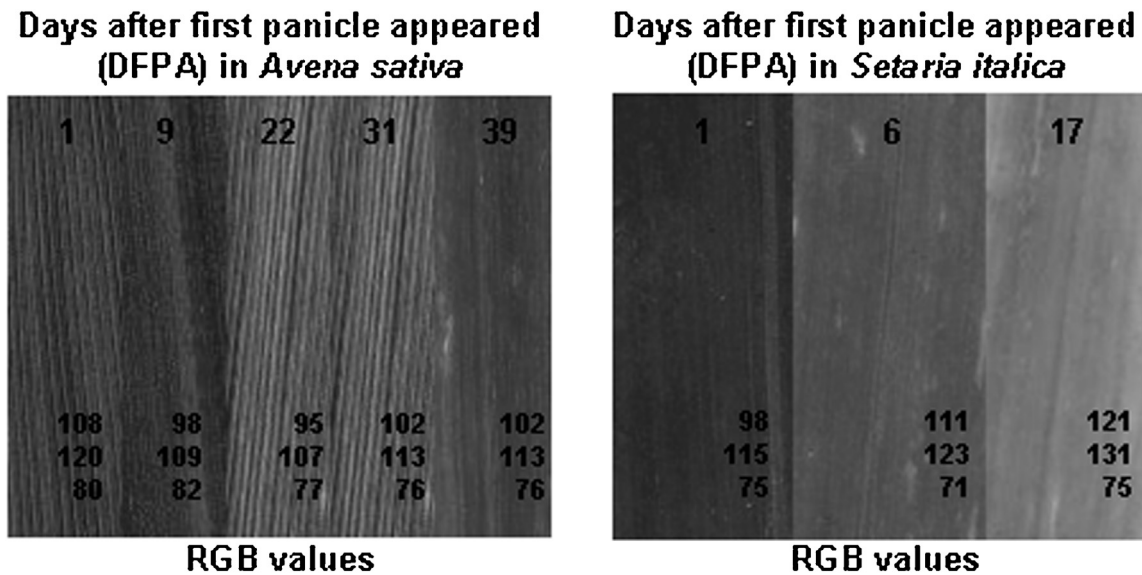


Fig. 1. Photographs show representative leaves at each day of measurement in both studied species: *Avena sativa* (left) and *Setaria italica* (right). Numbers on top of photographs represent the days after first panicle appeared (DFPA). In both cases measurements began when the first panicle became evident (day 1). In the lower part of each photograph the RGB values were added (average of ten measurements).

averaged. The values of initial fluorescence (F_0) were obtained illuminating the leaves with low light, and then a saturating light pulse was applied to obtain the maximum fluorescence (F_m). Subsequently the leaves were exposed to actinic light to reach steady state fluorescence (F_s). Next, a new pulse of saturating light was applied to obtain the value of maximum fluorescence of leaves adapted to light (F'_m). With these data three photosynthetic parameters were calculated (Eqs. (4)–(6)). The maximum photochemical quantum yield is the maximum photochemical efficiency of photosystem II (PSII) obtained in dark-adapted leaves (Rosenqvist and van Kooten, 2003), Eq. (4):

$$F_v/F_m = (F_m - F_0)/F_m \quad (4)$$

The quantum efficiency of PSII is the effective quantum yield of the open PSII reaction centers. It is explained as the efficiency of PSII under the actual degree of reaction center closure (leaves adapted to light) (Rosenqvist and van Kooten, 2003), Eq. (5):

$$\phi_{PSII} = (F'_m - F_s)/F'_m \quad (5)$$

The Stern-Volmer non-photochemical quenching parameter (NPQ) is related to non-radiative dissipation as heat, Eq. (6):

$$NPQ = (F_m - F'_m)/F'_m \quad (6)$$

An increase in this parameter indicates greater thermal dissipation in PSII and it positively correlates with zeaxanthin levels in leaves (Tambussi et al., 2002).

2.5. Statistical analyses

Analysis of variance and Pearson correlation analysis were performed using InfoStat software (Di Rienzo et al., 2010). Correlation significance was evaluated with Fisher's test at $\alpha=0.05$. Linear regression analysis and plots were performed with Sigma Plot 10.0 (Systat Software Inc., San Jose, CA).

3. Results and discussions

3.1. Pigment content variation between species

Statistically significant differences were observed for *Avena sativa* among dates for total chlorophyll (chlorophyll a + chlorophyll

b, i.e. Total Chl) concentrations and for chlorophyll a (Chl a) and b (Chl b) contents separately. Ten days after measurements began, we observed a slight increase in chlorophyll. Chlorophyll did not change appreciably up to 31 days after first panicle appeared (DFPA), displaying subsequently an important decrease (see Table A in Supplementary Material, SM hereinafter).

Total carotenoid content (xanthophylls + carotenes, i.e. Car) for *A. sativa* varied significantly over time. It doubled since the appearance of the first panicle until the tenth day of measurements, remaining then almost constant up to 22 DFPA. From this date to day 31 an increase was observed. During the final stages of natural senescence, from 31 DFPA to 39 DFPA, values began to fall. We also found that the ratio of total chlorophyll to total carotenoids (Total Chl/Car) was appreciably lower at the end of the experience. Chl a/b showed the same trend as Total Chl/Car as a function of time (see Table A in SM).

