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Chl Fluorescence Parameters and Leaf Reflectance Indices Allow Monitoring Changes in the Physiological Status of *Quercus ilex* L. under Progressive Water Deficit

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Abstract: Extreme droughts and heat events, frequently produced in Mediterranean climates, induce anomalies in the ecosystem–atmosphere CO_2 fluxes. In order to mitigate the consequences on forests and agriculture, managers must have a better knowledge of the ecosystem by monitoring plant status. Water status is commonly observed measuring water potential but when the extreme event is over, this parameter cannot show managers the recovery of other physiological processes such as photosynthesis. To address this problem, we have evaluated the *Quercus ilex* L. water status and photosynthetic capacity throughout an intense water scarcity event and a subsequent re-watering. Photosynthetic capacity was evaluated through chlorophyll fluorescence parameters and leaf reflectance indices. We found that all fluorescence parameters changed as water potential decreased and they did not completely recover after re-watering. Among the reflectance indices, the physiological reflectance index (PRI) varied similarly to fluorescence, obtaining a strong correlation with the non-photochemical quenching (NPQ). We proposed using PRI to detect the level of photosynthetic capacity in *Q. ilex*, as it is much easier-to-handle. We also concluded that intense droughts and heat stress not only might reduce photosynthetic capacity through changes in Chl fluorescence parameters during the stress period, but might also affect photosynthetic capacity once the plant water status is recovered.

Keywords: chlorophyll fluorescence; drought; water potential; *Quercus ilex*; reflectance

1. Introduction

Extreme climatic events, such as droughts and heat stress, can induce anomalies in the CO_2 fluxes between the ecosystem and the atmosphere, which may change the net carbon balance of an ecosystem [1,2]. Furthermore, the simultaneous combination of both stresses, drought and heat, entails a stronger C sink reduction than any other single extreme event [3]. Unfortunately, both stresses occur during the summer season of those areas under Mediterranean type-climates like the Mediterranean Basin [4]. In such ecosystems, climate change models also predict an increase of these extreme events [5], that would enhance water scarcity [6] leading to a higher down-regulation of net photosynthesis [7] and to a severe reduction in primary productivity [8]. Short-term consequences of this reduction are the increase of forest decline episodes [9] and, in agriculture, a more frequent use of irrigation for an affordable crop production [10]. *Quercus ilex* L., as a key tree species of the Mediterranean Basin might suffer both consequences. On the one hand this species is a well-spread oak of the Mediterranean Basin landscape [11] being also part of the so-called "dehesa" [12]. On the other hand, the oak is commonly used as producer of edible fungi of the genus *Tuber*, well appreciated in the haute cuisine [13,14].

In order to mitigate the consequences of extreme climatic events on forest and agriculture, managers must have a better knowledge of the ecosystem by monitoring plant status [15]. In this sense, the parameter water potential (Ψ) [16,17] is globally used to characterized plant water status (e.g., [18–20]). Nevertheless, once water scarcity is over, Ψ can only show managers the recovery of plants in terms of water, leaving unknown the recovery of other physiological processes such as photosynthesis. To avoid this problem, it is important to implement monitoring along with other easy-to-handle and efficient methods.

The development of hyperspectral remote sensing has enabled the use of leaf reflectance signals, both to estimate the leaf water concentration [21] and to quantify leaf chemistry in vegetation [22–24]. In this way, changes in reflectance indices such as the physiological reflectance index (PRI), based on changes in de-epoxidized forms of the xanthophyll cycle molecules [25,26], have been used to detect the effect of drought stress on photosynthetic capacity [27–30]. However, other indices as the normalized difference vegetation index (NDVI) that indicate changes in the chlorophyll pigment [31] did not always show a clear correlation with plant water stress [32]. Photosynthetic capacity changes are also accompanied by the emission of chlorophyll *a* fluorescence (ChlF) that regulates the switch between an efficient sunlight utilization and a thermal dissipation state [33,34]. This phenomenon is measurable as non-photochemical quenching (NPQ) of chlorophyll fluorescence [35], which under environmental stress, is associated to the down-regulation of maximum photochemical efficiency of photosystem II (FV/FM) [32,36–38].

Taking into account that plant status can be monitored easily and efficiently in terms of both water status and photosynthetic capacity, the aim of this work was to confirm the relationship of Ψ with fluorescence and reflectance parameters in order to find the best indices related to photosynthetic capacity able to complement a more established water status measurement. The relationship was studied during an intense soil water deficit period, under extreme temperature conditions, in *Q. ilex*, a species particularly important in Mediterranean forest landscape and agriculture. Additionally, plant recovery was also investigated by measuring plant status after re-watering. We hypothesized that most of the parameters used in this study would change with a decrease in water potential. We also hypothesized that after re-watering, plant water status would be completely recovered while photosynthetic capacity, estimated through ChlF and reflectance parameters, would not be completely recovered.

