



Full Length Article

Gas Exchange, Water Relations and their Relationships with Photochemical Reflectance Index in *Quercus ilex* Plants during Water Stress and Recovery

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Abstract

Diffusional limitations to photosynthesis (A), relative water content (RWC) and their association with photochemical reflectance index (PRI) were studied in holm oak (*Quercus ilex* L.) saplings subjected to water stress and subsequent water stress relief. RWC decreased sharply as drought progressed. Following rewetting, RWC gradually increased to pre-stress values. A , stomatal conductance (g_s), mesophyll conductance (g_m), total conductance (g_t), and PRI changed in parallel with RWC. A decrease in A resulted from coordinated suppression in leaf conductance. As water stress progressed, A measured in non-photorespiratory conditions ($A_{1\%O_2}$) became more than 3 times higher compared to that recorded at 21% [O_2], indicating that photorespiration likely increased in response to water stress. $A_{1\%O_2}$ was significantly correlated with all leaf conductances. $A_{1\%O_2}$ was limited more by g_s than by g_m , indicating that higher g_m favors photosynthesis over photorespiration. There was a significant linear correlation between RWC and PRI. Significant non-linear relationships were also found both between PRI and A and PRI and g_t . These data imply that PRI may clearly detect normal and stressed status of holm oak, but it is not very informative when plants are severely stressed. Overall, our data indicate that: a) PRI is an important indicator of leaf water status as evident from RWC; b) because leaf conductance did not limit photosynthesis when photorespiration was suppressed, holm oak, and in general sclerophyllous species, will respond to future elevated [CO_2] significantly more than mesophyllous plants; c) PRI can be used to predict changes in the performance of holm oak subjected to physiological water stress, whereas caution is needed under severe drought. © 2014 Friends Science Publishers

Keywords: CO_2 transport conductance; Drought; Leaf gas exchange; Photorespiration; Relative water content; Spectral reflectance index

Introduction

Drought is currently the major environmental factor limiting plant growth and yield in arid and semi-arid areas. Climate change is likely to increase the frequency of drought and mega-drought over large parts of the globe (Romm, 2011). To assess the ecological impact of increasing chronic water shortage and, consequently, implement forest management and breeding program for drought resistance, there is the need to develop and refine methods for early diagnostics on drought effects on forest vegetation. This, in turn, is based on real time detection of vegetation physiological status.

Water stress influences a number of physiological processes including carbon assimilation and water relations (Yordanov *et al.*, 2000; Chaves *et al.*, 2002; Centritto *et al.*,

2011a). As water stress develops, photosynthesis is increasingly reduced by total diffusional limitations, namely total leaf resistance to CO_2 diffusion, which in turn results from the product of its two series resistance components, i.e. stomata and mesophyll resistances (or by their inverse stomatal, g_s , and mesophyll, g_m , conductance) (Centritto *et al.*, 2003; Aganchich *et al.*, 2009). Diffusional limitations to photosynthesis are major component of leaf water-use efficiency (Flexas *et al.*, 2013) and increasingly used in phenotyping plant responses to water stress (Centritto *et al.*, 2009).

The pre-visual detection of water stress is a key issue to avoid yield loss. Several spectral indices have been developed based on changes in plant reflectance, which is sensitive to alterations in plant physiological status (Centritto *et al.*, 2003; Sun *et al.*, 2008). Among the several

remote sensing indices, the photochemical reflectance index (PRI) (Gamon *et al.*, 1992; Peñuelas *et al.*, 1995) has a sound functional basis and provides promising results. This index is based on the changes in the leaf reflectance at 531 nm due to alterations in de-epoxidation state of xanthophyll cycle during stress when reductions in photosynthetic efficiency occur under conditions of excess light (Gamon *et al.*, 1992). A good correlation has been observed between the PRI and photosynthetic activity at both the leaf (Gamon *et al.*, 1992) and canopy scales (Filella *et al.*, 1996) and across a range of environmental conditions (Whitehead *et al.*, 2005; Garbulsky *et al.*, 2011).

