Research Paper

High-frequency seasonal variation of leaf fluorescence and reflectance in Mediterranean forest species under natural environmental conditions

Sabrina Raddi[1]*, Federico Magnani[2]

Abstract - The strategies of two evergreen Mediterranean tree species (*Arbutus unedo* L. and *Quercus ilex* L.) to face annual temperature variability and extreme event responses has been monitored in continuum by leaf fluorescence and reflectance. The effect of chilling spells and the accumulation of cold days with chilling and freezing temperatures on winter photo-inhibition was discussed in terms of modulation capacity for fluorescence parameters to daily temperature and irradiance conditions. Throughout the winter *Q. ilex* resulted less photoinhibited than *A. unedo*, showing a more dynamic response in all fluorescence parameters and a higher non-photochemical quenching capacity. These characteristics effectively act to maintain a higher electron transport capacity in *Q. ilex* than in *A. unedo*.

Keywords - *Quercus ilex* L.; *Arbutus unedo* L.; photosynthesis; photochemistry; temperature stress.

Introduction

Gross primary production (GPP), the amount of atmospheric carbon fixed through the process of photosynthesis by living biomass, is the largest flux of CO_2 between the atmosphere and the Earth surface (Ciais et al. 2013), and has a key role as a primary driver of terrestrial productivity. A clear understanding of the adjustment of photosynthetic processes to surrounding conditions is therefore crucial in order to understand C sequestration and forest growth, and its response to the environment.

For plants, the adjustment of photosystem II (PSII) to environmental conditions is of vital importance in order to maintain an optimal photosynthetic electron flow to the Calvin cycle. This may be achieved through the dissipation of energy excess as heat, so minimizing the reactive oxygen species generated by surplus light (Horton et al. 1996, Logan et al. 2014).

Some of these adjustments occur at a rapid-time scale and are reversible in the range from minutes to hours, tracking the changes of light conditions (following for example sunflecks or temporary shading by clouds or overlapping branches). Other modifications occur at longer time-scale, involving also changes in foliar biochemical contents within days,

weeks or months. In response to severe meteorological events or biotic stresses, or more commonly to seasonal changes in light intensity, temperature and water or nutrient availability, slowly reversible changes occur in order to acclimate photosynthesis to on-going environmental conditions (Demmig-Adams et al. 2014). This double (short- and long-term) response should be accounted for in order to infer carbon assimilation from fluorescence parameters and environmental variables.

Chlorophyll fluorescence was discovered at the end of the XVII century (Müller 1874), as the capacity to re-emit at longer wavelength part the energy absorbed. It has since been investigated and quantified in order to understand the functioning of the photosystems and their regulatory mechanisms (Kautsky 1931). Chlorophyll molecules in plant leaves are organized into macromolecular complexes (called photosynthetic units), containing many chlorophyll molecules and reaction centers (RC). After a photon is captured by pigment molecules in RC, that act as antenna, the absorbed photon ('exciton') can move among the chlorophyll molecules of the photosynthetic unit (Engel et al. 2007). The exciton can have one of following fates occurring at different rates: (a) it can be used by photosystem II (PSII) to take an electron from water, producing

1 DAGRI, Università degli Studi di Firenze, Florence (Italy)
2 DISTAL, Alma Mater - Università di Bologna, Bologna (Italy)

*Corresponding author: * sabrina.raddi@unifi.it

oxygen; (b) it can undergo radiationless decay; (c) it can be re-emitted as a fluorescent photon, or (d) it can be quenched by regulated non-photochemical trapping centers (NPQ). When the potential flux of electrons exceeds the capacity to use them in photochemistry, NPQ activity increases, balancing supply with demand and resulting in diagnostic changes in fluorescence yield. In order to preserve the photosynthetic energy balance, partitioning of excitation energy between photochemical quenching (PQ), fluorescence and non-photochemical quenching (NPQ) processes is constantly adjusted in the leaf (Papageorgiou and Govindjee 2004).

Even if fluorescence is only a small part of the total changes in yield (1% - 2%), it is therefore useful to investigate the photosynthetic process and the fate of absorbed energy (for review of method and parameters see i.a. Lichtenthaler et al. 1986, Maxwell and Johnson 2000, Baker 2008, Pessarakli 2016).

Following several decades of laboratory and field studies, today passive measurements of solar induced chlorophyll fluorescence (SIF) can be used to estimate photosynthesis and vegetation GPP from space (Frankenberg and Berry 2018). SIF can be assessed from the filling-in of the spectrally wide atmospheric oxygen absorption bands at wavelengths around 687 nm and 760 nm (Davidson et al. 2003, Moya et al. 2004). Several sensors already have the capability to estimate SIF from space by means of high-spectral-resolution interferometers. Estimates at large spatial scale were first achieved by SCIAMACHY on Japanese GOSAT launched in 2009 (30×60 km^2, Frankenberg et al. 2011, Joiner et al. 2011, 2012) and EUMETSAT MetOp satellites launched in 2006 and 2012 with 0.5° resolution, later refined to 0.05° by Duveiller and Cescatti (2016) model. More recently, information has been obtained at a more detailed scale both by NASA OCO-2 (nadir 1.3×2.25 km^2, Frankenberg et al. 2014) and European Space Agency (ESA) TROPOMI sensor on the Sentinel 5 Precursor satellite (7×3.5 km^2, Köhler et al. 2018), with the aim of quantifying photosynthetic activity and GPP globally (Sun et al. 2018, Zhang et al. 2018). Following the results of SIFLEX (*Solar Induced Fluorescence EXperiment*) campaigns, ESA will launch in 2022 the *Fluorescence Imaging Spectrometer* (FLORIS) sensor onboard the FLEX satellite, with global coverage and a spatial resolution of just 300 m. Such efforts are highly relevant to get a better understanding of carbon and water cycles, in particular to reduce uncertainties in the estimate of GPP, to determine the cause-effect relationships between environmental factors and vegetation carbon sequestration, and to assess the anthropogenic influences on vegetation functioning (Magnani et al. 2009, Mohammed et al. 2014, ESA 2015).