2. Materials and Methods

2.1. Plant Material and Experimental Conditions

Seeds from holm oak (*Q. ilex* subsp. *rotundifolia*) ("Soria" provenance, 41°46′ N, 2°29′ W, 1074 m above sea level, Spain) were sown and cultivated in 2003 in 0.5 L containers inside a greenhouse under the same conditions with a mixture of 80% compost (Neuhaus Humin Substrat N6; Klasman-Deilmann GmbH, Geeste, Germany) and 20% perlite. After the first growth cycle, seedlings were transplanted to 25 L containers filled with the same mixture (4:1 compost:perlite) and cultivated outdoors since then at CITA de Aragón (41°39′ N, 0°52′ W, Zaragoza, Spain) under Mediterranean conditions (mean annual temperature 15.4 °C, total annual precipitation 298 mm). A slow-release fertilizer (15:9:12 N:P:K, Osmocote Plus, Sierra Chemical, Milpitas, CA, USA) was periodically added to the top 10-cm layer of substrate (3 g L^{-1} growth substrate). All plants were grown under the same environmental conditions and drip-irrigated every 2 days.

The experiment took place during the summer of 2017. Two weeks before the beginning of the experiment, ten potted plants (14 years old) were placed under a clear plastic roof that allowed the passing of 90% of PPFD (~1800 µmol photons m^{-2} s^{-1} at midday, during the experiment). The use of covers in water-stress experiments had the advantage of performing measurements in more controlled environmental conditions, avoiding re-watering by storms or unwanted rainfall events. Watering was stopped on 21 August 2017 and measurements in well-watered plants started on 22 August 2017. During the following days, measurements were performed every two or three days with increasing levels of drought stress. Drought stress was imposed during 20 days. Finally, after the last measurement under drought stressed conditions, plants were re-watered and measurements were performed again after 7 days.

Air temperature (T, °C) and relative humidity (RH, %) were measured at the experimental site using a Hobo Pro temp/RH data logger (Onset Computer, Bourne, MA, USA) located at 1.30 m above the soil surface. Measurements were recorded every 60 min during August and September of 2017. Mean diurnal (from dawn to sunset) T and RH, maximum T and minimum RH values for each day during this period are shown in Figure 1.

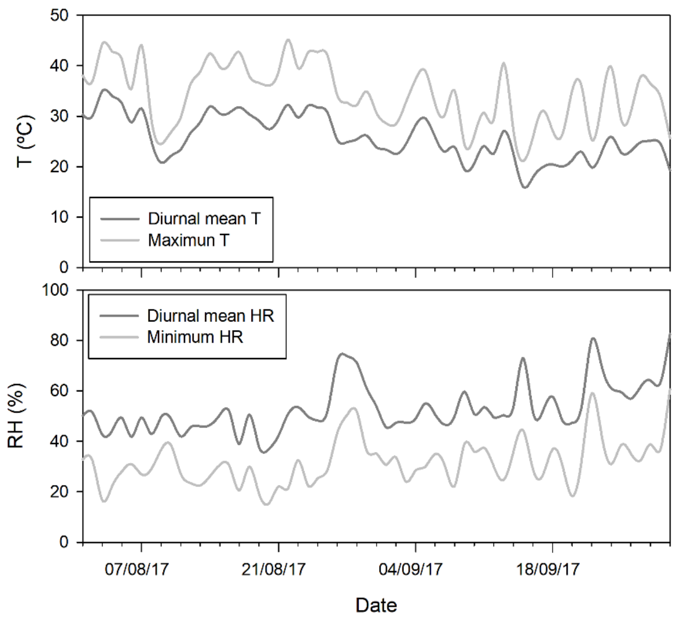

Figure 1. Atmospheric climatic conditions of the site during the experiment.

2.2. Water Potential Measurements

Predawn and midday leaf water potentials (Ψ_{PD} and Ψ_{MD}, MPa) were measured in shoots of holm oak (with leaves still attached to the shoots) with a Scholander pressure chamber following the methodological procedure described by Turner [39].