Holm oak (*Quercus ilex* L.) is frequently subjected to stressful environmental conditions. In previous studies on *Olea europaea* L. we demonstrated that reflectance indices measured at both leaf (Sun *et al.*, 2008) and canopy (Marino *et al.*, 2014) level were correlated to physiological parameters. In this study, diffusional limitations to photosynthesis and spectral reflectance were evaluated in holm oak saplings subjected to water stress and subsequent water stress relief. The objective was to evaluate whether photosynthesis traits and leaf water status could be correlated and, thus, remotely detected by PRI, in order to assess the sensitivity and applicability of this index for the detection of the physiological effects of plant water status.

Materials and Methods

The experiment was conducted in the greenhouse of the Institute of AgroEnvironmental and Forest Biology, National Research Council, Monterotondo (RM), Italy. Two-year-old plants of Holm oak (*Quercus ilex*) were grown in 15 dm³ pots filled with commercial soil. The plants were regularly watered to pot water capacity and fertilized with Hoagland solution once a week in order to supply mineral nutrients at free access rates (Magnani *et al.*, 1996; Centritto *et al.*, 1999). The dehydration treatment was initiated two months after the onset of the growing season. On the afternoon preceding the initiation of the experiment (day 0), 20 plants were fully irrigated and allowed to drain the excess water overnight. Then half of the seedlings were water-stressed by withholding water until seedlings showed symptoms of severe water stress, while the other half of the seedlings continued to be well-watered (pot capacity). On the afternoon of the Day 16 water-stressed plants were irrigated with 500 cm³ of water as a supplementary irrigation. On the evening of the Day 24, the water-stressed seedlings were re-watered daily to pot capacity over a 7-day recovery period.

Gas Exchange and Fluorescence Measurements

Leaf photosynthetic rates were measured by using a LI-6400-40 portable infrared gas-analyser (Li-Cor, Lincoln, NE), allowing simultaneous measurement of gas exchange and fluorescence. All gas exchange measurements were

made between 11.0 and 15.00 h, at saturating photosynthetic photon flux density (PPFD) (1300 $\mu\text{mol m}^{-2}\text{s}^{-1}$), with relative humidity ranging between 45-55%, and leaf temperature of 25°C. To reduce the diffusion gradient between the leaf cuvette and the surrounding air, which may have caused CO₂ and water vapour diffusion leaks through the chamber gaskets, two gasket rings separated by an air space were used as suggested by Rodeghiero *et al.* (2007) and described by Brilli *et al.* (2013). Instantaneous measurements of steady-state photosynthesis (*A*), stomatal conductance (*g_s*), and $\Delta F/F_m$ (i.e. the quantum yield of PSII in the light) were made on 5 plants per treatment after removing the effect of stomatal limitation on *A* as described by Centritto *et al.* (2003). Measurements of *R_d* (dark respiration) were also made at ambient CO₂ concentration in the dark on the same leaves. Mesophyll conductance to CO₂ diffusion (*g_m*) was calculated by using the variable *J* method (Harley *et al.*, 1992; Loreto *et al.*, 1992) based on the simultaneous measurements of gas-exchange and fluorescence parameters. According to the requirements of this method, a measurement of photosynthetic electron transport under low (1%) [O₂] was done to calibrate the system under non-photorespiratory conditions. The variable *J* method is quite sensitive to the estimation of Γ^* (CO₂ compensation point to photorespiration) and *R_d*. While the latter was measured, Γ^* used in the gas exchange algorithm was calculated by using the Rubisco specific factor as described by Centritto *et al.* (2009). Total conductance (*g_t*) was calculated as: $g_t = g_s g_m / (g_s + g_m)$.

Determination of Relative Water Content

Leaf samples for relative water content (RWC) were taken immediately after the gas exchange measurements and spectral measurements. Fully-developed leaves were detached and weighed to determine leaf fresh mass (*F_M*). After measuring leaf area, using a leaf area meter (LI 3100, LI-COR Inc., Lincoln, NE, USA), leaves were covered with a plastic bag and allowed to rehydrate with the cut-end under water in a dark cold room at 5°C for 18 h. Immediately after rehydration, each leaf was weighed to determine the saturated mass (*S_M*), and then each leaf was oven-dried at 80°C for 48 h to determine dry mass (*D_M*), while RWC was calculated as follows: $\text{RWC} = (F_M - D_M) / (S_M - D_M)$.