Total chlorophyll content decreased during natural senescence of *Setaria italica* but differences were not statistically significant. The same behavior was observed for Chl a and b and for the ratio between them. Variation in carotenoid content did not follow a clear trend despite significant differences among measurement dates. The Total Chl/Car ratio presented significant differences between the two previous dates (see Table A in SM).

As expected, pigment content data showed that the breakdown of chlorophyll started before that of carotenoids in both studied species. Regarding pigments, the best indicator of the processes of senescence in this work was the ratio of total chlorophyll to total carotenoids, which is an indicator of greenness in plants (Lichtenthaler and Buschmann, 2001). This ratio usually varies between 4.2 and 5 in sunny leaves. Lower values as found here are indicators of senescence, stress or damage in the photosynthetic machinery (see Table A in SM). Photographs of representative leaves on each date of measurement in both studied species were included to accompany the pigment data (Fig. 1). Additionally, RGB values were included as an index representing leaf senescence. Photographs and RGB values altogether provided an idea of the senescent level of leaves on each measurement day.

3.2. PRI variations between species and ontogeny: leaf and canopy level

PRI values at stand level were negative in both species and they were always much lower than the corresponding values at leaf level (Fig. 2). This pattern is consistent with previous results from literature (Barton and North, 2001). In our work this occurred because the spectral reflectance signature of canopy was contaminated with dry material reflectance (whose proportion increased as natural senescence progressed). The reflectance spectra of dry material had higher values at 570 nm than to 531 nm and for this reason, the canopy values of PRI were negative (see Eq. (1)). While the whole set of values for the stand level PRI were negative, leaf PRI values began positive and then fell to negative (more marked trend in *S. italica*). For *A. sativa* an inflection point was observed 22 days after first panicle appeared when stand level PRI values began to fall with a higher slope. This downfall was due to structural changes. It was confirmed by the stand level NDVI values, which began to fall at the same time i.e. 22 DFPA (see Fig. 3). This date was an inflection point from which carotenoid content in leaves increased, decreasing in turn the Total Chl/Car ratio; even though the total chlorophyll content in leaves was stable for ten days. In this sense, leaf PRI values were less sensitive to changes in pigment content and began to fall from 31 DFPA (see Fig. 2).

Leaf PRI values positively and significantly correlated with the stand level PRI values (Pearson correlation coefficient = 0.77, $p < 0.05$, Pearson's correlation coefficients were included in Table 1) as previous studies have shown in closed-canopy stands (Stylinski et al., 2002; Gamon et al., 2015 and references therein). The correlation observed supports the description of the canopy, at least for this index, in terms of the big-leaf hypothesis (Field, 1991). The big-leaf model builds from the concept that similar physical and physiological factors restrict the leaf and canopy functions in the same way (Field, 1991).

3.3. NDVI variations between species and ontogeny: leaf and canopy level

Stand level NDVI values were higher than their respective values at leaf level. This fact may be attributed to the enhanced multiple scattering and absorbing of multiple layers in the first case, which led to enhanced NDVI (Asner, 1998). For NDVI (as for PRI) stand level values were more sensitive to changes to senescence than the respective values at leaf level. NDVI at stand level decreased along time for *S. italica* while it remained practically unchanged at the leaf level. In the case of *A. sativa*, NDVI at stand level began to decrease appreciably from the day 22 on since first panicle appeared. Conversely, leaf NDVI values increased slightly over time, (Fig. 3). As the total chlorophyll content was unable to explain the changes observed (see section 3.1. and Table A in SM), we thought that in both species changes in stand level NDVI values were related to canopy structural changes. Undoubtedly an important factor was the increase in the content of dry material. In *A. sativa* these changes began on 22 DFPA while in *S. italica* changes occurred since the start of measurements. Total chlorophyll content began to decrease on 31 DFPA in *A. sativa*, while no significant changes were observed on the three measure dates for *S. italica*.

3.4. Relationship between pigment content and NDVI and PRI

No significant correlations were found between leaf NDVI and stand level NDVI with total chlorophyll content or with Total Chl/Car ratio for both studied species when data was analyzed separately and together. These results suggested that variation in the values of NDVI, both at leaf and stand level and for the two stud-

ied species were due to changes in canopy structure as already discussed.

On the other hand, leaf PRI correlated positively with Total Chl/Car ratio and with total chlorophyll content (Pearson's correlation coefficient: 0.73, and 0.75, respectively, both $p < 0.05$) when data of both species were combined (*A. sativa* and *S. italica*) (Table 1). At stand level no correlation was evident. For leaves of other species at different stages of senescence (*Paspalum dilatatum*, *Dactylis glomerata*, *Brassica napus L.*, *Liquidambar styraciflua* and *Rhus sylvestris*), together with the data for *A. sativa* and *S. italica*, we found a linear relationship between leaf PRI values and the log of total chlorophyll concentration (Leaf PRI = $-0.199 + 0.123 \times \log$ Total Chl, $R^2 = 0.78$, $p < 0.047$), and a linear relationship between leaf PRI and the log of Total Chl/Car ratio (Leaf PRI = $-0.076 + 0.116 \times \log$ Total Chl/Car, $R^2 = 0.80$, $p < 0.045$) (Figure A and B in the SM, respectively). Our results and previous work (Stylinski et al., 2002; Sims and Gamon, 2002) showed that the relationship between PRI and total chlorophyll content or PRI and the Total Chl/Car ratio at leaf level is robust within a species and among them. We suspected that at the stand level these relationships broke down when the canopy structure became more heterogeneous in terms of green and senescent material.