2.3. Chlorophyll Fluorescence

Chlorophyll fluorescence (ChlF) parameters were measured in fully developed current-year attached leaves of holm oak with a FMS II modulated fluorometer (Hansatech Instruments, Norfolk, UK). Initial ChlF in darkness (F_0) was measured at predawn by switching on the modulated light (0.6 kHz); leaf surface photosynthetic photon flux density was below 0.4 µmol m^{-2} s^{-1}. Maximal Chl fluorescence in darkness (F_M) was measured at predawn (20 kHz) with a 0.8-s pulse of 6000 µmol m^{-2} s^{-1} of white light. F_0 was measured in presence of far-red light that fully oxidizes the PSII acceptor side [40,41]. The Chl fluorescence at steady-state photosynthesis (F_S) was measured at mid-morning (8 h solar time) and midday (12 h solar time), and a second pulse of high-intensity white light was used to determine the maximum ChlF in the light-adapted state (F'_M). Leaves were then covered and the minimum ChlF after illumination in presence of far-red light (7 µmol m^{-2} s^{-1}) was determined (F'_0). The experimental protocol for the analysis of the ChlF quenching was essentially as described by

Genty et al. [42] with some modifications. These involved the measurements of F_0 and F'_0, which were measured in presence of far-red light (7 µmol m^{-2} s^{-1}) in order to fully oxidize the PSII acceptor side [40,41]. The dark-adapted, maximum potential PSII efficiency was calculated as F_V/F_M [43,44]. The actual (ϕ_{PSII}) and intrinsic (ϕ_{exc}) PSII efficiency were calculated as $(F'_M - F_S)/F'_M$ and F'_V/F'_M, respectively [42,45]. Photochemical quenching (qP) was calculated as $(F'_M - F_S)/F'_V$ according to van Kooten and Snel [46]. NPQ was calculated as $(F_M/F'_M) - 1$ [47].

2.4. Spectral Reflectance

Leaf reflectance was measured at mid-morning (8 h solar time) and midday (12 h solar time) in fully developed current-year attached leaves of holm oak with a visible/near-infrared spectroradiometer USB-2000 (Ocean Optics, Dunedin, FL, USA). A bifurcated fiber optic cable was connected to the spectroradiometer into one end and to a tungsten halogen light source LS-1-LL (Ocean Optics, Dunedin, FL, USA) into the other end. Leaf reflectance was expressed as spectral reflectance after standardization with white standard (Spectralon, Labsphere, North Sutton, NH, USA). Integration time was 200 ms. The physiological reflectance index (PRI) was calculated as $(R_{531} - R_{570})/(R_{531} + R_{570})$, where R_{531} and R_{570} represent, respectively, the reflectance at 531 and 570 nm [31,48,49]. The normalized difference vegetation index (NDVI) was calculated as $(R_{750} - R_{705})/(R_{750} + R_{705})$, where R_{750} and R_{705} represent, respectively, the reflectance at 750 and 705 nm [50,51]. The water index (WI) was calculated as R_{970}/R_{900}, where R_{970} and R_{900} represent, respectively, the reflectance at 970 and 900 nm [52,53]. The first derivate spectra were used to study the wavelength of the red-edge position [54,55].

2.5. Statistical Analysis

Data are expressed as means ± standard error of at least five single measurements, each one made on a different plant. Student's *t*-tests were used to compare the values measured for well-watered plants before the drought period and those measured 7 days after plants were re-watered. Values were considered statistically different when *p*-values were lower than 0.05. All statistical analyses were performed with SAS version 8.0 (SAS, Cary, NC, USA).

Data have been deposit in citaREA. The handle number is http://hdl.handle.net/10532/4065 and data are available online at https://citarea.cita-aragon.es/citarea/bitstream/10532/4065/1/Data.xlsx.

3. Results

3.1. Water Potential

Plants started the water deficit period with a predawn water potential (Ψ_{PD}) mean value of −0.1 ± 0.0 MPa. At this well-watered state, midday water potential (Ψ_{MD}) mean value was −2 ± 0.2 MPa. At the end of the dry period, plants reached Ψ_{PD} and Ψ_{MD} mean values of −7.0 ± 0.2 and −7.5 ± 0.2 MPa respectively. Seven days after plant re-watering Ψ_{PD} and Ψ_{MD} returned to less negative Ψ mean values, i.e., −0.6 ± 0.2 and −1.7 ± 0.4 MPa respectively, recovering the well-watered status.