As part of a research program started in 2013 on the diurnal and seasonal acclimation of photosystem II (PRIN project «*Effects of Global Change on Productivity and Radiative Forcing of Italian Forests*»), the chlorophyll fluorescence of two evergreen Mediterranean forest species (*Arbutus unedo* L. and *Quercus ilex* L.) has been monitored throughout the year. Two short-term (one month) trials were also carried out to evaluate the effects on photosynthetic parameters and spectral reflectance of water stress and nutrient supply. The evaluation of the response of coexisting Mediterranean species, such as *Q. ilex* and *A. unedo* – two species evolved under the Mediterranean climate (Quézel 1985, Blondel and Aronson 1999) - to the variability in meteorological conditions has important implications for climate-driven range dynamics and vegetation shifts (Liu et al. 2018).

The main aim of this study has been to describe the different strategies of evergreen Mediterranean tree species to face temperature variability during the year and their response to extreme events. This is particularly relevant in order to assess the acclimation and resilience capacity of these species in a climate change scenario, with a predicted increase in frequency and intensity of extreme climatic conditions (IPCC 2014). The Mediterranean area is actually considered a primary climate change hotspot (Giorgi 2006), because of the above average warming (Guiot and Cramer 2016). This could drive other ecologically important variables in complex or even counterintuitive ways with episodic or nonlinear responses (Jackson et al. 2009, Connor et al. 2018). Intra-annual chlorophyll fluorescence variability has been investigated together with some key aspects related to the response to severe winter conditions (low winter temperatures and frost days).

Materials and methods

Plant material and instrumentation
Well-irrigated five-year-old plants of *Arbutus unedo* L. (strawberry tree) and *Quercus ilex* L. (holm oak) about 1-2 m in height were grown outdoor in full sun in large (40 L) pots (70% peat, 30% pumice and fertilized by 120 g pot^{-1} slow release fertilizer Osmocote Exact Standard 12-14 months) by a commercial nursery of the Pistoia nursery district. Plants were then acclimated for two months to the experimental conditions in the Florence University nursery (Florence, 43.79 N, 11.17 E; 60 m a.s.l.). Chlorophyll *a* fluorescence and reflectance were measured in each species for more than a year, starting in September 2014 for strawberry trees (measurement period: 365

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days) and in September 2015 for holm oak (measurement period: 631 days). Measurements were carried out on healthy, fully expanded leaves from the last growth flush in the upper third of the crown. In order to minimize shadows, leaves were randomly chosen among those of the horizontal, South-facing and in full-light subset. Pulse-amplitude modulated fluorescence was monitored every 10 minutes by a multi-channel MONITORING-PAM fluorometer system (Heinz Walz GmbH, Effeltrich, Germany) with six MONI-head/485 fluorometers connected to a single data acquisition system (MONI-DA). The MONI-head delivers measuring light and saturating pulses to the sample through a window that transmits radiation in the range of 400–750 nm, situated at one end of the probe cylinder. Both the dim measuring light and the saturating light pulse (lasting 0.8 s with an intensity of more than 4000 µmol m^{-2} s^{-1}) were emitted by a blue LED source. The MONI-head employs PIN photodiodes to measure pulse-amplitude modulated (PAM) chlorophyll fluorescence at wavelengths longer than 625 nm. Using a photodiode protected by near-infrared filters, the MONI-head simultaneously measures the radiation (400-700 nm range) reflected by a 1.3 x 0.7 cm area of an optically diffuse Teflon sheet, 1 mm thick, mounted at the edge of the leaf clip, providing an estimate of incoming PAR (Photosynthetically Active Radiation). Air temperature is also measured inside the instrument head.
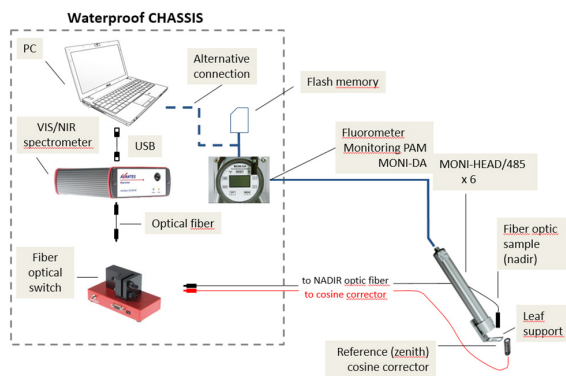

Figure 1 - Schematic representation for foliar fluorescence and reflectance measurement system. Fluorescence by Monitoring-PAM (Walz, Effeltrich, Germany) equipped with a MONI-DA acquisition system and MONI-HEAD/485 fluorometers. Reflectance VIS/NIR specrometers (Avan-Spec-2048-2) with optical fibers connected to (FOS-2-Inline, Avantes Eerbeck, The Netherlands). Further details in text.