Our results and previous studies supported the idea than the long-term signal of PRI, was influenced by pigments other than xanthophylls (Stylinski et al., 2002; Filella et al., 2009; Rahimzadeh-Bajgiran et al., 2012; Hmimina et al., 2013; Gamon and Bond, 2013; Soudani et al., 2014). The dynamics of chlorophyll content in leaves (or the Total Chl/Car ratio) could strongly question the usefulness of this index as a promising proxy of the photosynthetic radiation use efficiency or plant photosynthetic activity during the entire life cycle of plants. However, Sims and Gamon (2002) and later Filella and collaborators (2009) have already suggested that, as far as the photosynthetic light-use efficiency is correlated with carotenoid/chlorophyll ratio, PRI may still be used as an estimator of RUE during senescence or under stress conditions. It is necessary to note that this relationship may depend upon the operational definition of RUE, i.e. based on incident radiation, total absorbed radiation or radiation absorbed by green vegetation (Gitelson and Gamon, 2015). Additionally, recent researches have advanced in correcting PRI by the effect of seasonal variations in the content of pigments under controlled laboratory conditions (Rahimzadeh-Bajgiran et al., 2012; Hmimina et al., 2013).

3.5. Relationship between spectral indices

When data from the *A. sativa* and *S. italica* were considered together, stand level PRI did not correlate with stand level NDVI (Table 1). Leaf NDVI did not correlate with stand level NDVI (Table 1). However, if each species was analyzed separately, stand level PRI correlated with stand level NDVI (Pearson's correlation coefficient 0.97, $p < 0.01$ and 0.99, $p < 0.05$ for *A. sativa* and *S. italica*, respectively). *S. italica* showed higher values for stand level NDVI than *A. sativa*. Canopy PRI values were similar during the senescence process in both species (Figure C in SM). The relationship between NDVI and PRI values at stand level for *A. sativa* was $NDVI = 0.97 + 5.43 \times PRI$, $R^2 = 0.89$, $p < 0.04$ and for *S. italica*: $NDVI = 0.90 + 1.74 \times PRI$, $R^2 = 0.99$, $p < 0.003$.

Stylinski et al. (2002) found that canopy PRI did not significantly correlate with canopy NDVI values because they studied evergreen chaparral species. However, in our study both variables were related over the temporal and spatial scales when both species were considered separately. This behavior was expected in two annual grasses as those studied in this work. Although NDVI values for *S. italica* were higher than those for *A. sativa*, the first species showed lower values in the chlorophyll content (see Table A in SM). A possible explanation for the observed discrepancy may be

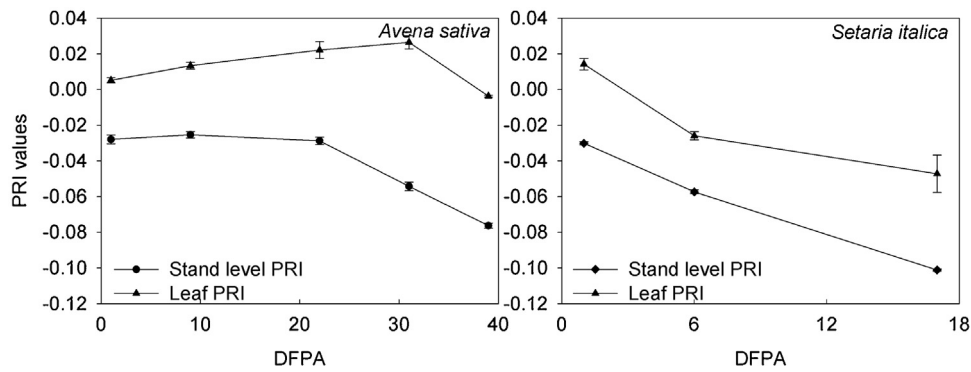


Fig. 2. Values of leaf and stand level PRI for both species. DFPA means days after first panicle appeared. Error bars are SE (n = 10).

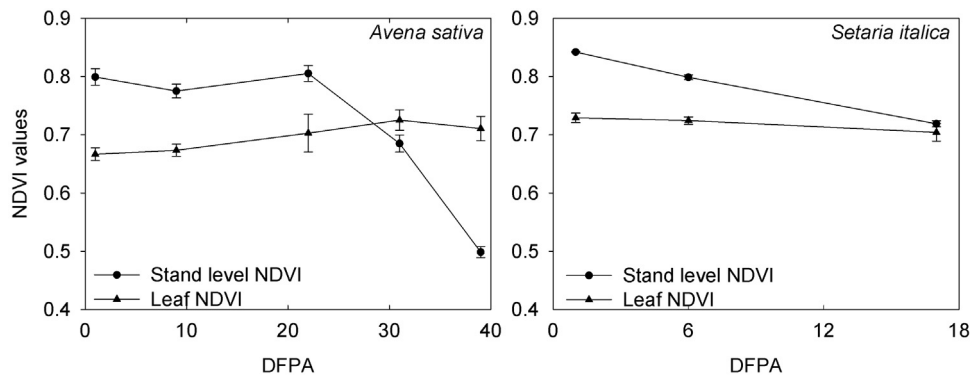


Fig. 3. Values of leaf and stand level NDVI for both species. DFPA means days after first panicle appeared. Error bars are SE (n = 10).