Photochemical Reflectance Index

The photochemical reflectance index (PRI) measurements were carried out in laboratory, by a portable spectroradiometer (Field spec FR 350-2500 nm, ASD Inc., Boulder, CO, USA). Fieldspec FR can be operated with different lenses that control the field of view, in this study an 8° lens was used to restrict the instrument field of view. PRI was calculated as $(R_{531} - R_{570}) / (R_{531} + R_{570})$ (Gamon *et al.*, 1992). Measurements were taken in dark room with two

incandescent lamps as the light source. Light source spectrum was sufficient and stable in the whole spectral range. Reflectance spectra were collected from a distance of 5.0 cm from the sample, which was fixed on a dark platform. Each spectral signature was recorded as the average of 100 scans to reduce instrumental noise. A further check of the stability of the reflected signal was performed by taking the white reflectance at the beginning of each sample. Reflectance spectra were pre-processed by using the ViewSpecPro (version 5.6, ASD, Inc.) software.

Statistics

The collection of experimental data was based on completely randomized design in five replications. Pearson's correlation analyses were performed to evaluate the correlation among the considered physiological variables using Excel (MS Office 2003). One-way ANOVA was performed using the Sigma Plot 10.0 software package (Systat software Inc, Chicago, IL, USA). to determine significant differences among photosynthetic parameters recorded at ambient (21%) and low (1%) [O₂] in the air.

Results

The changes in RWC, A , g_s , g_m , g_t and PRI over the drying cycle and recovery are presented in Fig. 1. As expected, RWC (Fig. 1a) was significantly ($P < 0.001$) affected by water availability. In fact, RWC decreased sharply as drought progressed (days 8-15). Then, following supplementary irrigation (day 16) and, finally, rewetting to pot water capacity (day 24), RWC gradually increased to pre-stress values. CO₂ assimilation (Fig. 1b), measured under natural and low [O₂] (21% and 1%, respectively), and diffusion conductance (Fig. 1c) were also dramatically affected by water stress ($P < 0.001$).

These gas exchange parameters changed in parallel with RWC, but their recovery was more apparent following rewetting on day 24, when they began to increase sharply. The response of PRI to the drying cycle and recovery was also significant ($P < 0.001$) and mirrored that of RWC (Fig. 1d).

We observed strong linear correlations between A and g_m ($r = 0.992$, $P < 0.001$) (a), between A and g_s ($r = 0.998$, $P < 0.001$) (Fig. 2b) and, consequently, between A and g_t ($r = 0.997$, $P < 0.001$) (Fig. 2c), when A was measured in natural [O₂]. It is noteworthy that A measured at low [O₂], i.e. in non-photorespiratory conditions ($A_{1\%O_2}$) (Fig. 1b), almost doubled the values measured at natural [O₂] (21%) before and after the stress imposition, indicating significant rates of photorespiration. However, during the water stress cycle, especially on day 20th, $A_{1\%O_2}$ was more than 3 times higher compared to A recorded at 21% [O₂]. Furthermore, $A_{1\%O_2}$ scaled significantly with all leaf conductance (Fig. 2d,e,f). Differently from A (Fig. 2a,b,c), the form of the responses of $A_{1\%O_2}$ to g_m , g_s , and g_t was saturating curve. It is worth noticing that when photorespiration is suppressed,