Leaf reflectance was also measured on the same leaves through a couple of optical fibers (P200-UV-VIS, Ocean Optics Inc. FL, USA) per sample, with one fiber pointing nadir to the same leaf area sampled by the fluorescence probe with a 28° field-of-view, while the other was equipped with a cosine corrector (CC3, Ocean Optics Inc. , FL, USA) so as to acquire a hemispherical reference signal. The latter was placed vertically on the same plane of the MONI-head leaf holder (as shown in Figure 1), in order to sense a comparable light environment to the leaf sample. Both fibers were connected through a fiber optical switch (FOS-2-Inline, Avantes, Eerbeck, The Netherlands) to a VIS/NIR spectrometer (AvanSpec-2048-2, Avantes), operated through a dedicated software for the acquisition of reflectance spectra (both leaf and reference) every 2 minutes.

Computation of environmental and fluorescence parameters

Among the environmental variables, daily mean temperature (T_{day} in °C), daily minimum and maximum temperature (T_{min} and T_{max}, resp., in °C), and daily mean and maximum value for photosynthetically active radiation (PAR$_{day}$ and PAR$_{max}$, µmol m^{-2} s^{-1}, resp.) were computed from long-term measurements. The number of frost days ($T_{min} < 0$°C) in each month and in the entire year (from 1 September to 31 August) was also calculated. Chilling and freezing periods were defined as days with $T_{min} \leq 15$°C and ≤ 0°C, respectively (Yadav 2010).

The temporal changes in photosynthetic processes were assessed from the following chlorophyll a fluorescence parameters computed after Porcar-Castell (2011).

- The operating quantum yield of photochemistry in photosystem II, φ_P, was estimated as: $(F_m' - F_t)/F_m'$ (Genty et al. 1989), where F_m' and F_t are the maximum and instantaneous fluorescence in light condition, respectively.

- The daily maximum quantum yield of PSII, F_v/F_m, defined as $(F_m - F_o)/F_m$ (Kitajima and Butler 1975), where F_m and F_o are the maximum and minimum fluorescence of a dark-adapted leaf, respectively. A value of $F_v/F_m \geq 0.75$ was deemed optimal and indicative of no sustained photoprotection/photoinhibition; for each month, the fraction of days above this threshold was also computed (ND$_{opt}$).

- Heat dissipation by PSII was estimated by the Stern-Volmer non-photochemical quenching (NPQ) parameter, computed as NPQ = $(F_m/F_m') - 1$ (Bilger and Björkman 1990). Sustained NPQ (NPQ$_S$) resulting from the overnight maintenance of photoprotection under stress conditions, was estimated as $(F_{mR}/F_m') - 1$ (Porcar-Castell 2011), where F_{mR} is the reference maximum value of F_m measured in the year during the night. In this long-term approach, the requirement of maintaining the same leaf area portion under observation during the season is essential for NPQ$_S$ determination (Logan et al. 2007).

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- The sustained photochemical quenching, qL_S, is the fraction of functional reaction centers (Porcar-Castell 2011) and it was computed as:

$$qL_S = (1/F_o' – 1/F_m')/(1/F_{oR} - 1/F_{mR})$$

- The electron transport rate ETR ($\mu mol\ m^{-2}\ s^{-1}$) at the leaf level is estimated as:

$$ETR = \alpha \cdot \beta \cdot PAR \cdot \varphi_P$$

where α, β, and PAR are leaf absorbance (set to α = 0. 83), energy partitioning to PSII (set to β = 0. 5), and photosynthetic photon flux density (in μmol photon $m^2\ s^{-1}$) and φ_P is the quantum yield of photochemistry in PSII (electron/photon; Baker 2008). ETR_{max} is the maximum electron transport rate under saturating light and was estimated by fitting a non-quadratic hyperbolic model to the diurnal response of ETR to PAR.

Foliar reflectance

Leaf reflectance at each wavelength (R_{wl}) was calculated as the ratio between spectral radiance reflected by the leaf surface and the radiance received by the surface itself (R_{wl} = Sample$_{wl}$ / Reference$_{wl}$).

The photochemical reflectance index (PRI) was originally developed by Gamon et al. (1992) to estimate the de-epoxidation of violaxanthin to zeaxanthin in the xanthophyll cycle, and as such was proposed to estimate photosynthetic light use efficiency at different scales, from leaf to canopy (for review see Garbulsky et al. 2011). The xanthophyll (VAZ) cycle is responsible for the development of NPQ, and acts as a photoprotective mechanism, preventing the oxidative damage of the photosynthetic apparatus when exposed to high light. Violaxanthin (V), prevailing in the dark, is converted upon illumination to antheraxanthin (A) and then to zeaxanthin (Z). The reaction begins when an excess of light induces a lowering of chloroplast lumen pH; Z binds to PSII proteins causing a conformational change that quenches the excess of energy as heat (Havaux and Niyogi 1999, Krause and Jahns 2004, Dall'Osto et al. 2010). In the dark, Z reverts back to V. Since A and Z show a higher absorption at 531 nm than V, reflectance decreases and a lower PRI is observed in high light (relative to low light) conditions (Middleton et al. 2012). Following Gamon et al. (1997), PRI was estimated as $(R_{531} - R_{570})/(R_{531} + R_{570})$, where R_{531} indicates reflectance at 531 nm (the waveband of the xanthophyll signal), and R_{570} indicates reflectance at 570 nm (the reference waveband).