Table 1

Pearson's correlation coefficients obtained for the linear correlations between optical indices, sun-induced fluorescence and photosynthetic parameters at stand and leaf level. Data from both species were analyzed together.

	SIF ₇₆₀ /a	sPRI	sNDVI	Fv/Fm	Φ _{ps2}	qP	NPQ	IPRI	INDVI	Chl
sPRI	0.88*									
sNDVI	0.45	0.60								
Fv/Fm	0.66	0.48	-0.10							
Φ _{ps2}	0.89*	0.82	0.43	0.82						
qP	-0.42	-0.06	-0.25	-0.21	-0.38					
NPQ	-0.41	-0.48	-0.92*		0.19	0.46				
IPRI	0.82	0.77	0.13	0.71	0.69	0.04	0.08			
INDVI	-0.39	-0.34	-0.13	-0.57	-0.70	0.47	0.32	-0.07		
Chl	0.49	0.40	-0.18	0.83*	0.60	0.10	0.34	0.75	-0.16	
Chl/Car	0.60	0.60	-0.08	0.89*	0.77	0.12	-0.20	0.73	-0.50	0.86*

Correlations obtained with $p < 0.05$ or better between different functional groups of the indices are shaded and marked as * when $p \leq 0.01$. sPRI are the values of PRI at stand level and IPRI indicates leaf values of PRI. Chl is the total chlorophyll content (Chl a + Chl b) and Car is the total carotenoid content (carotenes + xanthophylls).

the differences in canopy architecture. In fact, *A. sativa* had thinner leaves and, therefore, more erectophile leaves which produced a more uniform canopy. *S. italica*, on the other hand, had wider and longer blades resulting in a slight overturning of these leaves and consequently, in a more planophile architecture.

Then, the angle of leaf insertion (leaf inclination) as well as the leaf area index (Haboudane et al., 2004; Jacquemoud et al., 2009) seemed to affect the reflectance spectra of canopies more than the total chlorophyll content. The canopy architecture produced a much greater difference in near infrared which substantially influences the NDVI. In the additional set of data at the canopy level for *Cichorium intybus* the same effect was observed. The average chlorophyll content of *C. intybus* leaves was 22.00 ± 1.50 nmol/cm² of leaf (n=6). *S. italica* presented similar chlorophyll content in its leaves (at the beginning of senescence). However, the leaves

of the first species are erectophile as are the leaves of *A. sativa*. We thought that for these reasons the linear relationship found between NDVI and PRI for *C. intybus* presented a similar slope as that found for *A. sativa*. The linear regression for *C. intybus* was $NDVI = 1.06 + 7.92 \times PRI$, $R^2 = 0.98$, $p < 0.05$, data not shown. For all these reasons, it seems that the canopy structure, and not the content of pigments, drove the relationship between NDVI and PRI in the grasses evaluated under the conditions of this work.

3.6. Sun induced fluorescence (SIF) variation between species and ontogeny

The intensity of sun induced fluorescence extracted from the telluric O₂-A absorption band, i.e. F₇₆₀, as the values of the apparent fluorescence yield at 760 nm (SIF₇₆₀/a) were within the range

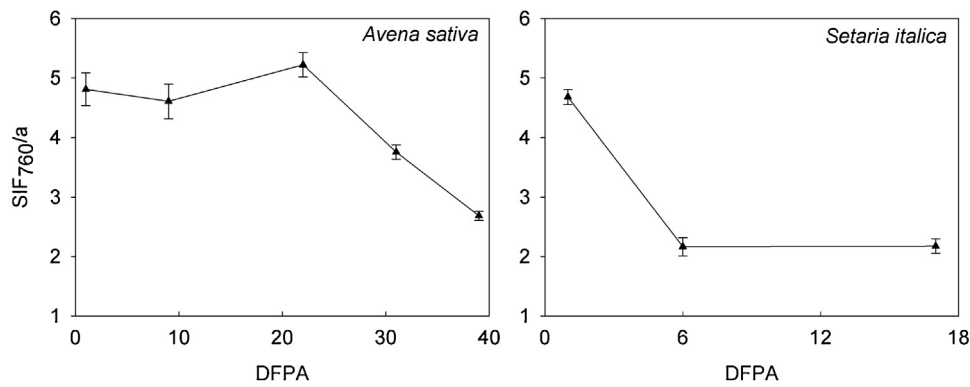


Fig. 4. Apparent fluorescence yield at 760 nm, $SIF_{760/a}$, for *Avena sativa* and *Setaria italica* under a PAR average intensity of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. DPAF means days after first panicle appeared. Error bars are SE ($n = 10$).