3.2. Chlorophyll Fluorescence Parameters

The maximum potential PSII efficiency (F_V/F_M) had an initial mean value of 0.81 ± 0.00 that remained constant from Ψ_{PD} = −0.1 to −3.0 MPa (Figure 2). Below −3.0 MPa, F_V/F_M started to decrease reaching a mean value of 0.48 ± 0.04 at Ψ_{PD} = −7 MPa. After plant re-watering, F_V/F_M mean value sifted up to 0.75 ± 0.01, value that was statistically different ($p < 0.05$) to the one measured at the beginning of the dry period when plants had a Ψ_{PD} = −0.1 MPa.

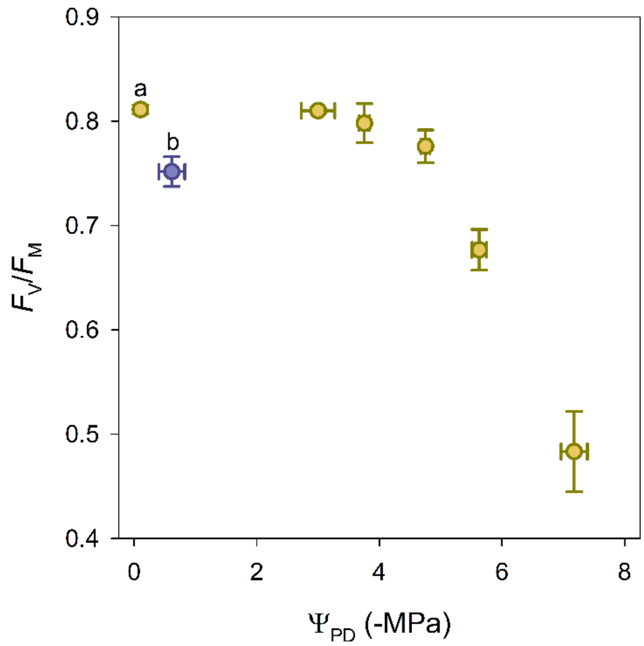

Figure 2. Relationship of predawn shoot water potential (Ψ_{PD}) with the maximum potential PSII efficiency (F_V/F_M). Yellow and violet circles are mean values ±SE ($n \geq 5$) measured during the water deficit period and after re-watering, respectively. Lowercase letters on top of the first yellow and violet mean points indicate statistically significant differences (Student's *t*-test, $p < 0.05$).

Mean values of actual (ϕ_{PSII}) and intrinsic (ϕ_{exc}) PSII efficiency and photochemical quenching (qP) measured at mid-morning were constant between Ψ_{PD} = −0.1 and −3.0 MPa. From −3.0 MPa to −7.0 MPa, the three parameters decreased: ϕ_{PSII} decreased from 0.48 ± 0.05 to 0.06 ± 0.01, ϕ_{exc} from 0.56 ± 0.06 to 0.21 ± 0.02 and qP decreased from 0.84 ± 0.03 to 0.31 ± 0.04 (Figure 3). At midday, the relation between Ψ_{MD} and these parameters, showed a continuous decrease of their mean values along the water potential range observed (from −2 to −7.5 MPa): ϕ_{PSII} decreased from 0.51 ± 0.00 to 0.08 ± 0.00, ϕ_{exc} from 0.59 ± 0.02 to 0.16 ± 0.03 and qP decreased from 0.87 ± 0.02 to 0.48 ± 0.04 (Figure 3). Mean values of ϕ_{PSII} and qP after re-watering shifted up, both at mid-morning and midday, reaching mean values above 0.2 and 0.5, respectively. On contrary, ϕ_{exc} did not show any recovery. Concerning the relations between Ψ and the non-photochemical quenching (NPQ), there were also no changes in the mean values of NPQ in the first sections of the curves (from Ψ_{PD} = −0.1 to −3.0 MPa and from Ψ_{MD} = −2.0 to −4.0 MPa) both measured at mid-morning and at midday. In the second sections (below Ψ_{PD} = −3.0 MPa and Ψ_{MD} = −4.0 MPa), mean values of NPQ increased from 1.2 ± 0.3 to 4.9 ± 0.6 at mid-morning and from 3.1 ± 0.0 to 6.1 ± 0.8 at midday. After plant re-watering, mean values of NPQ shifted down to 4.3 ± 0.3 at mid-morning and to 5.0 ± 0.7 at midday (Figure 3). Recovery values of these fluorescence parameters (excluding qP measured at midday) were statistically different ($p < 0.05$) to the ones measured at the beginning of the experiment (Figure 3).