Results

Environmental conditions

Environmental conditions were typical for the site, with an average annual temperature of 16.5°C and more than 50% of the days with high irradiance ($PAR_{max} > 1000\ \mu mol\ m^{-2}\ s^{-1}$) distributed from March to October (Figure 2). Over the measurement period, absolute minimum temperatures were -4°C, -6°C and -7°C for the three winter seasons, respectively (Figure 3); colder minimum temperatures were therefore experienced by *Q. ilex* than *A. unedo* over the monitoring period, which could partly explain observed differences among species in fluorescence parameters. Frost days occurred over a period of four-five months from November to February with 33, 16, 39 frost days in the three years. January was the coldest month, accounting for 36%, 63% and 46% of frost days in each year. Several consecutive frost days were observed on *A. unedo* in 2014/15 in two periods (at the end of December and of January), and on *Q. ilex* in 2015/16 at mid-January and 2016/17 during a prolonged intermittent period from early December to the end of January, with freezing temperature in about 50% of the days. Leaves emitted on lateral shoots at mid-November in *Q. ilex* (in both years) were successively killed by these frost events. Low chilling temperatures ($0°C < T_{min} \leq 8°C$) were also observed from September to May, well after leaf unfolding in early April for *Q. ilex* and end of January for *A. unedo*. The highest annual temperatures occurred in July, with the hottest two weeks in the second half of the month, when high T_{max} and low air relative humidity (RH < 25%) induced strong vapor pressure deficits (vpd > 4 kPa).
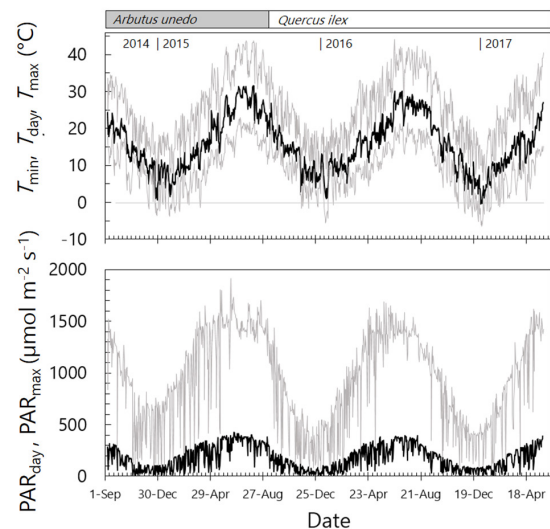

Figure 2 - Above. Daily temperature (*T*, °C): *Tmin* and *Tmax* (gray), *Tday* (black). Horizontal line, *Tday* = 0 °C. Below. Photosynthetic active radiation (PAR, μmol m⁻² s⁻¹): PAR*max* (gray), PAR*day* (black). Measurements on *Arbutus unedo* L. until 6-Sep 2015, then on *Quercus ilex* L.

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Table 1 - Average ± standard deviation of fluorescence parameters over the course of the study. *Fv/Fm* = maximum fluorescence yield. ETR*max* = maximum electron transport rate (µmol m^{-2} $^{s-1}$), NPQ$_S$ = sustained non photochemical quenching, qL$_S$ = fraction of functional reaction centers. Different letters indicate significant differences within species (*p* < 0.05). Italic bold, seasons without significant differences (*p* ≥ 0.05) between species (Wilcoxon (1945) rank sum test with continuity correction).

Season	*Fv/Fm*	ETR*max*	NPQ$_S$	qL$_S$
	Arbutus unedo L.			
spring (AMJ)	0.728 ± 0.038 b	47 ± 10 b	0.87 ± 0.56 b	0.58 ± 0.12 b
summer (JAS)	*0.744 ± 0.029 a*	*57 ± 8 a*	0.46 ± 0.25 c	0.51 ± 0.08 c
autumn (OND)	0.742 ± 0.048 a	36 ± 14 c	0.78 ± 0.61 b	0.57 ± 0.07 b
winter (JFM)	0.650 ± 0.049 c	31 ± 9 d	2.64 ± 0.89 a	0.76 ± 0.09 a
	Quercus ilex L.			
spring (AMJ)	0.742 ± 0.041 a	63 ± 18 a	1.24 ± 0.45 c	0.43 ± 0.05 b
summer (JAS)	*0.743 ± 0.032 a*	*59 ± 13 a*	0.51 ± 0.21 d	0.33 ± 0.04 c
autumn (OND)	0.690 ± 0.064 b	50 ± 16 b	1.84 ± 1.33 b	0.39 ± 0.05 d
winter (JFM)	0.636 ± 0.120 c	39 ± 16 c	4.56 ± 4.28 a	0.49 ± 0.11 a

Relationship between environmental and physiological parameters

Pooling the whole experimental period for the two species, significant relationships were detected between mean daily temperature (T_{day}) and fluorescence parameters (F_v/F_m: R = 0.63, R$^2_{adj}$ = 0.40; NPQ$_S$: R = -0.56, R$^2_{adj}$ = 0.31; ETR$_{max}$: R = -0.58, R^2 = 0.34; qL$_S$: R = -0.30, R$^2_{adj}$ = 0.10; all P < 0.001; n_{obs} = 996). A quadratic function increased model prediction for F_v/F_m, NPQ$_S$ and ETR$_{max}$ (but not qL$_S$) to R$^2_{adj}$ values of 0.61, 0.42 and 0.40, respectively (P < 0.001, n_{obs} = 996). The relationship between fluorescence parameters and radiation (PAR$_{max}$) showed significant (P < 0.001, n$_{obs}$ = 996) but lower R values than reported for temperature, with the best linear prediction found with ETR$_{max}$ (R = 0.53, R$^2_{adj}$ = 0.28), followed by F_v/F_m (R = 0.40, R$^2_{adj}$ = 0.17), NPQ$_S$ (R = -0.40, R$^2_{adj}$ = 0.16) and qL$_S$ (not significant).