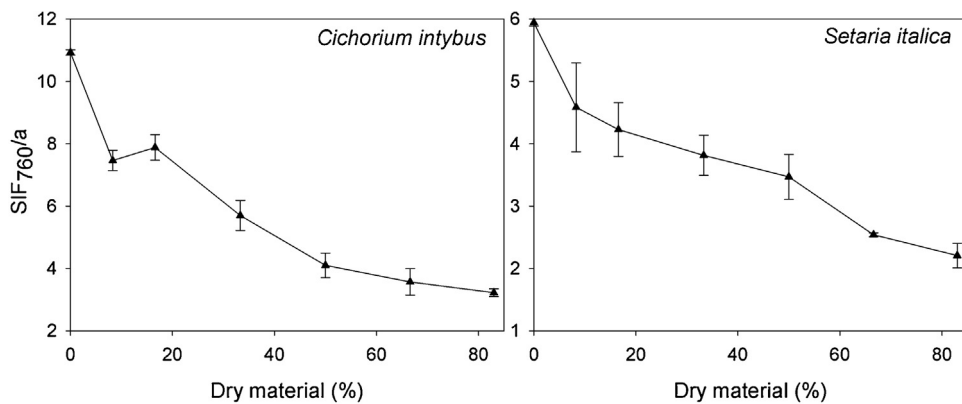


Fig. 5. Apparent fluorescence yield at 760 nm, $SIF_{760/a}$, for *Cichorium intybus* and *Setaria italica* under a PAR average intensity of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Error bars are SE ($n = 5$).

reported for different species (Meroni et al., 2009; Liu and Cheng, 2010; Liu et al., 2013). The apparent fluorescence yield at 760 nm showed the same global trend in the two studied species (*A. sativa* and *S. italica*). In *A. sativa*, $SIF_{760/a}$ remained approximately constant since the emergence of the first panicle (day 1) until day 22 DFPA, falling systematically afterwards (Fig. 4). Even though only three dates were measured for *S. italica*, it was possible to observe a slight declining trend in the apparent fluorescence yield (Fig. 4). The process of senescence in *S. italica* was faster than in *A. sativa* and, for this reason, it was not possible to extend the measures beyond three times in the first case. In both studied species, $SIF_{760/a}$ showed a behavior related to the pool of pigment content, which was confirmed by the significant correlation found with PRI (Pearson coefficient 0.88, $p < 0.01$); but it also related with the structure of the canopy, a fact that was evidenced by the correlation encountered between PRI and NDVI for each species separately (see section 3.5. Relationship between spectral indices). Therefore, fluorescence signal of canopy followed the behavior of two simultaneous effects: the decrease of Total Chl r /Car ratio along time with an increase in the proportion of non-photosynthesizing material occurring at the same time. The signal of fluorescence began to decay on 22 DFPA for *A. sativa* and so did the stand levels values of PRI and NDVI. On the other hand, in *S. italica* fluorescence signal fell systematically from the start of measurements as PRI and NDVI values did. In the other set of experiments carried out in canopies of *Cichorium intybus* and *Setaria italica* with different proportion of dry material, we could observe the same behavior in the fluorescence signal, i. e. a gradual decrease in this signal when more dried material was incorporated (see Fig. 5); even when Total Chl r /Car ratio was unchanged in the studied green plants.

3.7. Kautsky kinetics parameters behavior between species and ontogeny

The photosynthetic parameters derived from Kautsky kinetics showed the same trend along time as the apparent fluorescence yield at 760 nm, $SIF_{760/a}$. For *A. sativa*, they remained approximately constant at the beginning from day 1 to day 31 after first panicle appeared. After this day of measurement, the decrease for Φ_{PSII} was greater than for F_v/F_m (Figure C in SM). For *S. italica*, (although measurements were made only on 3 dates) we could observe that Φ_{PSII} values decreased slightly as a function of time while F_v/F_m did not show a clear tendency (Inset Figure D, SM). The photosynthetic parameter Φ_{PSII} , which represents the effective quantum yield of photochemical energy conversion in PSII, resulted a better indicator of disturbances taking place during the natural senescence, than the maximum photochemical efficiency of open reaction centers of PSII (F_v/F_m). It was previously reported in literature that Φ_{PSII} is a more sensitive parameter than F_v/F_m in the presence of stress situations (Iriel et al., 2014, 2015).

Non-photochemical quenching of variable chlorophyll fluorescence was estimated by NPQ, whose values increased after 22 DFPA for *A. sativa* (Figure E in SM) and from the very beginning of the measurements of *S. italica* (inset on Figure E, SM). The behavior of this parameter through time indicated a higher thermal dissipation at PSII as a consequence of the natural senescence processes. F_v/F_m and the non-photochemical parameter did not significantly correlate. Regarding Φ_{PSII} and NPQ, both correlated for *A. sativa* and *S. italica* but only when they were considered separately (Pearson coefficient = -0.99 $p < 0.05$, data not shown). This result agreed with Guo and Trotter (2004) who found that decreases in Φ_{PSII} among species were generally accompanied by increases in NPQ.

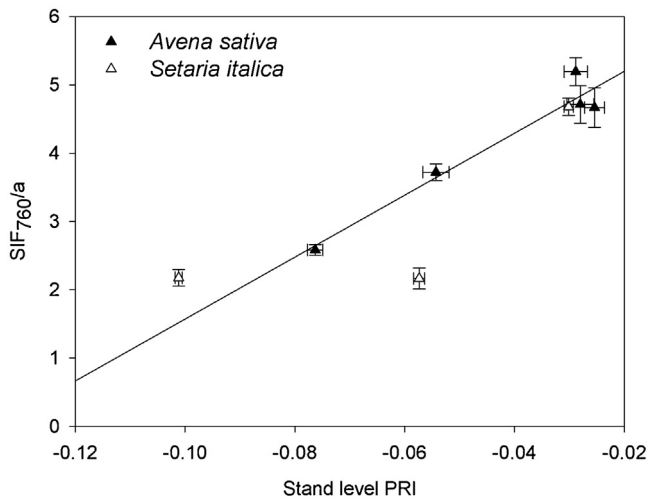


Fig. 6. Positive relationship between the apparent fluorescence yield ($SIF_{760/a}$) and stand level PRI for both species studied (*Avena sativa* and *Setaria italica*). Regression equations: $SIF_{760/a} = 5.75 + 39.92 \times PRI$, $R^2 = 0.78$, $p < 0.001$. Error bars are SE ($n = 10$).

