CHLOROPHYLL FLUORESCENCE FOR PHENOTYPING DROUGHT-STRESSED TREES IN A MIXED DECIDUOUS FOREST

SALVATORI E.[*], FUSARO L., MANES F.

Department of Environmental Biology, Sapienza University of Rome, Piazzale Aldo Moro, 5, 00185 Rome, Italy
[*]Corresponding Author: Telephone: +390649912451; email: elisabetta.salvatori@uniroma1.it

ABSTRACT – Among the environmental constraints affecting growth, productivity and health of crops and forests ecosystems, drought is known to be a key factor particularly in the Mediterranean area, where the typical dry and hot summer conditions may be exacerbated as a consequence of the foreseen Global Climatic Changes. Despite the drought stress response of forest trees has been intensively investigated, phenotyping for functional traits associated to drought adaptation in the field still remains particularly challenging, due to the intrinsic difficulties in screening tall trees in forest environment. In this work, we have applied a high-throughput phenotyping approach to investigate the drought response of two coexisting deciduous tree species (*Quercus cerris* L. and *Fraxinus ornus* L.) in a natural mixed forest (Circeo National Park, Central Italy). Our results have shown that the measurement of Chlorophyll fluorescence with the JIP-test application is particularly suitable for phenotyping the drought stress response of adult trees in the field. In particular, among the phenotypic traits investigated, the Total Photosynthetic Performance Index (PI_{TOT}) has proven to be the most suitable non-invasive marker of plant response to drought, which is able to provide reliable, fast and synthetic information on plant ecophysiological status.

KEYWORDS: CHLOROPHYLL FLUORESCENCE, JIP-TEST, DROUGHT STRESS, *QUERCUS CERRIS*, *FRAXINUS ORNUS*, PHENOTYPING

INTRODUCTION

During the last years, there has been a growing interest in plant phenotyping, due to the increasing demand for accurate and cost-effective protocols to characterize plant adaptation in resources-limited environments. Plant phenotyping, defined as "the act of determining the quantitative or qualitative values of a set of structural, physiological, or performance-related traits of a genotype in a given environment" (Dhondt et al., 2013), has in fact found many applications in different fields, ranging from plant breeding to precision agriculture (Rascher et al., 2011; Fiorani & Schurr, 2013), up to the mechanistic understanding of plant response to environmental stress factors (Granier & Tardieu, 2009). Plant phenotyping can be performed at any organizational level (canopy, individual, organ, tissue, cellular and subcellular level), and the phenotypic traits of interest can be measured at different spatial and temporal resolution. The throughput, i.e. the amount of units at the considered organizational level (e.g. individuals, leaves, etc.), that can be measured for a specific trait(s) at a given time, is also an important determinant of phenotyping systems (Dhondt et al., 2013). High throughput, non-invasive quantitative phenotyping methods are particularly required in plant stress analysis, but they often suffer from low resolution, or are based on expensive automated platforms, that are mostly designed for controlled growth conditions (Araus & Cairns, 2013).

In this frame, the measurement of Chlorophyll "a" fluorescence represents a relatively fast and low-cost approach for high throughput phenotyping of functional leaf traits, that can be easily applied in both controlled conditions and natural environments (Oukkarroum & Strasser, 2004; Rousseau et al., 2013; Murchie & Lawson, 2013; Gottardini

et al., 2014; Pollastrini et al., 2016). In particular, the measurement of Prompt Fluorescence (PF) and the application of the JIP-Test analysis (Strasser at al., 2004; Strasser et al., 2010), has proven very effective in detecting both early and late changes in plant physiological status under different environmental stress factors (Bussotti et al., 2011; Pollastrini et al., 2014; Salvatori et al., 2015; Fusaro et al., 2016). In fact, by measuring PF through standardized instrumental protocols, a rapid, *in vivo* ecophysiological screening of a large amount of photosynthetic samples is possible; the following application of the JIP-test on these measurements allows deriving high-resolution information regarding plant performance and stress tolerance (Desotgiu et al., 2012; Murchie & Lawson, 2013; Gottardini et al., 2014; Salvatori et al., 2014).

Among the environmental constraints affecting growth, productivity and health of crops and forests ecosystems, drought is known to be a key factor particularly in the Mediterranean area, where the typical dry and hot summer conditions may be exacerbated as a consequence of the foreseen Global Climatic Changes (Giorgi & Lionello, 2008; IPCC 2013). Despite the drought stress response of forest trees has been intensively investigated (Barbeta et al., 2015; Sperlich et al., 2015), phenotyping for functional traits associated to drought adaptation in the field still remains particularly challenging, due to the intrinsic difficulties in screening tall trees in forest environment (Pollastrini et al., 2016). In this work, we investigated the drought response of two coexisting deciduous tree species (*Quercus cerris* L. and *Fraxinus ornus* L.), under summer drought conditions occurring in the mixed broad-leaved forest of the Circeo National Park (Central Italy). A high-throughput phenotyping approach was applied, by measuring PF, gas exchanges, leaf water status and structural leaf traits on adult trees along one growing season (April-October). The results are discussed in the frame of the application of the parameters derived from the JIP-test analysis for fast and reliable phenotyping of drought stress in natural field conditions.

MATERIALS AND METHODS

Study area and sampling scheme

The study was conducted in the Circeo National Park, one of the most important Biosphere Reserves of UNESCO's MAB Programme in Italy, located about 100 km south of Rome, near the Tyrrhenian coast (41 12 00 N, 13 10 20 E). Its climate is of lower meso-Mediterranean thermo-type, and upper sub-humid ombrotype (Blasi et al., 1999), with mean annual temperature and rainfalls of 11.2 °C and 887 mm,

respectively (historical series 1957 – 2004) (Vitale et al., 2007). The study site is located in the plain forest of 3190 ha (Anselmi et al., 2004); it is a deciduous mixed wood (*Q. cerris*, *Q. frainetto* Ten., *Q. robur* L., *F. ornus* and *Carpinus betulus* L.), having Würmian sandy soils with pyroclastic material of volcanic origin (Dowgiallo & Bottini, 1998).

Structural and ecophysiological measurements were carried out on *Quercus cerris* (QC) and *Fraxinus ornus* (FO) from April to October 2005, according to the sampling scheme detailed in Table 1.

Stand structure and structural leaf traits

A plot of 400 m^2 surface, S facing and with 0% slope, was considered for the structural observations. The dominant tree layer, characterized by a canopy cover of 80% and a canopy height of 15m, was composed by *Q. cerris* (80%), *Q. frainetto* (20%), and *Q. robur*. The dominated tree layer, 40% canopy cover and 8m of height, was composed by *Ostrya carpinifolia* (80%) and *Fraxinus ornus* (20%); *Erica arborea*, *Acer campestre* and *Malus sylvestris* were also present.