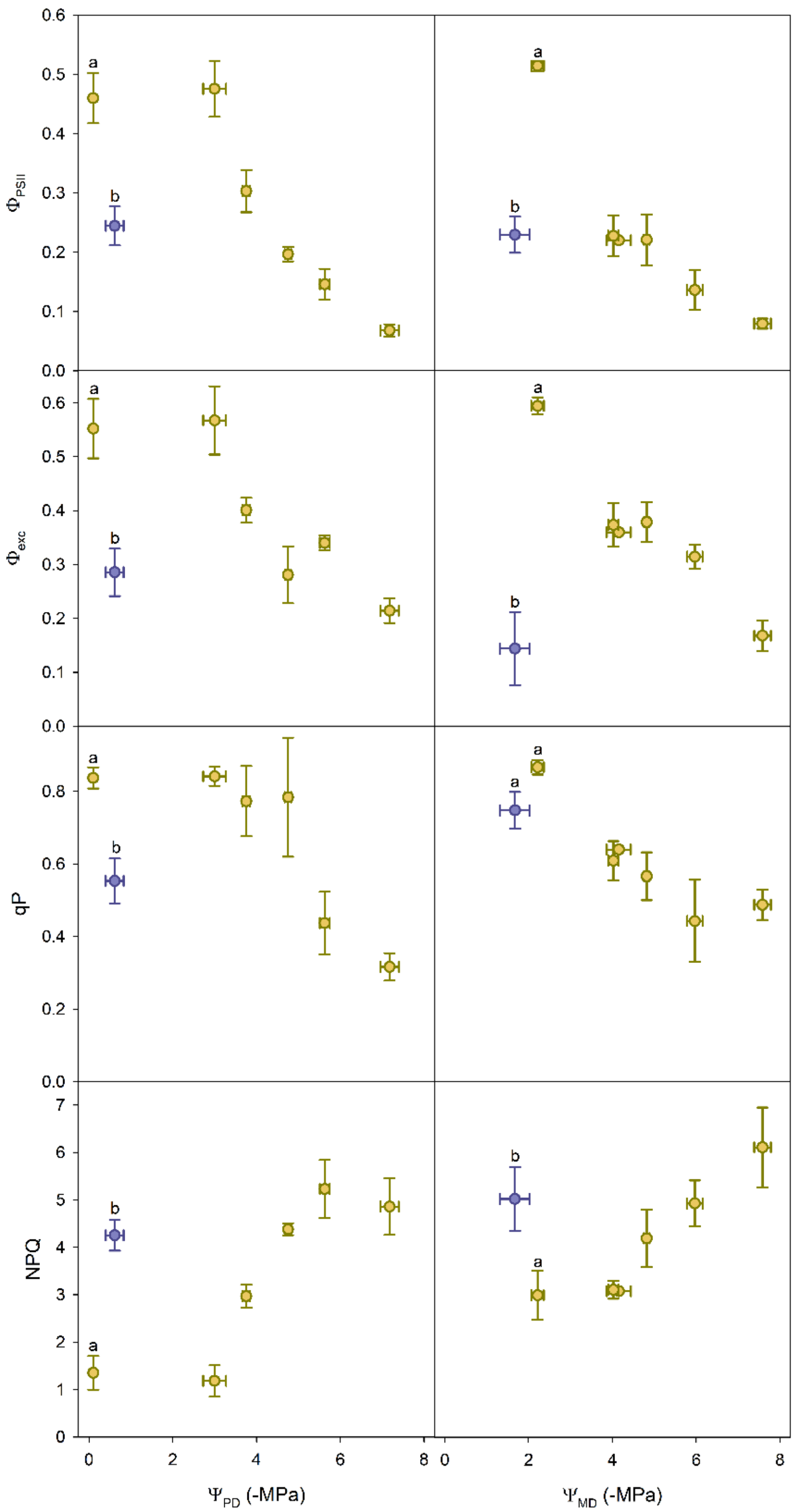

Figure 3. Relationships of predawn and midday shoot water potential (Ψ_{PD}, Ψ_{MD}) with chlorophyll fluorescence (ChlF) parameters: Actual (ϕ_{PSII}) and intrinsic (ϕ_{exc}) PSII efficiency, photochemical quenching (qP) and non-photochemical quenching (NPQ). ChlF parameters measured during mid-morning were associated to Ψ_{PD}, while those measured at midday were related to Ψ_{MD}. Yellow and violet circles are mean values ±SE ($n \geq 5$) measured during the soil water deficit period and after re-watering, respectively. Lowercase letters on top of the first yellow and violet mean points indicate statistically significant differences (Student's *t*-test, $p < 0.05$).