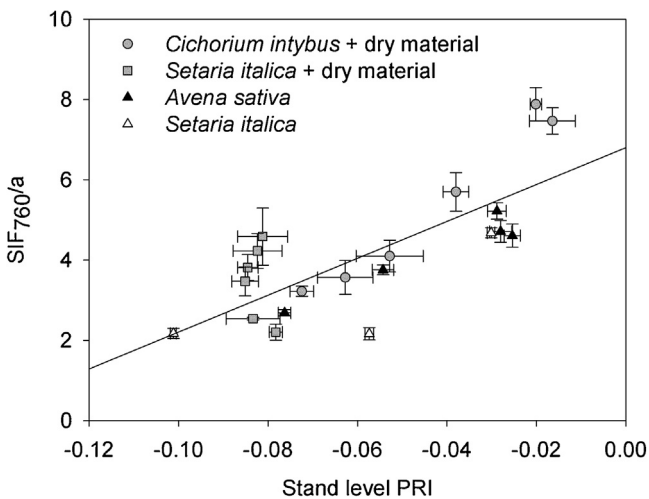


Fig. 7. Positive relationship between the apparent fluorescence yield ($SIF_{760/a}$) and stand level PRI for both species studied, *Avena sativa* and *Setaria italica*, together with data from the canopies of *Cichorium intybus* and *Setaria italica* with increasing proportion of dry plant material incorporated. Regression equation: $SIF_{760/a} = 6.8 + 45.9 \times PRI$, $R^2 = 0.59$, $p < 0.001$. Error bars are SE ($n = 3$).

3.8. Relationship between PRI and fluorescence descriptors (passive and active chlorophyll fluorescence)

We have found a positive correlation between stand level PRI and the apparent fluorescence yield at 760 ($SIF_{760/a}$) also obtained at stand level when data of both species were combined (Pearson coefficient 0.88, $p < 0.01$), see Table 1. The same regression model fit the data of both species (Fig. 6).

In Fig. 7, the apparent fluorescence obtained from the pot assemblies with incorporated dry material as a function of stand level PRI (*C. intybus* and *S. italica*) is shown together with those corresponding to the natural senescence of *A. sativa* and *S. italica*.

We found a significant positive correlation between stand level PRI values and the effective quantum yield of PSII (Φ_{PSII}) measured at leaf level when data of both species were combined (Pearson coefficient 0.82, $p < 0.05$) (Table 1 and Fig. 8). No significant correlations were found between PRI and the maximum quantum yield of PSII (F_v/F_m) (Table 1).

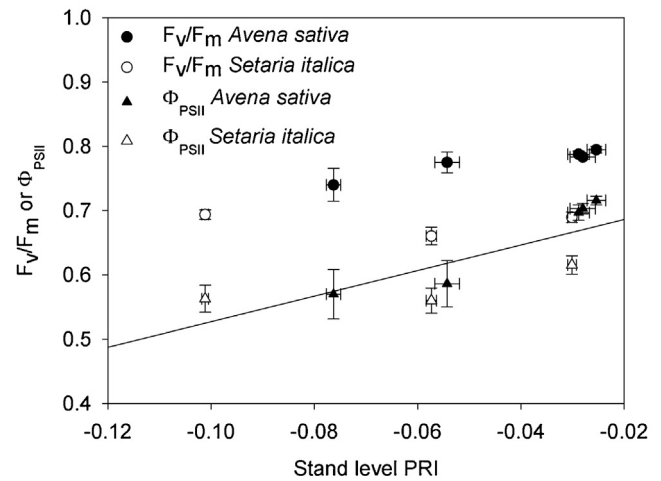


Fig. 8. Relationship between photochemical parameters (F_v/F_m and Φ_{PSII}) and stand level PRI. $\Phi_{PSII} = 0.73 + 1.98 \times PRI$, $R^2 = 0.65$, $p < 0.05$, data from *Avena sativa* and *Setaria italica* were represented by the same fitted line. The linear regression shown in the plot corresponds only to Φ_{PSII} . Error bars are SE ($n = 10$).

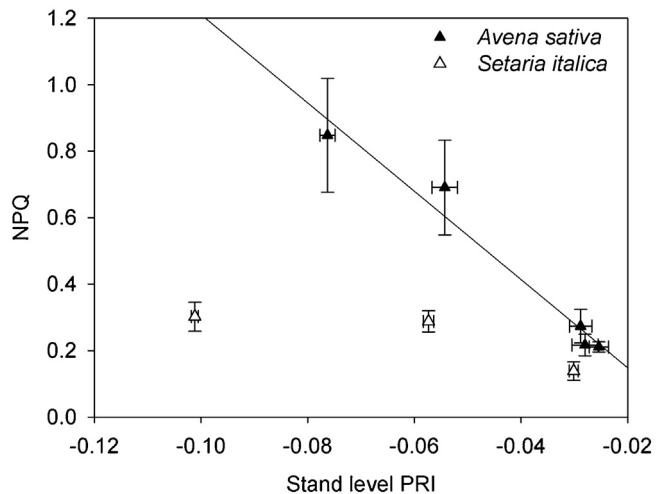


Fig. 9. Relationship between non-photochemical parameter (NPQ) and stand level PRI for both species studied (*Avena sativa* and *Setaria italica*). For *Avena sativa* the fitting equation is $NPQ = -0.12 - 13.28 \times PRI$, $R^2 = 0.97$, $p = 0.06$. Calculation of the Pearson's coefficients for the relationship NPQ vs. PRI of *Avena sativa* gave highly significant values: -0.98 , $p < 0.01$. No significant correlations were found when *Setaria italica* data were considered alone. Error bars are SE ($n = 10$).