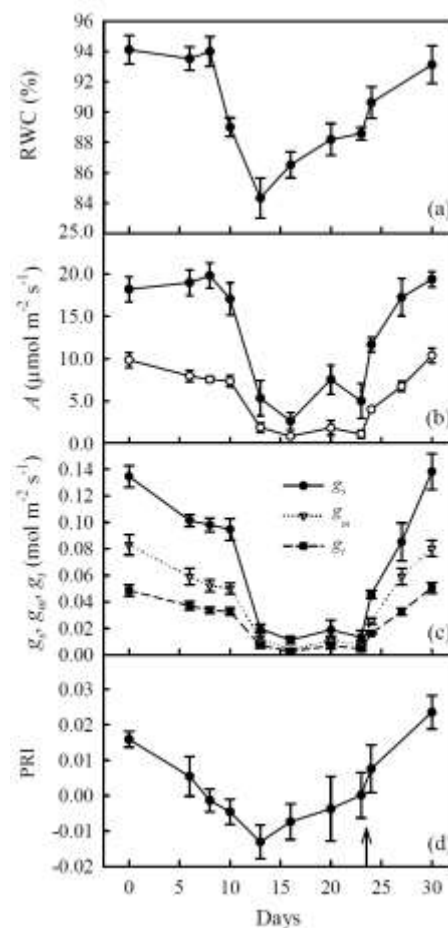


Fig. 1: Time course of (a) leaf relative water content (RWC), (b) net CO₂ assimilation (A) (measured in ambient air, ○, and in air with 1% [O₂], $A_{1\%O_2}$, ●), (c) stomatal (g_s), mesophyll (g_m) and total (g_t) conductances to CO₂ diffusion, and (d) photochemical reflectance index (PRI) in *Quercus ilex* saplings subjected to water stress and subsequent water stress relief. ↑ = end of the drying cycle. Data are mean of three plants per treatment (five leaves per tree) \pm SE

photosynthesis is more limited by g_s (Fig. 2e) than by g_m (Fig. 2d). Furthermore, the plot of g_m against g_s (Fig. 3), pooling together the data from control and water-stressed leaves, shows a good linear correlation ($r = 0.995$, $P < 0.001$).

There was a significant linear correlation between RWC and both PRI (Fig. 4a) and A (Fig. 4b), after pooling together control and water-stressed data. Similar linear relationships were also found between RWC and g_s , g_m and g_t (Fig. 4c), (Pearson's correlation coefficients: $r = 0.843$ - $P < 0.005$, $r = 0.84$ - $P < 0.005$, $r = 0.842$ - $P < 0.005$, respectively). Furthermore, our data show non-linear relationship between PRI and A ($r = 0.736$, $P < 0.05$) (Fig. 5a). It can be seen that PRI changes in parallel with A only at higher values of photosynthetic rate. A similar non-linear relationship was observed between PRI and g_t ($r = 0.762$, $P < 0.01$), where these two parameters changed in parallel when g_t was above 0.035 (Fig. 5b).