Species differed within each season for fluorescence parameters (F_v/F_m, ETR$_{max}$, NPQ$_S$ and qL$_S$) except for F_v/F_m and ETR$_{max}$ in summer (Wilcoxon sum rank test P > 0.05). In each season, NPQ$_S$ was higher and qL$_S$ lower in *Q. ilex* than in *A. unedo*. ETR$_{max}$
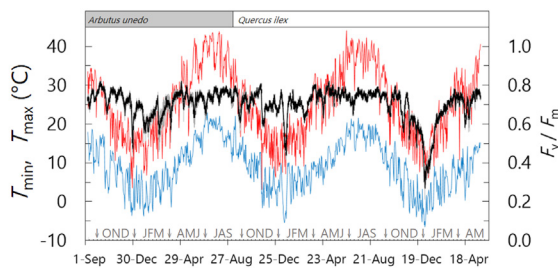

Figure 3 - Daily measurements of *Fv/Fm* (black) from Sep 2014 to May 2017. Seasons are identified by the capital letter of the months (OND, October November December; JFM, January February March; AMJ, April May June; JAS, July August September). Concurrent changes in air temperature (*T*, °C; daily maximum, in red, and minimum, in blue) are presented as a reference. In the upper part of the graph the measurement period for *A. unedo* and *Q. ilex* is indicated.

was also higher in *Q. ilex* in all of the seasons except summer, while F_v/F_m was higher in *A. unedo* in autumn and winter, but lower in spring (Table 1).

Temperature response dynamics

The time-course of temperature and F_v/F_m for both species is presented in Figure 3. In each season, F_v/F_m appears to rapidly acclimate to temperature changes, mainly decreasing in response to chilling spells and recovering when temperatures rose again. F_v/F_m acclimated to temperature more rapidly (in just a few days) in *A. unedo* than *Q. ilex* (often more than a week).

In mild winters, such as 2015 and 2016, the two species showed similar F_v/F_m minimum values (*A. unedo*, 0.48; *Q. ilex*, 0.45). In *A. unedo* minimum values occurred both after 7 consecutive frost days at the end of December 2014 and 2 days after the year absolute T_{min} = -4°C in January. *Q. ilex* showed the minimum value in F_v/F_m after 10 consecutive frost days at the end of January 2016 (F_v/F_m = 0.48) and again at the beginning of January 2017 (F_v/F_m = 0.28). The severe frosts of winter 2017 had a particularly severe effect and were followed by a long period of photo-inhibition lasting more than two months until spring (Figure 3). This could be the result of the accumulation of cold days with chilling and freezing temperatures: even if T_{min} values of the 10 days before the F_v/F_m minimum were similar in 2016 and 2017 (P > 0.8), temperatures averaged over a longer period (20-40 days) showed colder (P < 0.05) conditions in 2017.

The dynamics of the recovery to optimal F_v/F_m values (F_v/F_m ≥ 0.75) are also worth considering. In autumn (both species) or in winter (only *Q. ilex*), F_v/F_m recovered to its optimal value following warm days (T_{day} > 8°C), while later in the season (April-May) a higher temperature was required (T_{day} of

about 14°C). In late spring and early summer (June until mid-July) both species became sensitive to high temperatures, which induced a drop of F_v/F_m below 0.7 in association with the first T_{max} peaks (and drops of RH below 30%). From the end of the summer (mid-September) both species responded again to drops in temperature, resulting in a reduction in F_v/F_m (Figure 3). Overall, in both species F_v/F_m followed T_{day} changes according to a quadratic function (Q. ilex, $R^2 = 0.65$; A. unedo, $R^2 = 0.53$, $P < 0.001$). While similar values of F_v/F_m were observed above $T_{day} = 10°C$, chilling (or freezing) temperatures ($T_{day} < 10°C$) induced a higher depression in F_v/F_m in Q. ilex than in A. unedo, with F_v/F_m values below 0.5 and 0.6 at $T_{day} = 0°C$, respectively (Figure 4).

The fraction of days with sub-optimal F_v/F_m values (< 0.75) within each month and season is shown in Figure 5. The frequency of days with sub-optimal F_v/F_m progressively increased in Q. ilex from spring (48%) to winter (91%), while A. unedo progressively decreased this frequency from winter (100%) to autumn (48%). Q. ilex (but not A. unedo) still showed optimal F_v/F_m values in favorable winter days (JFM), but never in December. In early summer (July) both species showed an increase in the frequency of days with sub-optimal F_v/F_m values, extended also to June in A. unedo (Figure 5).