As others have shown (Guo and Trotter, 2004; Filella et al., 2009; Weng et al., 2006; Weng et al., 2010), we found a strong correlation between PRI values obtained at leaf level with F_v/F_m and Φ_{PSII} in our results when data of both species were considered together (Pearson's correlation coefficient: 0.71 and 0.69, respectively, both $p < 0.05$) (Table 1). When we included in the analysis data from leaves of other species at different stages of senescence (*Paspalum dilatatum*, *Dactylis glomerata*, *Brassica napus* L, *Liquidambar styraciflua* and *Rhus sylvestris*), we also found a significant correlations between leaf PRI and the two photochemical parameters considered, $F_v/F_m = 0.76 + 1.46 \times PRI$, $R^2 = 0.76$, $p < 0.05$; $\Phi_{PSII} = 0.64 + 1.65 \times PRI$, $R^2 = 0.78$, $p < 0.05$ (Figures F and G in the SM, respectively).

As mentioned before, NPQ can provide valuable information about the non-radiative deactivation pathway. There was no single correlation between PRI and NPQ when both species were considered together (Fig. 9). In *A. sativa* higher values of thermal dissipation were reached at the end of senescence. On the other hand,

even though we only performed measurements on three dates in *S. italica*, the values obtained for NPQ never overcame the value of 0.3. The metabolic pathway of the studied species may explain the observed differences. In fact, *Setaria italica*, a C4 species, has lower photoprotection requirements, as it shows higher tolerance to conditions of high irradiance and heat and water deficiency. Accordingly, low values for the non photochemical quenching parameters are expected for this species. On the other hand, *Avena sativa* is a C3 species with lower resistance to extreme conditions, presenting a high need of photoprotection and, accordingly, greater values of NPQ. Similar observations were made earlier by Brugnoli and colleagues in sun and shade leaves of C3 and C4 plants (Brugnoli et al., 1998). More recent works confirmed these results, Guo and Trotter (2004) found that decreases in Φ_{PSII} among species were generally accompanied by increases in NPQ and broadly associated with decreases in PRI. Nichol and collaborators (2006) in a dense experimental mangrove canopy found similar results.

The correlation between SIF_{760}/a with PRI was higher than correlations between PRI and the photosynthetic parameters (Φ_{PSII} and NPQ). So, PRI would be a good proxy of SIF_{760}/a . Previous articles have reported positive relationships between PRI and RUE in herbaceous at leaf (Gamon et al., 1997; Guo and Trotter, 2004) and canopy (Strachan et al., 2002; Cheng et al., 2013) scales (Garbulsky et al., 2011). Based on these results and ours (Fig. 8), we assume that increases in the values of PRI (less negative values) and in the effective quantum yield of PSII (Φ_{PSII}) would indicate greater values of RUE.

Regarding the apparent sun-induced fluorescence yield at 760 nm (SIF_{760}/a), it should be inversely related to RUE but only if non-photochemical quenching remains constant. Liu and Cheng confirmed this behavior in wheat and maize at leaf and canopy levels (Liu and Cheng, 2010). However, in our work the values of NPQ increased over time (Figure E in SM). In a recent review, the authors argue that SIF and quantum yield of photochemistry in PSII vary concomitantly in response to stress under high-light conditions (measurements carried out during the midday as in our work) (Porcar-Castell et al., 2014). This behavior is explained in that changes in quantum yield of photochemistry of PSII are dominated by the non-photochemical quenching, whilst the photochemical quenching remained constant (Porcar-Castell et al., 2014). As mentioned before, we observed increases in NPQ values along time in both studied species while the Photochemical Quenching Coefficient (qP), a parameter calculated as $(F_m' - F_s)/(F_m' - F_0)$ (Lagorio, 2011) and related with photochemical quenching, remained close to 1 in both cases (data not shown). For all these reasons, under our conditions of measurements, we would expect a positive correlation between SIF_{760}/a and Φ_{PSII} and hence with RUE.

3.9. Relationship between active and passive chlorophyll fluorescence parameters

Photosynthetic parameters derived from Kautsky kinetics (active fluorescence) obtained at leaf level correlated with apparent sun induced fluorescence yield (passive fluorescence) obtained at stand level (Table 1). The best correlation was found between SIF_{760}/a and Φ_{PSII} (Pearson coefficient: 0.89 $p < 0.01$). These two parameters showed the behavior in steady state conditions with plants adapted to ambient light. In a very comprehensive review Porcar-Castell et al. (2014) argued that fluorescence yield was related to photochemical yield and hence to RUE.