Leaf Area Index (LAI, m^2 m^{-2}) was measured through a LAI-2000 Plant Canopy Analyzer (Li-Cor, Lincoln, NE, USA) (Jonckheere et al., 2004). It measures simultaneously diffuse radiation by means of a fisheye light sensor in five distinct angular bands, with central zenith angle of 7°, 23°, 38°, 53° and 68°. The LAI estimate is obtained through the calculation of the so called "gap fraction", by taking measurements above the canopy/outside the stand (A readings) and below the canopy/inside the stand (B readings) and calculating the difference between the incident radiation above and below the canopy (Welles, 1990). The B measurements were recorded in 9 evenly spaced sampling points within the plot, while the A measurements were instead collected in an open area close to the experimental plot, at the beginning and at the end of each cycle, in order to take into account changes in light conditions during the measurements (Manes et al., 2010). Measurements were taken once a week during spring, and once a month during summer (Table 1). For each sampling day, three replicates LAI measurements were taken. All measurements were taken at dusk, by applying a 90° view cap on the lens.

The Leaf Mass per Area (LMA, mg cm^{-2}), considered an index of sclerophylly (Bussotti., 2008), was calculated for QC and FO as the ratio between leaf dry weight (DW) and leaf area (LA). LMA was determined on a biweekly basis from May to October (Table 1), on 15 sun-exposed leaves, collected from the upper/outer portion of the crown of 4 representative adult trees per species.

Leaf water status

Leaf-level relative water content at midday (RWC, %) was measured on the same leaves collected for LMA determination (Table 1). The RWC was calculated as follows:

$$RWC = ((FM – DM)) / ((TM – DM))$$

Where FM is leaf fresh mass, DM is leaf dry mass, and TM is leaf turgor mass, measured as water saturated leaf weight after 10–12 h in water saturating conditions (petiole in water). Leaf water potential at predawn (Ψ_{PD}, MPa), an indicator of average soil water potential in the root zone, was measured with a portable pressure chamber (Scholander bomb, PMS Instruments, Oregon, USA) on 4 individual plants per species. Ψ_{PD} was measured on a biweekly basis from July to September 2005 (Table 1).

Table 1. Sampling scheme for ecophysiological and structural measurements.

Date of sampling	DOY	LAI	LMA	RWC	Ψ_{PD}	Gas exchanges	Chlorophyll fluorescence
19-Apr-05	109	X	-	-	-	-	-
25-Apr-05	115	X	-	-	-	-	-
01-May-05	121	X	-	-	-	-	-
08-May-05	121	X	X	-	-	-	-
15-May-05	135	X	X	X	-	X	X
22-May-05	142	X	X	-	-	-	-
29-May-05	149	X	-	-	-	-	-
07-Jun-05	158	X	X	X	-	X	X
13-Jun-05	164	-	X	-	-	-	-
22-Jun-05	173	X	X	X	-	X	X
29-Jun-05	180	X	X	-	-	-	-
05-Jul-05	186	-	-	X	X	X	X
17-Jul-05	198	-	X	X	X	X	X
02-Aug-05	214	X	-	-	-	-	-
19-Aug-05	231	-	-	X	X	X	X
27-Aug-05	239	X	X	X	X	X	X
11-Sep-05	254	-	-	X	-	X	X
25-Sep-05	268	X	X	X	X	X	X
10-Oct-05	283	-	-	X	-	X	X

Where the header "Type of Measurement" spans the columns LAI, LMA, RWC, Ψ_{PD}, Gas exchanges, Chlorophyll fluorescence.

Gas exchanges measurements

Net photosynthesis (Pn, $\mu molCO_2$ m^{-2} s^{-1}), leaf transpiration (E, mmolH$_2$O m^{-2} s^{-1}), stomatal conductance (gs, mmolH$_2$O m^{-2} s^{-1}) and sub-stomatal CO_2 concentration (Ci, ppm) were simultaneously recorded *in vivo* by a portable open system

CIRAS I (PP Systems, Hitchin, UK), on sun exposed leaves growing on the upper/outer portion of the tree crowns. The measured values were used to calculate the ratio between sub-stomatal and external CO_2 concentration (Ci/Ca, dimensionless). Environmental parameters such as irradiance (PAR, μmol photons m^{-2} s^{-1}), relative humidity (RH, %), leaf-to-air Vapour Pressure Difference (VPD, mbar) and air temperature (Tair, °C), were also recorded by the instrument. The Water Use Efficiency (WUE, $\mu molCO_2$ mmolH$_2$O^{-1}), was also calculated. Gas exchanges were measured on a biweekly basis from May to October 2005, on the same days and individuals as LMA and leaf water status (Table 1). In each day, 2 to 4 measurements cycles were carried out from 8:00 to 14:00 h GMT +1, and the number of sampled leaves varied from a minimum of 60 to a maximum of 100 per species, depending on the photoperiod.

Chlorophyll "a" fluorescence measurements and OJIP test

Direct Chlorophyll "a" fluorescence was measured *in vivo*, on sun exposed leaves still attached on the plants, with a portable Plant Efficiency Analyser (PEA, Hansatech Ltd, UK), during the same dates, hours and on the same individuals considered for gas exchanges (16 to 40 leaves per sampling, depending on the photoperiod) (Table 1). The prompt fluorescence transient (Strasser et al., 2000, 2004), was recorded on leaf sample areas of 4 mm in diameter, dark adapted for 40 minutes with specific leaf clips, and then exposed to a saturating red light pulse (peak 650 nm, 3000 μmol photons m^{-2} s^{-1}), of 1 s duration. When plotted on a logarithmic time-scale, this fast fluorescence signal exhibits a series of steps, labelled as O (F_0, when all the reaction centres of PSII are open, 20-50 μs), J (2 ms), I (30 ms) and P (Fm, when all the PSII reaction centres are fully reduced). The first part of the transient curve (O–J) is called "single turnover region" (Strasser et al., 2004). It expresses the photochemical events and represents a single event of reduction of Q_A. The J–I–P region of the fluorescence transient is called multiple turnover region: in particular, the I-P region reflects the velocity of ferredoxine reduction downstream the PSI. The JIP-test is a tool that translates this polyphasic fluorescence transient to a constellation of biophysical parameters, that quantify the single steps of the photochemical pathway through both PSII and PSI (Strasser et al., 2010). For the JIP-test analysis, the data recorded by the fluorimeter were processed by the software Biolyzer (Bioenergetics Lab., Geneva, CH), in which the fluorescence value at 50 μs was considered as the basal fluorescence F_0 (Strasser et al., 2000). The following JIP-test parameters are considered in this study:

- $F_v/F_m = \Psi_{Po} = TR_0/ABS = (F_m-F_0)/F_m$, maximum quantum yield of PSII primary photochemistry measured on dark-adapted samples. F_v/F_m expresses the probability that an absorbed photon will be trapped by the PSII reaction centre.

- J-Phase: $\Psi_{Eo} = ET_0/TR_0 = 1 - V_J = 1 - (F_{2ms} - F_0) / (F_m - F_0)$, expresses the probability that the energy of a trapped excitation is used for electron transport beyond Q_A. V_J is the relative variable fluorescence at 3 ms, calculated as $V_J = (F_J - F_0)/(F_m - F_0)$.

- I-P phase $\Delta V_{I-P} = 1 - V_I = 1 - (F_m - F_{30\,ms}) / (F_m - F_0)$, represents the amplitude of the I-P phase of the fluorescence transient OJIP (Oukkarroum et al., 2009). It is regarded as a measure of the efficiency of electron flux through PSI to reduce the final acceptors of the electron transport chain, i.e. ferredoxin and NADP, and it is also related to the activity and/or quantity of PSI (Ceppi et al., 2012). V_I is the relative variable fluorescence at 30 ms, $V_I = (F_I - F_0)/(F_m - F_0)$.