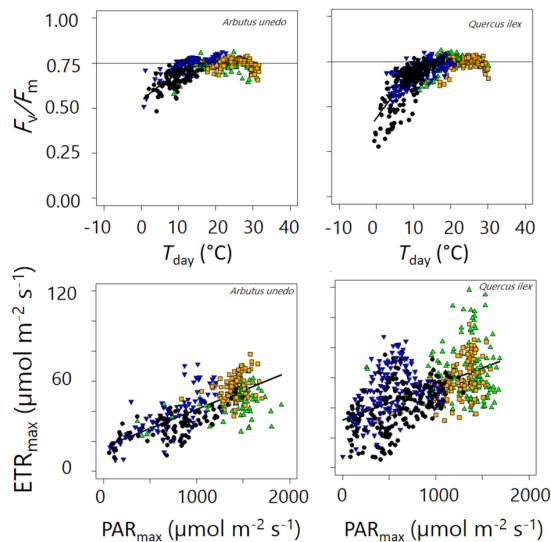Considering other fluorescence parameters, low temperatures resulted in an increase in sustained non-photochemical quenching (NPQ_S), which reached the maximum values in winter ($NPQ_s > 4$), while in summer never exceeded 1.2. PRI peaked at 0.03 at temperatures between 14 and 21°C, decreasing at both higher and lower temperatures to values of -0.10 and -0.15, respectively. Only a weak quadratic relationship with T_{day} was observed, however ($R^2_{adj} = 0.28$). As expected (Porcar-Castell et al. 2012), a strong negative linear relationship was observed at seasonal scale between NPQ_S and PRI ($R = -0.78$, $R^2_{adj} = 0.621$, $P < 0.001$). An overall linear and positive relationship between T_{day} and ETR_{max} was also observed in both species, which was stronger in A. unedo than in Q. ilex (A. unedo: $R = 0.800$, $R^2 = 0.632$; Q. ilex: $R = 0.571$, $R^2_{adj} = 0.325$; $P < 0.001$, $n_{obs.} = 365$; 631, resp.), with no difference in slope ($P > 0.05$), but a 70% higher intercept in Q. ilex.

Response to solar radiation

Both fluorescence parameters and PRI showed a lower correlation with radiation than with temperature, except for ETR_{max} (overall, $R^2_{adj} = 0.57$; A. unedo: $R^2_{adj} = 0.59$; Q. ilex: $R^2_{adj} = 0.29$, $P < 0.001$; Figure 4). It should be noted, however, that PAR_{max} and T_{day} were linearly correlated ($R = 0.755$, $R^2_{adj} = 0.569$, $P < 0.000$), making the interpretation of results more dif-

Figure 4 - Above. Relationship between *Tday* (°C) and *Fv/Fm: Arbutus unedo*, y = -0.00045·x^2 + 0.0198x + 0.538 (R^2 = 0.53, p < 0.000, nobs. = 365); *Quercus ilex* L., y = -0.00071x^2 + 0.0304^x + 0.4348 (R^2 = 0.65, P < 0.000, *nobs*. = 630). Below. Relationship between PARmax (μmol m$^{-2 s-1}$) and *ETRmax* (μmol m-2 s-1): *Arbutus unedo* L., y = 0.025 x + 15.96 (R^2 = 0.59, P < 0.000, nobs. = 365); *Quercus ilex* L. , y = 0.023 x + 31.77 (R^2 = 0.29, p < 0.000, *nobs*. = 630). Black circles (winter), green triangles (spring), orange squares (summer), blue down-pointing triangles (autumn).
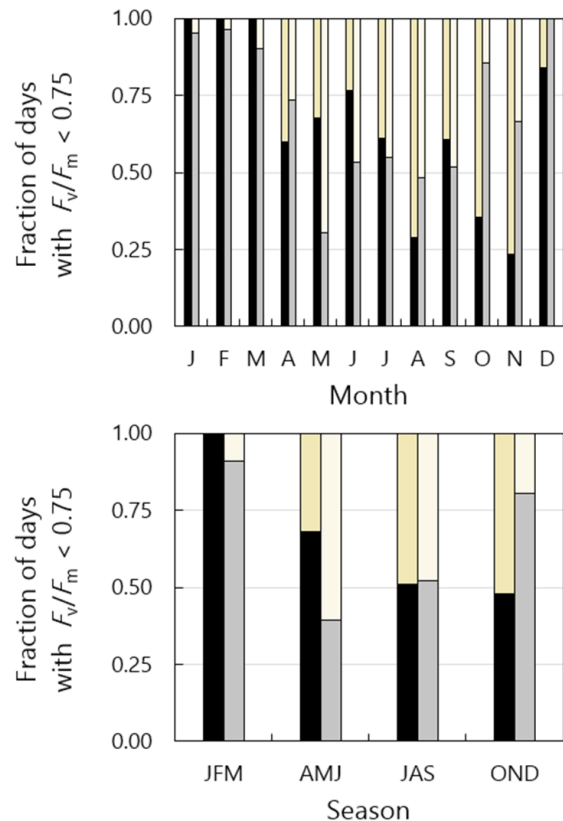

Figure 5 - Fraction of days in each month (above) or season (below) showing photoprotection (*Fv/Fm* < 0.75) (*A. unedo* in black; *Q. ilex* in gray). Complement to 1 (colored bars) is *NDopt*, the fraction of days with optimal *Fv/Fm* (≥ 0.75).