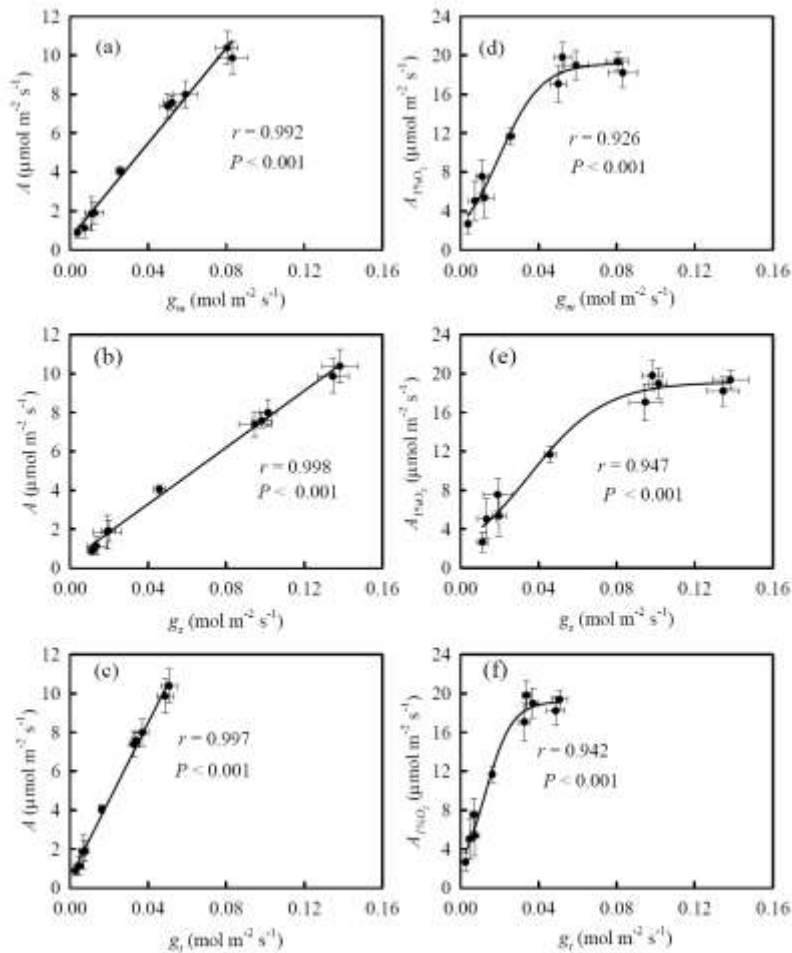


Fig. 2: Relationships of photosynthesis (A), measured in ambient air (a, b, c) and in air with 1% $[\text{O}_2]$ ($A_{1\% \text{O}_2}$) (d, e, f), and mesophyll conductance (g_m) (a, d), A and stomatal conductance (g_s) (b, e), and total conductance (g_t) (c, f) in *Quercus ilex* saplings after pooling together data collected during the water stress cycle and subsequent water stress relief. Data are mean of three plants per treatment (five leaves per tree) \pm SE

Discussion

The decrease in A under physiological water stress (Alvino *et al.*, 1994; Centritto *et al.*, 2000) is normally attributed to coordinated suppression in g_m and g_s , which in turn results in decreased g_t (Centritto *et al.*, 2009; Flexas *et al.*, 2013; Pallozzi *et al.*, 2013). The linear correlations between A and leaf conductance to CO_2 (Fig. 2) shown herein clearly indicate that increased transport limitations induced A inhibition. Consistently with previous observations (Centritto *et al.*, 2003; Flexas *et al.*, 2008), g_m and g_s were linearly correlated (Fig. 3). Despite, this finding could be due not to an interaction between g_m and g_s , but rather to similar responses to environmental factors, it demonstrates once again that CO_2 transport between the ambient air around leaves and the Rubisco sites inside the chloroplasts is a major limiting factor for assimilation rate in plants with different leaf structure (Terashima *et al.*, 2011; Brilli *et al.*, 2013), grown in different environment (Shi *et al.*, 2008; Feng *et al.*,

2013) or exposed to stress conditions (Loreto *et al.*, 2009; Velikova *et al.*, 2011; Pallozzi *et al.*, 2013)

While the impact of water stress on g_s has been largely explained as a result of decreased leaf turgor and of root-generated chemical signals (Chaves *et al.*, 2002; Ashraf and Harris, 2013), the main determinants of g_m inhibition under water stress are not yet clear (Flexas *et al.*, 2008; Loreto and Centritto, 2008). However, specific aquaporin-facilitated CO_2 diffusion, namely COOporins, may play an important role in g_m regulation as recently hypothesized by Kaldenhoff (2012). Besides, water stress can impose structural alterations in the leaf such as increased cell wall thickness and greater degree of cell wall lignification that improve its tolerance to low water potentials, but they may result in greater limitations to CO_2 diffusion chloroplasts decreasing g_m (Niinemets *et al.*, 2006). Finally, the maximal value of g_m of about $0.09 \text{ mol m}^{-2} \text{s}^{-1}$ (Fig. 1c), found in this study and that is in keeping with previously observed g_m values ranging between 0.06 - $0.12 \text{ mol m}^{-2} \text{s}^{-1}$ in leaves of *O.*

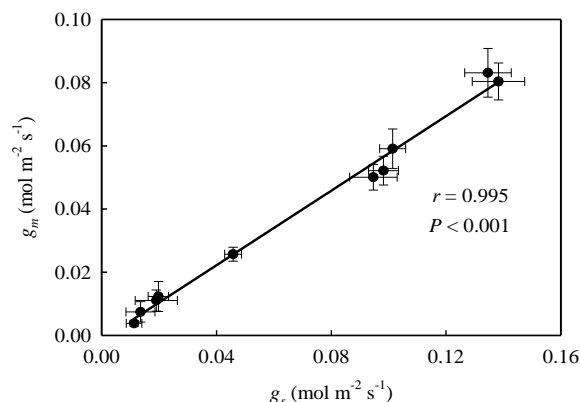


Fig. 3: Linear relationship between mesophyll (g_m) and stomatal (g_s) conductances in *Quercus ilex* saplings after pooling together data collected during the water stress cycle and subsequent water stress relief. Data are mean of three plants per treatment (five leaves per tree) \pm SE

europaea (Centritto *et al.*, 2003) and *Q. ilex* (Loreto *et al.*, 1992; Niinemets *et al.*, 2005), confirms that sclerophytic leaves have lower g_m than mesophytic ones (Flexas *et al.*, 2008; Loreto and Centritto, 2008).