- Performance Index Total: $PI_{TOT} = (RC/ABS)[\varphi_{Po}/(1-\varphi_{Po})][\Psi_{Eo}/(1-\Psi_{Eo})]\,[\delta_{Ro}/(1-\delta_{Ro})])$, the potential performance index for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors, $RC/ABS = \varphi_{Po}(V_J/M_0)$, where M_0 is the slope of the curve at the origin of the relative variable fluorescence rise dV/dt_0. It is a measure of the rate of the primary photochemistry $M_0 = 4(F_{300\mu s}-F_0)/(F_m- F_0)$. δ_{Ro} represents the efficiency by which an electron can move from the reduced intersystem electron acceptors to the end acceptors beyond the PSI, namely ferredoxine and $NADP^+$, $\delta_{Ro} = (1-V_I)/(1-V_J) = (F_m-F_I)/(F_m-F_J)$ (Strasser et al., 2010).

Statistical analysis

Physiological, structural and environmental parameters were analyzed by using the STATISTICA 7 software package (StatSoft Inc., Tulsa, OK, USA). For each species, a one-way Analysis of Variance (ANOVA) was applied. Significant differences between means were determined through the post hoc Student–Neuman–Keuls test at $p \leq 0.05$. Normality and homogeneity of variance (Levene's test) requirements were previously tested, and data transformed when necessary. Data in figures and tables are expressed as Means ± S.E.

Results and Discussion

Leaf Area Index and Leaf Mass per Area

Figure 1 shows the seasonal trend of LAI ($m^2\ m^{-2}$) measured in the deciduous forest, and of the LMA ($mg\ cm^{-2}$) of QC and FO. Both parameters increased sharply starting from early May (DOY 128), and reached a plateau already on DOY 142 (22nd of May) in correspondence with the end of active phenological phase. The highest measured LAI was $5.29 \pm 0.28\ m^2\ m^{-2}$ (on DOY 214), a value that is in the range of what previously reported for deciduous oaks stands (Cutini et al., 1998; Le Dantec et al., 2000). Interestingly, the LMA values of both species slightly increased in September (DOY 268), after the summer drought (7.60 ± 0.32 and $5.91 \pm 0.18\ mg\ cm^{-2}$ for QC and FO, respectively), coherently to what reported by Castro-Díez et al. (1997) along an ecological gradient with increasing drought stress conditions.

Water availability, predawn leaf water potential and midday Relative Water Content

In the year 2005, total rainfalls in the Circeo National Park were 878.5 mm, and were distributed mainly in late winter and autumn months (Fig. 2, a). For the same study area, Vitale et al. (2007) reported a yearly precipitation value of 884.70 mm averaged for the historical series 1957-2002, and a similar distribution of rainfalls along the seasons. On the basis of these data, the year 2005 can be therefore considered as an "average year" for what concerns water availability in the plain forest, with little or no precipitations during the summer months. In parallel with the gradual increase of water shortage, Ψ_{PD} and RWC progressively decreased in both species, reaching a minimum on DOY 231 (August 19), in correspondence with the drought peak. However, QC showed relatively higher Ψ_{PD} values than FO as drought progressed during summer: at the beginning of July, Ψ_{PD} was -0.48 ± 0.04 MPa in QC and -2.70 ± 0.36 MPa in FO, and, on DOI 231, Ψ_{PD} was -1.03 ± 0.22 MPa and -5.73 ± 0.14 in QC and FO, respectively. The latter species was therefore experiencing severe water stress (Mitchell et al., 2013). Such a different water availability in the root zone between QC and FO could be explained taking into account their different root growth dynamic: Chiatante et al. (2006) have in fact shown that taproot biomass decreases under water stress condition in FO, but not in a coexisting *Quercus* species (*Q. pubescens* Wild.), and, in mature forest stands, QC has been reported to increase fine root growth as the soil dries, thus increasing water uptake under seasonal drought conditions (Montagnoli et al., 2012). These structural differences could also explain the different seasonal decline
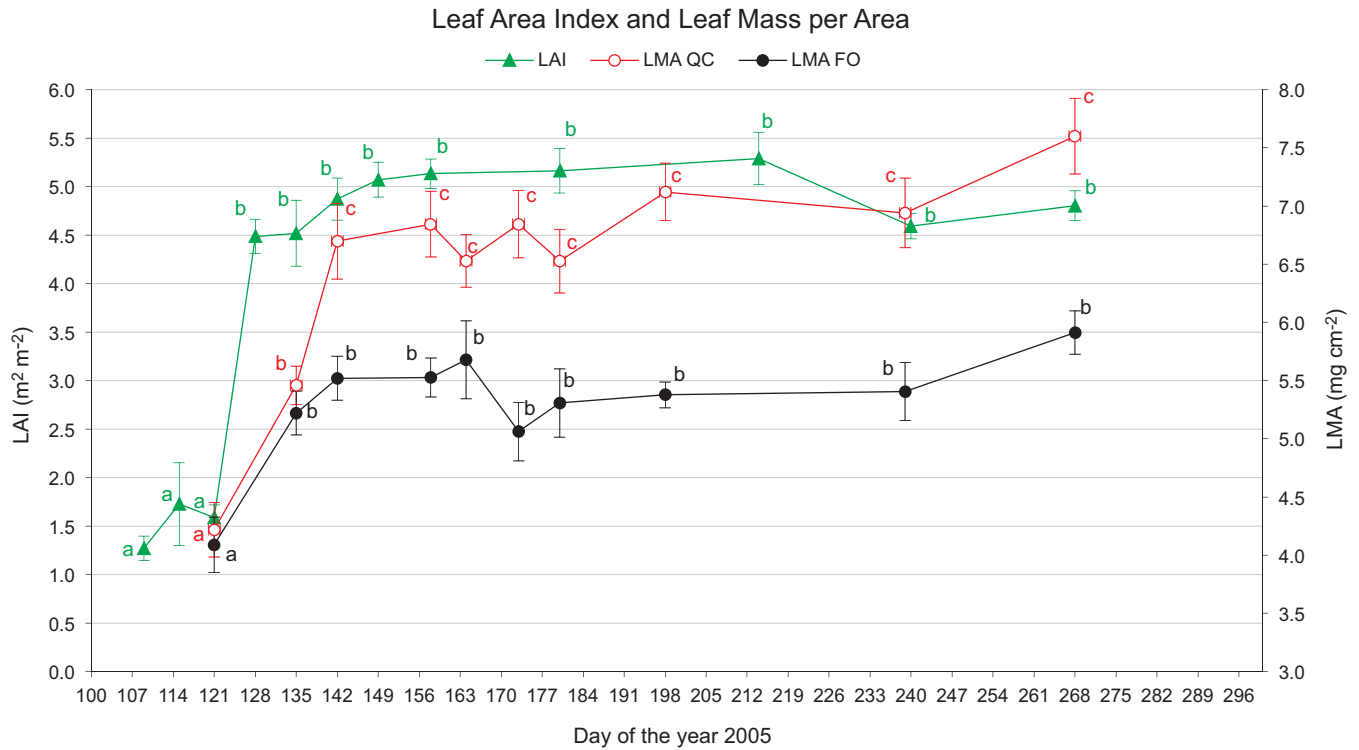
Leaf Area Index and Leaf Mass per Area