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ficult. As already reported for temperature, regression lines of ETR_{max} as a function of PAR_{max} for the two species were parallel (no significant slope difference) and had a higher intercept (almost double) in oak. High irradiance did not appear to have a lasting photo-inhibitory effect. In *A. unedo*, measurements of F_v/F_m, NPQ_S and qL_S taken after days with saturating light conditions (PAR > 1000 µmol m^{-2} s^{-1} in spring and summer; PAR > 500 µmol m^{-2} s^{-1} in autumn and winter) did not differ from the overall seasonal average. *Q. ilex* showed a higher sensitivity to high solar radiation, as saturating light conditions resulted in higher F_v/F_m (and lower NPQ_S) values in autumn and winter but no significant deviation from the overall average was observed in spring and summer.

Discussion

Long-term observations of chlorophyll fluorescence and reflectance has allowed the investigation at daily scale of the annual dynamics of leaf photochemistry several fluorescence parameters and reflectance indices in response to temperature and light environment in two co-existing Mediterranean evergreen species (Gasparini and Tabacchi 2011). Although our experimental setup did not allow a truly paired comparison between species, it provided useful information on their response to temperature stresses differing in severity and frequency. In comparison with previous studies which periodically measured leaf photochemistry on a monthly basis (e.g. Aranda et al. 2005), our approach could track its daily response, making it possible to determine seasonal changes in stress sensitivity, the outcome of the first stressful events (i.e. first autumn frosts or first summer high temperatures), and the cumulative effect of cycles of stressful conditions. On the other hand, had the research focus been on the evaluation of variability of physiological traits at population level, this time-intensive monitoring approach would not have been be the best choice.

Under cool and cold Mediterranean climate, hot summer periods (associated with drought and high radiation), but also cold winter days, might be challenging for evergreen species (Larcher 2000, 2003). Generally, summer drought is considered the most important growth-limiting factor under Mediterranean-climate, but the effect of low temperatures should also considered (Specht 1981, Öquist and Huner 2003). A variety of structural, anatomical and functional traits help plants to face stressful conditions (Cherubini et al. 2003, Bussotti et al. 2015, Niinemets and Keenan 2014, Gratani et al. 2018), and the role of photoprotection should not be overlooked.

Photosynthesis requires fine regulation mechanisms to effectively cope with environmental variability in radiation, temperature, water and nutrient availability. The balance between growth and protection is essential for assuring survival in perennial life-form. Thus, a competitive strategy should assure a high rate of electron transport to the Calvin cycle under favorable conditions and avoid damages by hazardous reactive oxygen species formation by dissipating any excess energy as heat through NPQ. The non-photochemical quenching of excitation energy relies on physiological processes regulated at different timescales from pico-seconds to weeks (Garcia-Plazaola et al. 2017, Porcar-Castell et al. 2014). The decrease (or down-regulation) in F_v/F_m, sometimes referred to as 'photoinhibition', is often the result of the maintenance overnight of a sustained photo-protective state, in response to light levels in excess of the Calvin cycle capacity. In reality, down-regulation of F_v/F_m may be caused both by an increase in sustained NPQ and by damaged (photo-inactivated) reaction centers (Porcar-Castell et al. 2014).

The F_v/F_m reduction associated with a temperature decrease and the ensuing recovery indicate the high modulation capacity of this parameter in order to ensure an adequate photoprotection and flexible acclimation of photosynthesis processes throughout the year. Only under the first hot days in late spring/early summer did F_v/F_m reverse its response to temperature, showing an enhanced photo-protection/photo-inhibition state in response to a temperature (and vpd) increase (Lange et al. 1985, Ripullone et al. 2009, Martínez-Vilalta et al. 2003). Under such conditions, high non-stomatal limitations to photosynthesis (Grassi et al. 2009) and growth inhibition (Campelo et al. 2018) are not always linked to a reduction in carbon assimilation (Crescente et al. 2002). In the present study, under conditions of full water availability both species had rather constant values of F_v/F_m and ETR_{max} over the summer. This is consistent with previous studies, which for many Mediterranean evergreens under field conditions reported a maximum carbon assimilation over an air temperature range of 10-20°C, with the higher limit determined by the onset of summer drought (Flexas et al. 2014, Gratani et al. 2018). In both species examined in the present study, a high thermostability at short-term time scale has also been observed for F_v/F_m over a wide range of leaf temperatures, from 5°C to 48°C (Méthy et al. 1997, Bilger et al. 1987). In response to stress evergreen species change both their foliar biochemical contents (increasing antioxidants and xanthophyll pigments; Verhoeven 2014) and the composition of PS II antenna but also in-