3.3. Reflectance Indices

The physiological reflectance index (PRI) measured at mid-morning remained constant from a well-hydrated state (Ψ_{PD} = −0.1 MPa) to a value of Ψ_{PD} c.a. −3.0 MPa. Below this Ψ_{PD} value, PRI measured at mid-morning dropped from 0.01 ± 0.00 to −0.02 ± 0.00, value that remained constant at more negative values of Ψ_{PD}. PRI measured at midday followed a similar pattern: a constant mean value of 0.01 ± 0.01 from −2.0 to −4.0 MPa and a drop to c.a. −0.04 ± 0.01 at Ψ_{MD} = −7.5 MPa (Figure 4). After plant re-watering both mid-morning and midday PRI mean values were −0.02 ± 0.01, values statistically different ($p < 0.05$) to those measured at the beginning of the dry period.

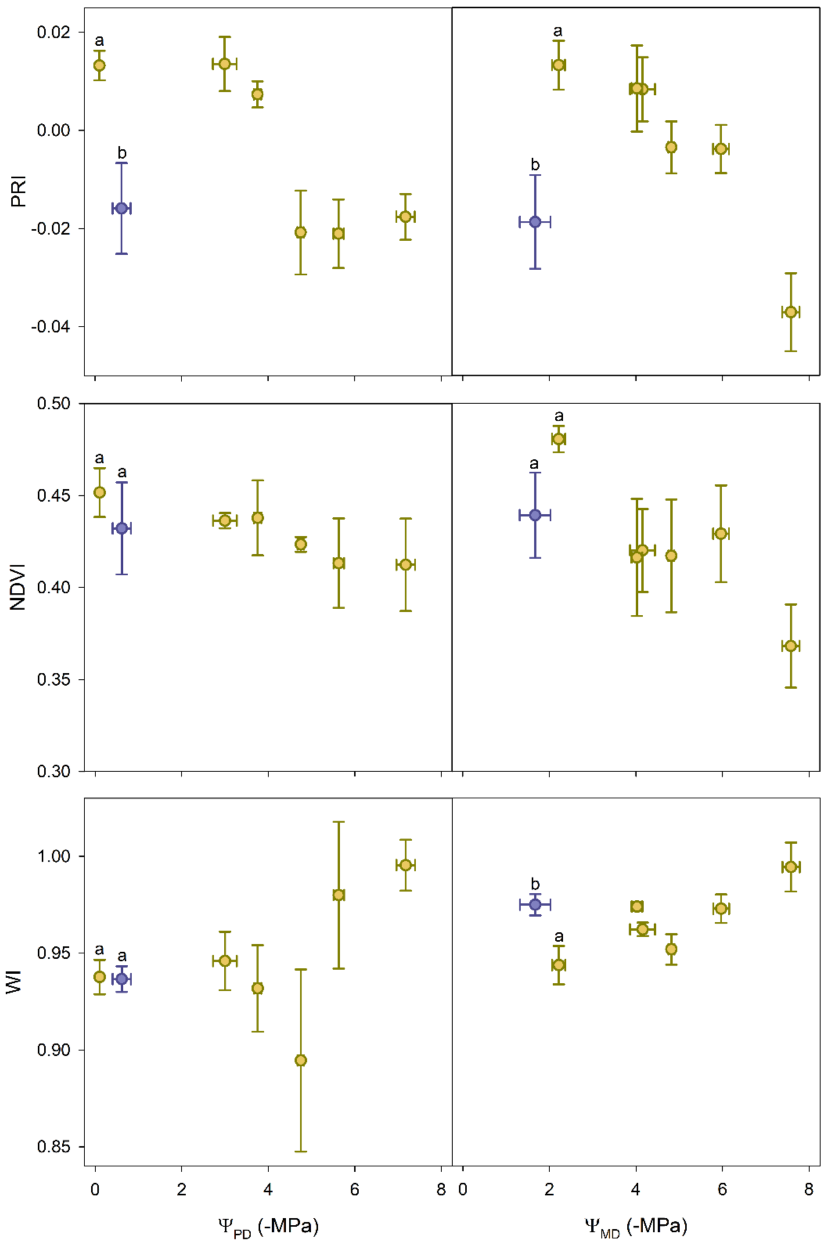

Figure 4. Relationships of predawn and midday shoot water potential (Ψ_{PD}, Ψ_{MD}) with reflectance indices: physiological reflectance index (PRI), normalized difference vegetation index (NDVI) and water index (WI). Reflectance indices measured during mid-morning were associated to Ψ_{PD}, while those measured at midday were related to Ψ_{MD}. Yellow and violet circles are mean values ±SE ($n \geq 5$) measured during the soil water deficit period and after re-watering, respectively. Lowercase letters on top of the first yellow and violet mean points indicate statistically significant differences (Student's *t*-test, $p < 0.05$).