Figure 1. Seasonal trend of Leaf Area Index (m² m⁻²), and Leaf Mass per Area (LMA, mg cm⁻²) of *Quercus cerris* (QC) and *Fraxinus ornus* (FO) measured in the plain forest of the Circeo National Park from April to October 2005. Mean ± S.E., n = 3 for LAI measurements and n = 15 leaves for LMA. For each parameter, different letters indicate statistically significant differences between day of measurements.

of RWC values observed in the two species (Fig 2, b): in fact, in correspondence of drought peak (DOY 231), QC maintained higher RWC values than FO (65% and 50% in QC and FO, respectively). The latter species adopted a drought-tolerant behavior, as suggested by the pronounced leaf dehydration followed by fast recovery after the late summer rainfalls (DOY 239), when Ψ_{PD} values also returned to optimal values (-0.52 ± 0.01 MPa) (Fig. 2, a).
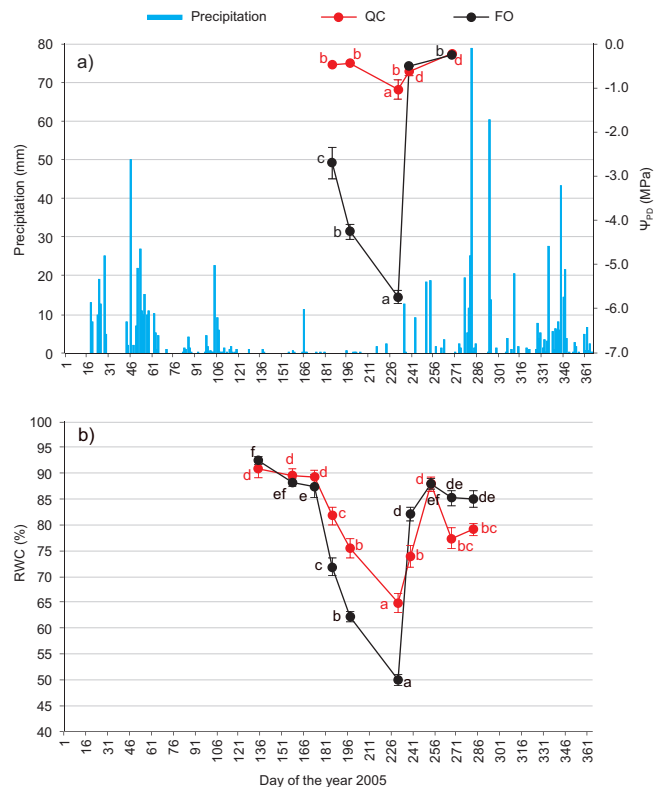

Figure 2. a) Yearly trend of daily precipitation (mm), and predawn leaf water potential values (Ψ_{PD}, MPa) measured during summer 2005 on *Quercus cerris* (QC) and *Fraxinus ornus* (FO). Mean ± S.E., n = 4 leaves for Ψ_{PD}; b) Leaf Relative Water Content at midday (RWC, %), measured from May to September 2005 on *Quercus cerris* (QC) and *Fraxinus ornus* (FO). Mean ± S.E., n = 15 leaves. For each parameter, different letters indicate statistically significant differences between day of measurements.

Gas exchanges

Figure 3 shows the daily values of the environmental parameters Tair, RH, VPD and PAR, simultaneously recorded during the gas exchanges measurements. Average PAR values during the sampling period were never below 1400 µmol photons m^{-2} s^{-1}. The highest values of Tair and VPD, as well as the lower values of RH, were recorded during DOY 173, 186, 198 and 231, influencing, together with drought, the trend of gas exchanges (Figure 4). It can be noticed that both species showed a progressive reduction of Pn, gs and WUE during drought, but this reduction was more severe in FO than in QC (Fig 4 a, c). In fact, on DOY 231, in correspondence with the maximum drought conditions (Fig 2, a), QC was able to maintain a certain degree of CO_2 assimilation, with Pn and gs values of 2.15 ± 0.26 µmolCO_2 $m^{-2}s^{-1}$ and 27.06 ± 2.75 mmolH_2O m^{-2} s^{-1}, respectively (Fig. 4, a). This is in agreement with previous studies (Tognetti et al., 1996; Anselmi et al., 2004; Vitale et al., 2007; Grossiord et al., 2014), in which QC adopted a non conservative use of water resources, maintaining a significant gas exchange rate also when its midday RWC was markedly reduced. During the same day, FO showed instead an almost complete stomatal closure (gs = 7.52 ± 0.61 mmolH_2O m^{-2} s^{-1}), which lowered Pn values close to the CO_2 compensation point (0.07 ± 0.08 µmolCO_2 $m^{-2}s^{-1}$) (Fig. 4, c), coherently with the few studies reporting the water stress response of this species in both field and pot conditions (Tretiach, 1993; Nardini et al., 2003; Gortan et al., 2009; Fusaro et al., submitted). Moreover, the Ci/Ca ratio increased significantly in FO, reaching the peak of 0.97 ± 0.04, while in QC this ratio was never higher than 0.60 (Fig. 4 b, d). This indicates that, differently from QC, the reduction of photosynthesis in FO can be attributed to both stomatal and
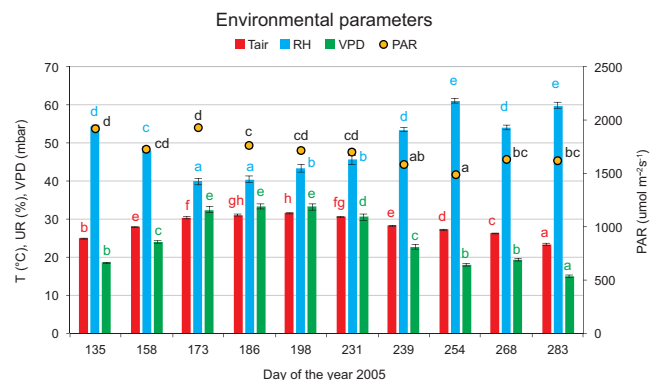
non stomatal limitations (Flexas et al., 2014). It is in fact known that at moderate leaf water deficit (RWC down to around 70%), photosynthesis is mainly limited by stomatal closure, without any significant decline in mesophyll capacity (Cornic and Fresneau, 2002). Instead, when prolonged drought leads to leaf dehydration values markedly below 70% RWC, or other stresses are superimposed (e.g. high temperatures, high light), CO_2 fixation and electron transport are also affected (Giardi et al., 1996; Grassi & Magnani, 2005). The increase of Ci/Ca ratio in FO suggests an enhancement of photorespiration rate, to sustain the photochemical flux at lower gs (Manes et al., 2006; Flexas & Medrano, 2002), and avoiding oxidative burst and functional damage (Carvalho, 2008), thus allowing a fast recovery of gas exchanges just after the late summer rainfalls (Fig. 4, c).