The about two-fold increase in $A_{1\%O_2}$, observed before and after the drying cycle (Fig. 1b), is an indirect indication of significant photorespiration rates. This result is in keeping with the findings of early literature (Filella *et al.*, 1998; Peñuelas *et al.*, 1998; Ogaya and Peñuelas, 2003) showing that *Q. ilex* has high photorespiration rates. Moreover, the higher stimulation in photosynthesis measured in non-photorespiratory conditions caused by water stress (about 300%) indicates that photorespiration likely increased as water stress progressed (Voss *et al.*, 2013). It was demonstrated that photorespiration seems to provide a 'safety-valve' for excess energy to avoid photochemical damage under high (Peñuelas and Llusià, 2002) and low (Tsonev *et al.*, 2003) temperature conditions when CO_2 assimilation is inhibited. A similar effect, which prevents the accumulation of reactive oxygen species, has recently been proposed also for other abiotic stress, including water deficit (Voss *et al.*, 2013). Moreover, Peñuelas and Llusià (2002) showed that the leaf-internal CO_2 pool provided by photorespiration supports the Calvin cycle and isoprenoid biosynthesis, even when stomata are closed.

Furthermore, the finding that $A_{1\%O_2}$, which scaled non linearly with leaf conductance, was limited more by g_s than by g_m (Fig. 2) is consistent with the hypothesis that higher g_m , by reducing the internal CO_2 gradient leads to higher $[CO_2]$ at the Rubisco sites, is a major player in favoring photosynthesis over photorespiration (Sage, 2013). In addition, the saturating responses of $A_{1\%O_2}$ to diffusional conductance implies that when photorespiration is suppressed, photosynthesis is no longer limited by CO_2 transport even at rather low leaf conductance (i.e., values of

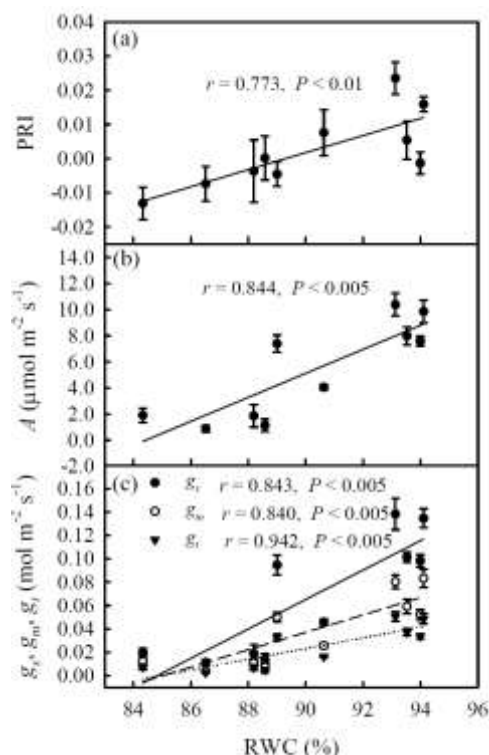


Fig. 4: Linear relationships between (a) relative water content (RWC) and photochemical reflectance index (PRI), (b) RWC and photosynthesis (A), and (c) RWC and stomatal (g_s), mesophyll (g_m) and total (g_t) conductances in *Quercus ilex* plants. Data are mean of three plants per treatment (five leaves per tree) \pm SE

g_m , g_s , and g_t of about 0.06, 0.10 and 0.04 $mol\ m^{-2}\ s^{-1}$, respectively). This indicates that sclerophyllous vegetation type, which is characterized by low leaf conductance (Centritto *et al.*, 2011b), will respond to future elevated $[CO_2]$ significantly more than mesophyllous plants.

RWC, a parameter universally used to detect plant water status, affected in parallel PRI, leaf conductance and CO_2 assimilation (Fig. 1). In fact the linear correlations found between RWC and all these parameters (Fig. 4), demonstrated the importance of water in determining in processes associated to carbon assimilation (Centritto *et al.*, 2011a; Ashraf and Harris, 2013), and that leaf water status, as described by RWC, can be detected by spectral reflectance indices (Sun *et al.*, 2008).