The NPQ, instead, did not correlate with apparent sun induced fluorescence yield. Working with *Trifolium repens* L. exposed to the action of ozone (chlorophyll degradation connected to senescence-like mechanisms have been reported as a common effect of ozone exposure) Meroni et al. (2008) found that in the early phase of stress, SIF at 760 nm decreased significantly in treated plants with

respect to controls after 1 day of treatments. At the same time, plants showed a significant decrease in Φ_{PSII} from day 4 on fumigation with ozone. A significant decrease in F_v/F_m was experienced by treated plants after 21 days of fumigation (Meroni et al., 2008).

It should be noticed that the results of this section are strictly valid for the experimental set up used in this work. In fact, Julitta et al. (2016) recently pointed out in their detailed and exhaustive comparison of four commercial field spectroradiometers that different devices yielded diverse absolute values for the retrieved fluorescence in the red and in the far-red. This fact, however, does not invalidate the usefulness of the measured SIF/a as a proxy of photosynthetic RUE, given the high Pearson's correlation coefficient found here between SIF/a and Φ_{PSII} (Pearson coefficient: 0.89 in Table 1). The parameter Φ_{PSII} (measured in this work under the same light conditions as SIF) is one of the most widely accepted fluorescence parameters related to the physiological state of a plant (Maxwell and Johnson, 2000).

4. Conclusions

Our results showed that two chlorophyll fluorescence signals Φ_{PSII} and the apparent sun-induced fluorescence yield at 760 nm (SIF_{760}/a) correlated with the canopy PRI values in the two grasses assessed along senescence. This correlation may be explained in conjunction with the decrease of chlorophyll content along senescence. In fact, during the natural aging process, the photosynthetic activity diminished (decrease in F_v/F_m and Φ_{PSII}), the chlorophyll content decreased and consequently its fluorescence also dropped. Even when significant changes occurred in the Total Chl/Car ratio along senescence in both studied species, the correlation observed between PRI and the chlorophyll fluorescence parameters might indicate the usefulness of this reflectance index as a proxy of photosynthetic RUE. The relationships found between stand level PRI values and the fluorescence estimators (Φ_{PSII} and SIF_{760}/a) were positive and linear in both cases. These results, together with those obtained in previous works, would indicate lower values of RUE when PRI or fluorescence estimators decreased under stress conditions. In this case, the PRI values became more negative.

The use of a modulated fluorometer allows a rapid measurement of the Φ_{PSII} . However, these determinations are restricted to the leaf level. SIF_{760}/a has the additional advantage of allowing proximal sensing of the photosynthetic activity of the whole canopy by means of a portable spectroradiometer. Our experiments were carried out throughout the process of natural senescence for the two studied species, showing that the relationships found applied not only to healthy vegetation (as already proposed by some authors) but also to senescent plants or under stress conditions. These results were supported by experiments conducted also at stand level where we modified artificially the proportion of dry matter content of canopy.

A noteworthy result derived from the present study was the fact that significant linear correlations between leaf PRI and photosynthetic parameters as F_v/F_m and Φ_{PSII} , the latter derived from chlorophyll fluorescence, were obtained from leaves of different plant species considered together (*Setaria italica*, *Avena sativa*, *Paspalum dilatatum*, *Dactylis glomerata*, *Brassica napus* L, *Liquidambar styraciflua* and *Rhus sylvestris*). The independence of this correlation of the type of species considered, suggested a sort of generality that, although it should be verified for additional species, resulted very promising for the remote monitoring of RUE. In fact, this work showed that measuring leaf PRI along senescence may give information about the physiological state of the plant. Furthermore, leaf PRI would allow comparison of plant health of different species in diverse ontogenic states. The strong correlation found in this work as in previous studies between leaf and stand level PRI points in the

same direction. The positive correlation among PRI, F_v/F_m and Φ_{PSII} may be easily rationalized in terms of an increase in the reflectance at 570 nm, due to chlorophyll losses (reduction in PRI value) as leaf senesces and drops its photosynthetic activity (decrease in F_v/F_m and Φ_{PSII}). Moreover, Total Chl/Car ratio correlated with F_v/F_m and Φ_{PSII} because during the senescence process the decrease in photochemical activity was accompanied by a diminution of total chlorophyll while carotenoids increased. Although the results presented here should be verified for additional species, this work represents a promising starting point.

We found that (1) Total Chl/Car ratio was the best pigment indicator of senescence in both grasses species. (2) Stand level NDVI patterns were related to changes in the structure of canopies and not to the pigment content. (3) Stand level PRI patterns suggested changes both in terms of the structure of canopies and the pigment content of leaves. (4) NDVI and PRI values at stand level highly correlated for each species separately due to differences in the structures of both canopies. Accompanying these effects, (5) a decrease in the apparent sun-induced fluorescence yield at 760 nm and Φ_{PSII} was noticed. A strong increment in NPQ was evidenced in *Avena sativa*, a C3 species, which has lower resistance to extreme conditions and, therefore, higher requirements of photoprotection. While in *Setaria italica*, a C4 species, NPQ values were lower than in the other grass in accordance with its higher tolerance to extreme environmental conditions. (6) The high significant correlation between SIF_{760}/a and PRI would indicate that fluorescence signal was affected simultaneously by the two factors affecting PRI, Total Chl/Car ratio and changes in the structure of canopies.

Beyond the temporal dynamics, the underlying reason supporting the high correlation between stand-level PRI and leaf PRI is the use of homogenous canopies that may be considered as a big leaf. Remarkably, it was shown here that the optical properties of the canopy may be described by the optical properties of individual leaves even when senescent material is present.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jplph.2016.05.010>.

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