OJIP Chlorophyll fluorescence

Consistently to what observed for gas exchanges, the maximum quantum yield of PSII primary photochemistry, F_v/F_m, was not affected by drought in QC, and the electron transport probability (J-phase, Ψ_{Eo}) was only slightly reduced in this species (Fig. 5, a). Both parameters instead decreased significantly in FO and, in particular, Fv/Fm dropped to values as low as 0.54 ± 0.02 on DOY 231 (Fig 5, c). This transient reduction of F_v/F_m and J-phase, which recovered just after late-summer rainfalls, indicates the activation of a typical down-regulation of PSII photochemistry, considered as a photoprotective mechanism adopted in drought stressed plants (Oukkarroum et al., 2009; Guidi & Calatayoud, 2014). Concurrently, in FO also the amplitude of the I-P phase (ΔV_{I-P}) decreased markedly on DOY 231 (Fig 5, c). The I–P phase is a proxy for efficiency of reducing final acceptors beyond PSI, and correlates with the content of active PC and P700 (Ceppi et al., 2012). The reduction of the I-P phase thus indicates that, in FO, water stress also affected PSI photochemistry. This parameter has been previously reported to be very sensitive to severe drought (Oukkarroum et al., 2009; Pollastrini et al., 2014), as well as to other oxidative stress factors (Desotgiu et al., 2013; Fusaro et al., 2015; 2016; Salvatori et al., 2015).

Finally, the photosynthetic Performance Index (PI_{TOT}) further highlights the different behaviour of QC and FO under drought (Fig. 5 b, d). PI_{TOT} is a synthetic multi-parametric expression, that combines four parameters related to photosynthetic activity: (i) the density of reaction centres; (ii) the quantum yield of primary photochemistry; (iii) the probability of electron transport between PSII and PSI; (iv) the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors (Strasser et al. 2004; 2010). PI_{TOT} is

Figure 3. Environmental parameters recorded simultaneously with the gas exchanges measurements. Tair, air temperature (°C); RH, relative air humidity (%), VPD, Vapour Pressure Difference between leaf and air (mbar), PAR, Photosynthetic Active Radiation (µmol photons $m^{-2}s^{-1}$). Mean ± S.E., $60 \le n \le 100$. For each parameter, different letters indicate statistically significant differences between day of measurements.
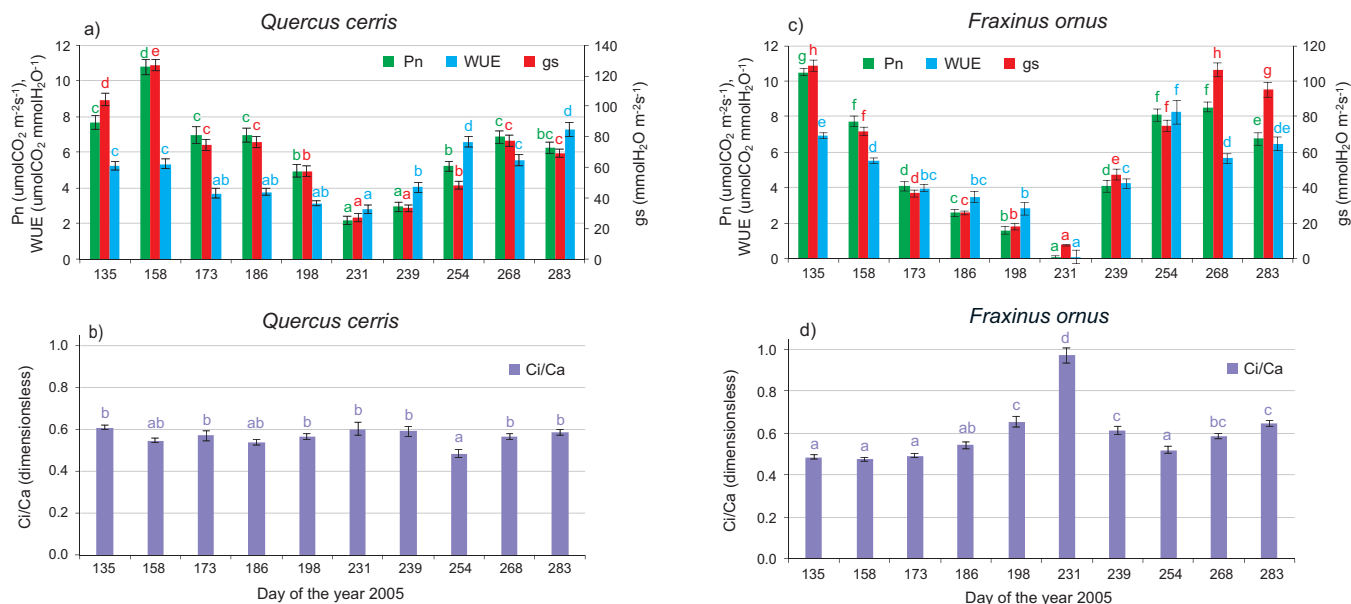
Figure 4. Trend of leaf-level exchanges measured on *Quercus cerris* (QC, left panel) and *Fraxinus ornus* (FO, right panel) from May to October 2005. a) and c) Pn, net photosynthesis ($\mu molCO_2$ m^{-2}s^{-1}); gs, stomata conductance to water vapour ($mmolH_2O$ m^{-2}s^{-1}), WUE, Water Use Efficiency ($\mu molCO_2$ $mmolH_2O$); b) and d) Ci/Ca, ratio between substomatal and atmospheric CO_2 concentration (dimensionless). Mean ± S.E., $60 \leq n \leq 100$. For each parameter, different letters indicate statistically significant differences between day of measurements.

known to be affected by many kind of different environmental stresses, such as high temperature (Oukkarroum et al., 2012), ozone (Bussotti et al., 2011; Gottardini et al., 2014; Salvatori et al., 2013; 2015; Fusaro et al., 2016), drought (Strasser et al., 2010; Goltsev et al., 2012), or heavy metals contamination (Bernardini et al., 2016 a, b). Interestingly, although in both species PI_{TOT} was significantly reduced on DOY 231, this reduction was as high as -86.4% in FO, and only of -40.2% in QC, in respect to the values measured on DOY 158 (at full leaf development and non-limiting water availability). Moreover, while QC kept its PI_{TOT} values rather constant during the early response to drought stress (DOY 186 and 198), in FO PI_{TOT} decreased progressively, concurrent to the decrease of RWC and gas exchanges (see Figures 2 and 4).

The measurement of Chlorophyll fluorescence appears therefore as a reliable diagnostic tool able to provide a set of sensitive indicators for phenotyping drought response of different tree species in natural field conditions.