The normalized difference vegetation index (NDVI) at mid-morning slightly decreased from a mean value of 0.45 ± 0.01 (at $\Psi_{PD} = -0.1$ MPa) to 0.41 ± 0.02 (at $\Psi_{PD} = -7.0$ MPa). The decrease at midday was a bit more remarkable falling from 0.48 ± 0.01to 0.37 ± 0.02. Mean values after re-watering reached 0.43 ± 0.02 both at mid-morning and at midday (Figure 4). These values were not statistically different ($p < 0.05$) to those measured at the beginning of the dry period.

The mean value of water index (WI) slightly increased towards lower values of Ψ from 0.94 ± 0.01 to 0.99 ± 0.01, both at mid-morning and at midday. After plant re-watering, mean values of WI were

0.94 ± 0.01 at mid-morning (not statistically different to the one measured at Ψ_{PD}= 0.1, $p < 0.05$) and 0.97 ± 0.01 at midday (statistically different to the one measured at Ψ_{PD}= 0.1, $p < 0.05$).

Finally, the red-edge position did not show any variation with changes in water potential (data not shown).

4. Discussion

4.1. Response to Water Scarcity

The enhanced levels of soil water scarcity imposed in this study caused a severe reduction in shoot water potential, both at predawn (Ψ_{PD}) and at midday (Ψ_{MD}), reaching values close to those found in natural stands during summer drought [56]. This reduction in has been successfully related to the variation of several chlorophyll fluorescence (ChlF) parameters (maximum potential PSII efficiency (F_V/F_M); the actual (ϕ_{PSII}) and intrinsic (ϕ_{exc}) PSII efficiency; photochemical (qP) and non-photochemical (NPQ)), confirming similar results found in previous studies [32,57]. As these fluorescence parameters can be considered a proxy of the photosynthesis capacity [33,34], the Ψ_{PD} value from which the parameters started to change (c.a. −3 MPa) could be the threshold from which CO_2 assimilation rates of *Q. ilex* could significantly decreased, reducing the primary productivity and inducing anomalies in the tree–atmosphere CO_2 fluxes [1,2]. In fact, Peguero et al. [58] measured negligible CO_2 assimilation rates at −3 MPa for *Q. ilex*, showing also similar results in F_V/F_M, ϕ_{PSII} and qP. This threshold, obtained with fluorescence parameters, was also detected in *Robinia pseudoacacia* and *Amorpha fruticosa* [57], which initial decrease in F_V/F_M also matched the beginning of negligible CO_2 assimilation rates. However, F_V/F_M in *Quercus suber* did not show a clear variation with Ψ_{PD} [58], which implies that not always Chl fluorescence parameters worked as photosynthesis capacity proxies.

Changes in ChlF have also been related to changes in reflectance indices in previous studies [31,49,59,60]. Our investigation confirmed such relation as we found an increase in the values of NPQ with a decrease in the physiological reflectance index (PRI) during the progressive increment in water scarcity ($R^2 = 0.80$, $p < 0.05$, Figure 5). Other works have also found good correlations between PRI and NPQ for *Vitis vinifera* [61] and *Quercus coccifera* [31]. This relation can be expected because both parameters reflect in many cases photoprotection processes related to pH and/or de-epoxidation of the xanthophyll cycle [49]. In fact, PRI has been directly correlated with various photosynthetic-related variables [62–64]. Due to the relationship in Figure 5, the variation of Ψ_{PD} with PRI was similar to those found for the ChlF parameters, including the detection of the same threshold point at $\Psi_{PD} = -3$ MPa (Figure 4). Variations in PRI with changes in holm oak water status have been also found in Tsonev et al. [64] and Zhang et al. [32]. However, these authors stated that values of PRI at severe drought remained quite stable. Our results confirmed partially this statement as PRI values measured at mid-morning remained constant below c.a. $\Psi_{PD} = -4.5$ while PRI measured at midday continued decreasing (Figure 4). The other reflectance indices used in this study (normalized difference vegetation index (NDVI); water index (WI); red-edge position) showed weaker and noisier relationships with Ψ. Furthermore, these indices did not show correlations with fluorescence parameters (data not shown), indicating a worse relation with photosynthesis capacity. In fact, previous studies in holm oak showed that WI was not very sensitive to changes in leaf water content during the first steps of the drying process [21] and NDVI was not correlated with leaf-level net photosynthesis rates [32,65]. On contrary, red edge measured in pine was strongly correlated with chlorophyll concentration [66] and it was able to distinguish healthy pines from those undergoing decline [67]. Despite of NDVI and position of red edge to be highly related to changes in chlorophyll concentration [54,55,68], the lack of drought-induced changes in Chl concentration showed in *Q. ilex*, (as reported by Peguero-Pina et al. [58]), may help to explain the lack of relationship between NDVI and the red-edge position with fluorescence parameters.