PRI was firstly developed to track fast changes in the xanthophyll cycle occur during stress (Gamon *et al.*, 1992). However, it has been found in a number of studies that PRI can track the photosynthetic activity at both leaf and canopy levels (Garbulsky *et al.*, 2011; Marino *et al.*, 2014), as well as under water stress conditions (Naumann *et al.*, 2008; Sun *et al.*, 2008; Osório *et al.*, 2012; Marino *et al.*, 2014). However, because photosynthesis is primarily limited by diffusional limitations (Fig. 2), we wanted to test whether PRI could detect changes in the total leaf conductance as well as in photosynthesis under water stress conditions in a

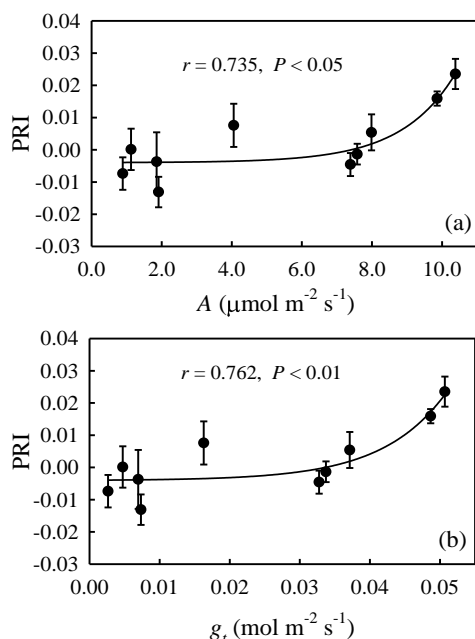


Fig. 5: Non-linear relationships between photochemical reflectance Index (PRI) and (a) photosynthesis (A) and (b) total diffusion conductance (g_t) in *Quercus ilex* plants. The measurements were made between 10:00 and 16:00 h. Data are mean of three plants per treatment (five leaves per tree) \pm SE

sclerophyllous species such as *Q. ilex*. The non-linear relationships found between PRI and both A and g_t (Fig. 5), show that PRI changes in parallel with these two parameters only at higher values of A and g_t . This also implies that PRI may clearly discriminate normal and stressed status of holm oak trees. However, PRI is not very informative under severe stressed conditions, when photosynthesis becomes increasingly limited by metabolic factors (Flexas *et al.*, 2008; Loreto and Centritto, 2008). Probably, at severe drought stress when photosynthesis is significantly inhibited, the efficiency of xanthophyll cycle activity is low and other biochemical mechanisms may play a more important role in photosynthetic down-regulation. Similarly, Osório *et al.* (2012) have not observed a statistically significant correlation between PRI and non-photochemical quenching (NPQ) in *C. siliqua* seedlings subjected to water stress. They hypothesised that in this case, non-regulated quenching mechanisms may play a more important role in PSII photoprotection under stress than xanthophyll cycle-mediated thermal dissipation. In support of this Porcar-Castell *et al.* (2012) reported that in boreal Scots pine PRI and NPQ have been highly correlated during most of the year, but decoupled in early spring when the photosynthesis was deeply down-regulated. They conclude that PRI leads to an underestimation of NPQ under conditions of severe stress in overwintering Scots pine. Such a mechanism most likely occurs also in species experiencing severe drought, because NPQ can be enhanced by mechanisms that do not directly involve the xanthophyll cycle pigments (Demmig-Adams and Adams, 2006).

In conclusion, taken together our data indicate that: a) increased leaf conductance, and in particular g_m , are major players in favoring photosynthesis over photorespiration, especially in sclerophyllous plants; b) *Q. ilex* has significant rates of photorespiration under ambient $[\text{CO}_2]$, and because under non-photorespiratory conditions photosynthesis is not limited by CO_2 transport even at rather low g_t , sclerophyllous plants will likely be more responsive to future elevated $[\text{CO}_2]$ than mesophyllous species; c) PRI can be used to predict changes in leaf water status of *Q. ilex*. Furthermore, changes in the quantity of radiation reflected in the visible region are affected by the amount of energy assimilated by the photosynthetic antenna and, in turn, by the photosynthetic status. Thus, PRI can be overall used to predict photosynthetic performance of *Q. ilex* subjected to physiological water stress. However, the results should be interpreted with caution under very severe drought, when photosynthesis is strongly inhibited likely by the increasing contributions of biochemical limitations.

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