CONCLUSIONS

The increase in frequency, duration, and severity of drought stress associated with climate change can exert detrimental effects on forests trees in many regions of the world (Allen

et al., 2010; Williams et al., 2013). In particular, both intraspecific and interspecific differences in drought tolerance can affect the species functional performance, as well as the composition, structure, and biogeography of mixed forest ecosystems, as well as competition for available water (Cavin et al., 2013; Nardini et al., 1999; Grossiord et al., 2014). Phenotyping ecophysiological traits under drought is therefore fundamental for understanding potential changes in the functioning of forest ecosystems and in the Ecosystem Services that they provide, particularly in regions such as the Mediterranean one, where severe climate changes are expected to occur (Di Filippo et al., 2010; Matesanz & Valladares, 2014). Our results have confirmed that the measurement of Chlorophyll fluorescence with the JIP-test application allows a fast and non-destructive monitoring of the drought stress response of adult trees in the field. In particular, among the phenotypic traits investigated in the current study, PI_{TOT} has proven to be the most suitable non-invasive marker of plant response to drought which, being related to the overall performance of the photosynthetic apparatus (both PSII and PSI), is able to provide reliable synthetic information on plant ecophysiological status.
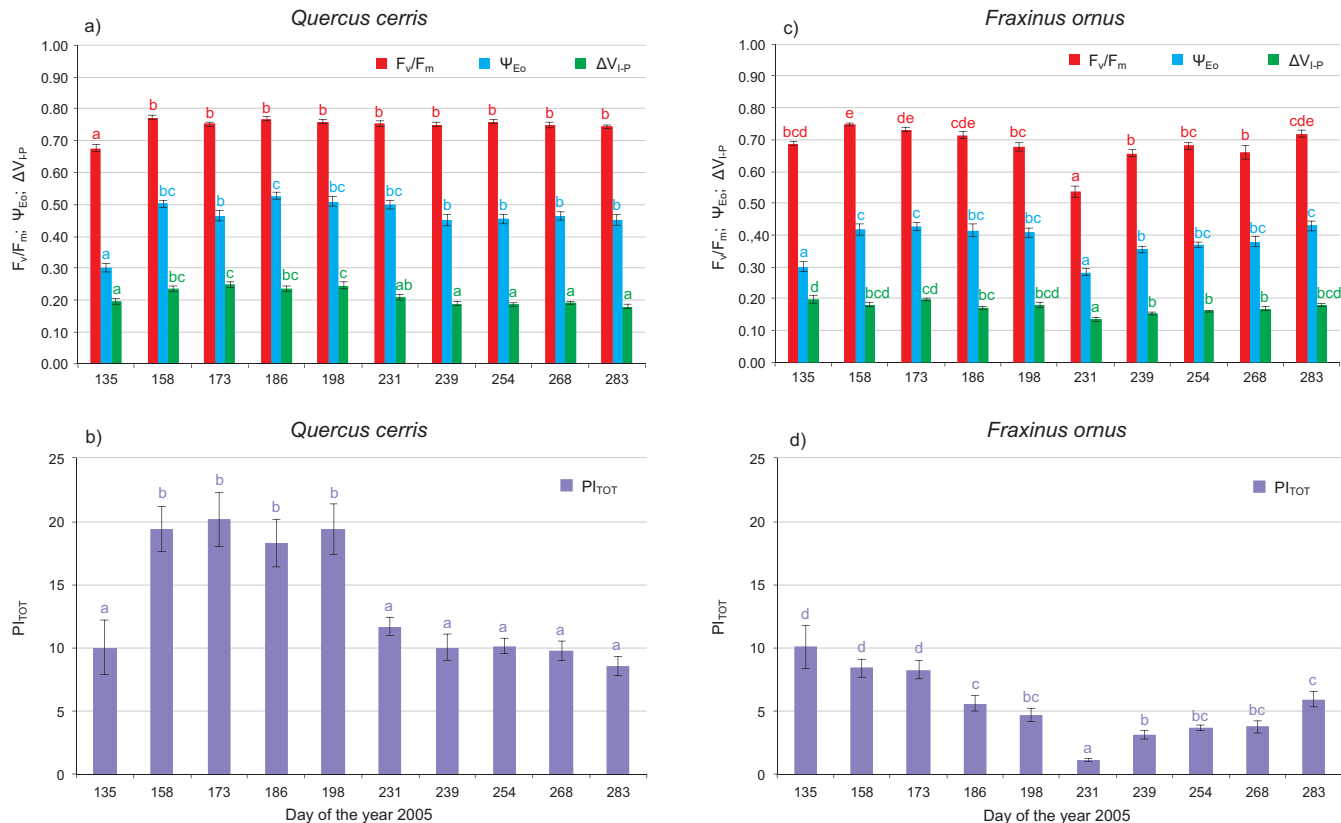
ACKNOWLEDGMENTS

Figure 5. Trend of the main chlorophyll fluorescence parameters measured on *Quercus cerris* (QC, left panel) and *Fraxinus ornus* (FO, right panel) from May to October 2005. a) and c) F_v/F_m, maximum quantum yield of primary photochemistry; Ψ_{Eo} or J-Phase, expresses the probability that the energy of a trapped excitation is used for electron transport beyond Q_A; ΔV_{I-P} or I-P phase, represents the amplitude of the I-P phase of the fluorescence transient OJIP; b) and d) PI_{TOT}, total Photosynthetic Performance Index. Mean ± S.E., $16 \leq n \leq 40$. For each parameter, different letters indicate statistically significant differences between day of measurements.

activities, and Dr. S. Zerunian for the meteorological data supply.

References

Allen C.D., Macalady A.K., Chenchouni H., Bachelet D., McDowell N., Vennetier M., Kitzberger T., Riglingh A., Breshears D.D., Hogg E.K., Gonzalez P., Fensham R., Zhang Z., Castro J., Demidova N., Lim J-H., Allard G., Running S.W., Semerci A., Cobb N., 2010. A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. Forest Ecology and Management 259, 660-684.

Anselmi S., Chiesi M., Giannini M., Manes F., Maselli F., 2004. Estimation of Mediterranean forest transpiration and photosynthesis through the use of an ecosystem simulation model driver by remotely sensed data. Global Ecology and Biogeography 13, 371-380.

Araus J.L., Cairns J.E., 2014. Field high-throughput phenotyping: the new crop breeding frontier. Trends in Plant Science 19(1), 52-61.

Barbeta A., Mejìa-Chang M., Ogaya R., Voltas J., Dawson T.E., Peñuelas J., 2015. The combined effects of a long-term experimental drought and an extreme drought on the use of plant-water sources in a Mediterranean forest. Global Change Biology 21, 1243-1225.

Bernardini A., Salvatori E., Di Re S., Fusaro L., Nervo G., Manes F., 2016. Natural and commercial *Salix* clones differ in their ecophysiological response to Zn stress. Photosynthetica 54(1), 56-64.

Bernardini A., Salvatori E., Guerrini V., Fusaro L., Canepari S., Manes F., 2016. Effects of high Zn and Pb concentrations on *Phragmites australis* (Cav.) Trin. Ex. Steudel: Photosynthetic performance and metal accumulation capacity under controlled conditions. International Journal of Phytoremediation 18(1), 16-24.

Blasi C., Carranza, M.L., Filesi L., Tilia A., Acosta A., 1999. Relation between climate and vegetation along a Mediterranean-Temperate boundary in central Italy. Global Ecology and Biogeography 8, 17-27.

Bussotti F., 2008. Functional leaf traits, plant communities and acclimation processes in relation to oxidative stress in trees: a critical overview. Global Change Biology 14, 2727-2739.