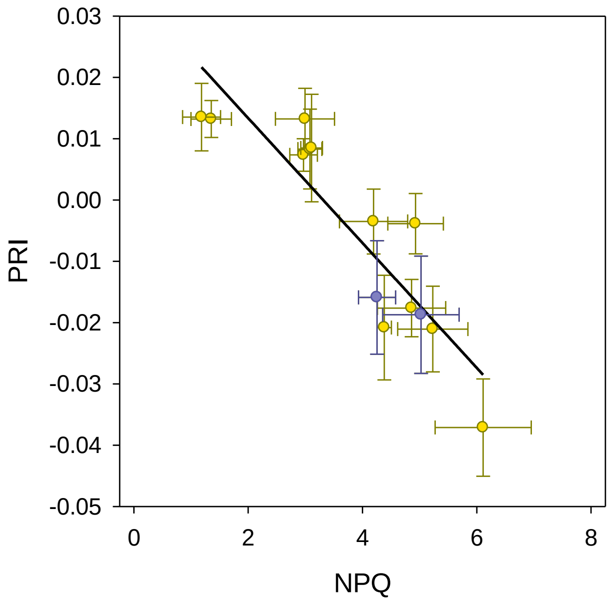

Figure 5. Relationship between mean values (±SE) of the non-photochemical quenching (NPQ) and the physiological reflectance index (PRI) measured along the water scarcity period (yellow) and after re-watering (violet). Mid-morning and midday mean values are both included.

4.2. Recovery from Water Scarcity

The level of drought stress imposed in this study can induce in *Q. ilex* an 80% loss of xylem conductivity [69]. In spite of this possible loss, Ψ after re-watering indicated a complete recovery of plant water status, consistent with other species in previous reports [57,69–71]. On contrary, the photosynthetic capacity, estimated through Chl fluorescence parameters, did not show a complete recovery, indicating a possible reduction in the CO_2 fixation rates after drought stress. With respect to this, Galmés et al. [72] also showed a net photosynthesis partial recovery in 10 other Mediterranean plant species after a severe drought. Concerning the reflectance indices, the partial recovery of PRI was similar to the partial recovery of NPQ, supporting the relation found in Figure 5. The other reflectance indices (NDVI, WI, red-edge) showed in most cases a complete recovery, probably due to their lower change, confirming again the lack of relationship with Chl fluorescence parameters previously found during the drought period.

The non-recovery of ChlF activity after an intense water stress event in the evergreen *Q. ilex* might imply, not only a reduction in primary productivity during the stress event, but also a reduction after the stress event when plants were water recovered. Water efficiency of the ecosystem in these cases will be very low and the income flux of CO_2 would be reduced until a new leaf growth.

5. Conclusions

Chlorophyll fluorescence parameters, as estimators of photosynthetic capacity, varied with a decrease in water potential during a drought-simulated period in *Quercus ilex*. One on these parameters, the non-photochemical quenching (NPQ), was correlated with the physiological reflectance index (PRI), which values also changed with the increase of water scarcity. Both parameters had a threshold point around −3 MPa that might indicate the beginning of negligible net photosynthesis rates. After re-watering, plant water status was completely restored while fluorescence parameters and PRI did not recover the initial values. That is, intense droughts and heat stress not only might reduce the photosynthetic capacity of holm oak during the stress period, but also might affect the photosynthetic capacity once the plant water status is recovered. Taking into account that PRI showed the same trend than ChlF parameters and that measuring PRI is much easier-to-handle than ChlF, we propose to use PRI to detect the level of photosynthesis capacity in *Q. ilex*, and in this way, to complement the measurements of Ψ during drought events.

Author Contributions: D.S.K., O.M., E.G.P. and J.J.P.P. conceived and designed the experiments; D.S.K., J.J.P.P. and O.M. performed the experiments; J.J.P.P. and O.M. analyzed the data; D.S.K. wrote the initial draft; All authors contributed to the discussion of the results and to the writing of the final version of the manuscript.

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