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crease energy dissipation through cyclic electron transport (Öquist and Huner 2003), in particular during the chilly/frosty winter period. In our study, the strong reduction of F_v/F_m was mainly related to the increase in sustained non-photochemical quenching during the winter (NPQ$_S$, R = -0.85). This latter was strongly associated to PRI, suggesting that also seasonal changes in xanthophyll (VAZ) pools or xanthophyll de-epoxidation state occurred in both species (Baraldi et al. 2008, Ripullone et al. 2011, Corcuera et al. 2005; Camarero et al. 2012). In their de-epoxidated state (Z+A) xanthophylls prevent damages to the thylakoid membranes, quenching excess energy through heat (NPQ$_s$ increase) and thus reducing electron flow in parallel with Calvin cycle limitations (ETR$_{max}$ reduction). García-Plazaola and colleagues (2017) observed in *Q. ilex* an emergency mechanism associated with the lutein cycle (LxL), which supplements the VAZ cycle in response to prolonged environmental stresses, both in winter and in summer. Although *A. unedo* had a lower content of lutein than holm oak (García-Plazaola et al. 2004), in the former species the LxL cycle was involved in photo-protection from salinity-induced water stress over the summer (Fusaro et al. 2014). Regulatory mechanisms via non-photochemical quenching could allow photosynthesis (and eventually growth) in seasons when water is abundant but temperatures are sub-optimal (Miller 1982), and thus reward the construction and maintenance costs. In evergreens, greater resistance to frost and freezing-induced cavitation may be obtained by thicker leaves (Lo Gullo & Salleo 1993), but higher allocation of structural carbohydrates and nitrogen to cell wall than to photosynthetic apparatus (González-Zurdo et al. 2016) adds construction costs at the expenses of carbon gain (van Ommen Kloeke et al. 2012). In both species, ETR$_{max}$ and F_v/F_m values were still high during the autumn (even if lower than in spring and summer), consistent with the high carbon assimilation capacity found in this season (Gratani and Varone 2004, Beyschlag et al. 1987, Ogaya and Peñuelas 2003). In autumn (and winter, if temperatures are not too low) a dynamic response to temperature and light allows evergreen species to effectively exploit these seasons for carbon assimilation (Oliveira and Peñuelas 2004, Ripullone et al. 2009). This is particularly relevant, since in autumn under natural conditions photosynthesis could still drive growth, in terms of new shoots flushing and elongation, stem enlargement (Susmel et al. 1976, Gratani et al. 1992, Martin 2012), or mobilizing processes to storage reserves (Canadell et al. 1999, Zavala et al. 2011). In both species, a minor annual peak in stem growth is sometimes ob-

served during the autumn (Martin 2012, Campelo et al. 2018, Castell et al. 1994), and brachyblasts can be emitted (Orsham 1989, Pereira et al. 1987); although a strong variability among years and sites exists, related to environmental conditions (Milla et al. 2010). Winter ETR$_{max}$, even if reduced in comparison with other seasons, still responded dynamically to radiation and temperature, taking advantage of warm sunny days, adjusting dynamically together with the other physiological parameters (F_v/F_m, NPQ$_S$ and PRI), similarly to what observed by Zhang (2017). Oliveira and Peñuelas (2004) reported a positive relationship between F_v/F_m and light-saturated carbon assimilation capacity (A_{sat}) in winter, maintained as long as leaves are not subjected again to favourable conditions that enhanced F_v/F_m more rapidly than photosynthetic capacity. In the present study, the positive correlation between F_v/F_m and ETR$_{max}$ was maintained also in the response to the lowest T_{min} (-7°C), but after this frost event F_v/F_m recovered more rapidly in response to the temperature increase than ETR$_{max}$, which showed a lag of 10 days until also daily maximum temperature increased. The positive electron transport capacity (ETR$_{max}$) observed at sub-freezing temperatures in both species, if not quenched through photochemistry or cyclic electron transport, might induce damaging side reactions, exposing leaves to the risk of oxidation by reactive oxygen species (Murchie and Harbinson 2014, Verhoeven et al. 2018). Low winter temperatures and frost events in particular (Aranda et al 2005, Sperlich et al. 2014) can thus limit the survival of evergreen species not only at high latitudes or altitudes (Nichol et al. 2019, Williams et al. 2003), but also under Mediterranean climate (Alessi et al. 2018). For surviving and at the same time maintaining growth (and thus a high competitive capacity) in harsh environments, evergreen species have developed acclimation mechanisms to cope with cold spells, so limiting the damages induced by low temperatures in combination with high irradiance (and eventually low water availability by frozen soil, Sutinen et al. 2001). This allows the plant to maintain a positive carbon assimilation both in the summer, also in presence of high leaf temperatures (Gratani et al. 2000, Grassi et al. 2009), and in warm autumn days, so prolonging the period of active photosynthesis. In association with other traits (e.g. capacity to recover from drought- or frost-induced xylem cavitation; Salleo et al. 1997, Cherubini et al. 2003), this could result in a selective advantage over deciduous species (Blondel et al. 2010, Barbeta et al. 2019), if temperatures are not too extreme (Gentilesca et al. 2017, Pollastrini et al. 2018).

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Conclusions

From an ecological point of view, the two evergreen species displayed slightly different strategies; under the sub-humid Mediterranean climate at the study site, both species showed the highest photoinhibition in winter, with a high temperature dependence (Fernández et al. 2008) but with different strategies and acclimation capacity. On average, *A. unedo* was more photoinhibited than *Q. ilex* throughout the winter, despite the less negative temperatures experienced, and to a lesser extent also in spring and autumn, maintaining a chronic photoinhibition for at least 3 months. *Q. ilex* has a more dynamic response, with a high plasticity (range/max) in all fluorescence parameters, and appears to have a higher non-photochemical quenching capacity in all seasons, complemented by a lower fraction of open reaction centers. These characteristics effectively maintain a high electron transport capacity in *Q. ilex*, and protect the photosystems by dissipating the excess energy as heat and rapidly reducing the fraction of functional reaction centers. In the summer, under optimal water conditions, the differences between *Q. ilex* and the more thermophilous *A. unedo* become not significant.

Acknowledgments

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S. Raddi[1] *, F. Magnani[2]

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