Bussotti F., Desotgiu R., Cascio C., Pollastrini M., Gravano E., Gerosa G., Marzuoli R., Nali C., Lorenzini G., Salvatori E., Manes F., Schaub M., Strasser R.J, 2011. Ozone stress in woody plants assessed with chlorophyll a fluorescence. A critical reassessment of existing data. Environmental and Experimental Botany 73, 19-30.

Carvalho M.D., 2008. Drought stress and reactive oxygen species. Plant Signal Behavior 3, 156-165.

Castro-Díez P., Villar-Salvador P., Pérez-Rontomé C., Maestro-Martínez, Montserrat-Martí G., 1997. Leaf morphology and leaf chemical composition in three *Quercus* (*Fagaceae*) species along a rainfall gradient in NE Spain. Trees 11, 127 -134.

Cavin L., Mountford E.P., Peterken G.F., Jump A.S., 2013. Extreme drought alters competitive dominance within and between tree species in a mixed forest stand. Functional Ecology 27, 1424-1435.

Ceppi M.G., Oukkaroum A., Çiçeka N., Strasser R.J., Schansker G., 2012. The IP amplitude of the fluorescence rise OJIP is sensitive to changes in the Photosystem I content of leaves: a study on plants exposed to magnesium and sulfate deficiencies, drought stress and salt stress. Physiologia Plantarum 144, 277-288.

Chiatante D., Di Iorio A., Sciandra S., Scippa G.S., Mazzoleni S., 2006. Effect of drought and fire on root development in *Quercus pubescens* Willd. And *Fraxinus ornus* L. seedlings. Environmental and Experimental Botany 56, 190-197.

Cornic G., Fresneau C., 2002. Photosynthetic carbon reduction and carbon oxidation cycles are the main electron sinks for photosystem II activity during a mild drought. Annals of Botany 89, 887-894.

Cutini A., Matteucci G., Scarascia-Mugnozza G., 1998. Estimation of leaf area index with the Li-Cor LAI 2000 in deciduous forests. Forest Ecology and Management 105, 55-65.

Desotgiu R., Pollastrini M., Cascio C. , Gerosa G., Marzuoli R., Bussotti F., 2013. Responses to ozone on *Populus* "Oxford" clone in an open top chamber experiment assessed before sunrise and in full sunlight. Photosynthetica 51(2), 267-280.

Desotgiu R., Pollastrini M., Cascio C., Gerosa G., Marzuoli R., Bussotti F., 2012. Chlorophyll a fluorescence analysis along a vertical gradient of the crown in a poplar (Oxford clone) subjected to ozone and water stress. Tree Physiology 32(8), 976-986.

Dhondt S., Wuyts N., Inze D., 2013. Cell to whole-plant phenotyping: the best is yet to come. Trends in Plant Science 18(8), 429-439.

Di Filippo A., Alessandrini A., Biondi F., Blasi S., Portoghesi L., Piovesan G., 2010. Climate change and oak growth decline: Dendroecology and stand productivity of a Turkey oak (*Quercus cerris* L.) old stored coppice in Central Italy. Annals of Forest Science 67(7), 706.

Dowgiallo G., Bottini D., 1998. Pedological aspects of the Circeo National Park. In: Stanisci, A., Zerunian, S. (Eds.), Flora e Vegetazione del Parco Nazionale del Circeo, pp. 33-46. Ministero per le Politiche Agricole, Gestione ex A.S.F.D, Sabaudia.

Fiorani F., Schurr U., 2013. Future Scenarios for Plant Phenotyping. Annual Review of Plant Biology 64, 267-91.

Flexas J., Diaz-Espejo A., Gago J., Gallé A., Galmés J., Giulías J., Medrano H., 2014. Photosynthetic limitations in Mediterranean plants: A review. Environmental and Experimental Botany 103, 12-23.

Flexas J., Medrano H., 2002. Drought inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revised. Annals of Botany 89, 183-189.

Fusaro L., Gerosa G., Salvatori E., Marzuoli R., Monga R., Kuzminsky E., Angelaccio C., Quarato D., Fares S., 2016. Early and late adjustments of the photosynthetic traits and stomatal density in *Quercus ilex* L. grown in an ozone-enriched environment. Plant Biology 18(Supp 1), 13-21.

Fusaro L., Salvatori E., Manes F., submitted. Effects of nitrogen deposition, drought and their interaction, on functional and structural traits of *Fraxinus ornus* L. and *Quercus ilex* L. Submitted to Plant Biosystems.

Fusaro L., Salvatori E., Mereu S., Marando F., Scassellati E., Abbate G., Manes F., 2015. Urban and peri-urban forests in the metropolitan area of Rome: Ecophysiological response of *Quercus ilex* L. in two green infrastructures in an ecosystem services perspective. Urban Forestry and Urban Greening 14(4), 1147-1156.

Giardi M.T., Cona A., Geiken B., Kučera T., Masojidek J., Mattoo A.K., 1996. Long-term drought stress induces structural and functional reorganization of photosystem II.

Planta 199, 118-125.

Giorgi F., Lionello P., 2008. Climate change projections for the Mediterranean region. Global and Planetary Change 63, 90-104.

Goltsev V., Zaharieva I., Chernev P., Kouzmanova M., Kalaji H.M., Yordanov I., Krasteva V., Alexandrov V., Stefanov D., Allakhverdiev S.I., Strasser R.J., 2012. Drought-induced modifications of photosynthetic electron transport in intact leaves: Analysis and use of neural networks as a tool for a rapid non-invasive estimation. Biochimica Et Biophysica Acta-Bioenergetics 1817, 1490-1498.

Gortan E., Nardini A., Gascó A., Salleo S., 2009. The hydraulic conductance of *Fraxinus ornus* leaves is constrained by soil water availability and coordinated with gas exchange rates. Tree Physiology 29(4), 529-539.

Gottardini E., Cristofori A., Cristofolini A., Nali C., Pellegrini E., Bussotti F., Ferretti M., 2014. Chlorophyll-related indicators are linked to visible ozone symptoms: Evidence from a field study on native *Viburnum lantana* L. plants in northern Italy. Ecological Indicators 39, 65-74.

Granier C., Tardieu F., 2009. Multi-scale phenotyping of leaf expansion in response to environmental changes: the whole is more than the sum of parts. Plant, Cell and Environment 32, 1175-1184.

Grassi G., Magnani F., 2005. Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. Plant, Cell and Environment 28, 834-849.

Grossiord C., Gessler A., Granier A., Pollastrini M., Bussotti F., Bonal D., 2014. Interspecific competition influences the response of oak transpiration to increasing drought stress in a mixed Mediterranean forest. Forest Ecology and Management 318, 54-61.

Guidi L., Calatayud A., 2014. Non-invasive tools to estimate stress-induced changes in photosynthetic performance in plants inhabiting Mediterranean areas. Environmental and Experimental Botany 103, 42-52.

IPCC, 2013: Summary for Policymakers. In: Stocker T.F., Qin D., Plattner G.-K., Tignor M., Allen S.K., Boschung J., Nauels A., Xia Y., Bex V., Midgley P.M. (Eds.), Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jonckheere I., Fleck S., Nackaerts K., Muys B., Coppin P., Weiss M., Baret F., 2004. Review of methods for in situ leaf area index determination. Part I. Theories, sensors and hemispherical photography. Agricolture and Forest Meteorology 121, 19-35.

Le Dantec V., Dufrêne E., Saugier B., 2000. Interannual and spatial variation in maximum Leaf Area Index of temperate deciduous stands. Forest Ecology and Management 134, 71-81.

Manes F., Ricotta C., Salvatori E., Bajocco S., Blasi C., 2010. A multiscale analysis of canopy structure in *Fagus sylvatica* L. and *Quercus cerris* L. old-growth forests in the Cilento and Vallo Diano National Park. Plant Biosystems 144(1), 202-210.

Manes F., Vitale M., Giannini M., Puppi G., 2006. Different ability of three Mediterranean oak species to tolerate progressive water stress. Photosynthetica 44(3), 387-393.

Matesanz S., Valladares F., 2014. Ecological and evolutionary responses of Mediterranean plants toglobal change. Environmental and Experimental Botany 103, 53-67.

Mitchell P.J., O'Grady A.P., Tissue D.T., White D.A., Ottenschlaeger M.L., Pinkard E.A., 2013. Drought response strategies define the relative contributions of hydraulic dysfunction and carbohydrate depletion during tree mortality. New Phytologist 197(3), 862-872.

Montagnoli A., Terzaghi M., Di Iorio A., Scippa G.S., Chiatante D., 2012. Fine-root morphological and growth traits in a Turkey-oak stand in relation to seasonal changes in soil moisture in the Southern Apennines, Italy. Ecological Researches 27, 1015-1025.

Murchie E.H., Lawson T., 2013. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. Journal of Experimental Botany 64(13), 3983-3998.

Nardini A., Lo Gullo M.A., Salleo S., 1999. Competitive strategies for water availability in two Mediterranean *Quercus* species. Plant, Cell & Environment 22(1), 109-116.

Nardini A., Salleo S., Trifilo P., Lo Gullo M.A., 2003. Water relations and hydraulic characteristics of three woody species co-occurring in the same habitat. Annans of Forest Science 60, 297-305.

Oukarroum A., Schansker G., Strasser R.J., 2009. Drought stress effects on photosystem I content and photosystem II thermotolerance analyzed using Chl a fluorescence kinetics in barley varieties differing in their drought tolerance. Physiologia Plantarum 137, 188-199.

Oukarroum A., Strasser R.J., Schansker G., 2012. Heat stress and the photosynthetic electron transport chain of the lichen Parmelina tiliacea (Hoffm.) Ach. in the dry and the wet state:

differences and similarities with the heat stress response of higher plants. Photosynthesis Researches, 111, 303-314.

Oukkarroum A., Strasser R.J., 2004. Phenotyping of dark and light adapted barley plants by the fast chlorophyll a fluorescence rise OJIP. South African Journal of Botany 70(2), 277-283.

Pollastrini M., Desotgiu R., Camin F., Ziller L., Gerosa G., Marzuoli R., Bussotti F., 2014. Severe drought events increase the sensitivity to ozone on poplar clones. Environmental and Experimental Botany 100, 94-104.

Pollastrini M., Holland V., Brüggemann W., Bussotti F., 2016. Chlorophyll a fluorescence analysis in forests. Annali di Botanica 6, 23-37.

Rascher U., Blossfeld S., Fiorani F., Jahnke S., Jansen M., Kuhn A.J., Matsubara S., Märtin L.L.., Merchant A., Metzner R., Müller-Linow M., Nagel K.A., Pieruschka R., Pinto F., Schreiber C.M., Temperton V.M., Thorpe M.R., van Dusschoten D., Evan Volkenburgh E., Windt C.A, Schurr U., 2011. Non-invasive approaches for phenotyping of enhanced performance traits in bean. Functional Plant Biology 38, 968-983.

Rousseau C., Belin E., Bove E., Rousseau D., Fabre F., Berruyer R., Guillaumès J., Manceau C., Jacques M.-A-, Boureau T., 2013. High throughput quantitative phenotyping of plant resistance using chlorophyll fluorescence image analysis. Plant Methods 2013, 9:17.

Salvatori E., Fusaro L., Gottardini E., Pollastrini M., Goltsev V., Strasser R.J., Bussotti F., 2014. Plant stress analysis: Application of prompt, delayed chlorophyll fluorescence and 820 nm modulated reflectance. Insights from independent experiments. Plant Physiology and Biochemistry 85, 105-113.

Salvatori E., Fusaro L., Mereu S., Bernardini A., Puppi G., Manes F., 2013. Different O3 response of sensitive and resistant snap bean genotypes (Phaseolus vulgaris L.): The key role of growth stage, stomatal conductance, and PSI activity. Environmental and Experimental Botany 87, 79-91.

Salvatori E., Fusaro L., Strasser R.J., Bussotti F., Manes F., 2015. Effects of acute O3 stress on PSII and PSI photochemistry of sensitive and resistant snap bean genotypes (Phaseolus vulgaris L.), probed by Prompt Chlorophyll "a" fluorescence and 820 nm Modulated Reflectance. Plant Physiology and Biochemistry 97, 368-377.

Sperlich D., Chang C.T., Peñuelas J., Gracia C., Sabaté S., 2015. Seasonal variability of foliar photosynthetic and morphological traits and drought impacts in a Mediterranean mixed forest. Tree Physiology 35, 501-520.

Strasser R.J., Srivastava A., Tsimilli-Michael M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: YunusM., Pathre U., Mohanty P. (Eds), Probing photosynthesis: Mechanisms, Regulation and Adaptation, pp. 558. Taylor & Francis, London - New York.

Strasser R.J., Tsimilli-Michael M., Alaka S., 2004. Analysis of the Fluorescence Transient. In: Papageorgiou G.C. and Govindjee (Eds), Chlorophyll a Fluorescence: A Signature of Photosynthesis, vol. 19, pp. 321-362. Springer, Dordrecht, The Netherlands.

Strasser R.J., Tsimilli-Michael M., Qiang S., Goltsev V., 2010. Simultaneous in vivo recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant *Haberlea rhodopensis*. Biochimica Et Biophysica Acta-Bioenergetics 1797, 1313-1326.

Tognetti R., Raschi A., Béres C., Fenyvesi A., Ridder H.W., 1996. Comparison of sap flow, cavitation and water status of *Quercus petraea* and *Quercus cerris* trees with special reference to computer tomography. Plant, Cell and Environment 19, 928-938.

Tretiach M., 1993. Photosynthesis and transpiration of evergreen Mediterranean and deciduous trees in an ecotone during a growing season. Acta Oecologica 14, 341-360.

Vitale M., Anselmi S., Salvatori E., Manes F., 2007. New approaches to study the relationship between stomatal conductance and environmental factors under Mediterranean climatic conditions. Atmospheric Environment 41, 5385-5397.

Welles J.M., 1990. Some indirect methods of estimating canopy structure. Remote Sensing Reviews 5, 31-43.

Williams A.P., Allen C.D., Macalady A.K., Griffin D., Woodhouse C.A., Meko D.M., Swetnam T.W., Rauscher S.A., Seager R., Grissino-Mayer H.D., Dean J.S., Cook E.R., Gangodagamage C., Cai M., McDowell N.G., 2013. Temperature as a potent driver of regional forest drought stress and tree mortality. Nature Climate Change 3, 292-297.