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 et 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 m² 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 15 m, was composed by Q. cerris (80%), Q. frainetto (20%), and Q. robur. The dominated tree layer, 40% canopy cover and 8 m 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, m² 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 sclerophyll (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:

$$RWC = \frac{(FM - DM)}{(TM - DM)}$$

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 ($\Psi_{pd}$, 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. $\Psi_{pd}$ was measured on a biweekly basis from July to September 2005 (Table 1).

<table>
<thead>
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<th>Date of sampling</th>
<th>DOY</th>
<th>LAI</th>
<th>LMA</th>
<th>RWC</th>
<th>$\Psi_{pd}$</th>
<th>Gas exchanges</th>
<th>Chlorophyll fluorescence</th>
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Gas exchanges measurements

Net photosynthesis ($P_n$, μmol CO$_2$ m$^{-2}$ s$^{-1}$), leaf transpiration ($E$, mmol H$_2$O m$^{-2}$ s$^{-1}$), stomatal conductance ($g_s$, mmol H$_2$O m$^{-2}$ s$^{-1}$) and sub-stomatal CO$_2$ concentration ($C_i$, 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 ($C_i/C_a$, 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, μmol CO$_2$ mmol H$_2$O$^{-1}$), was also calculated. Gas exchanges were measured on a biweekly basis from May to October 2005, on the same dates, hours and on the same 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 (Pm, 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 QA. 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:
\[
\frac{F_v}{F_m} = \Psi_{po} = TR_o/ABS = (F_m - F_o)/F_m, \text{ maximum quantum yield of PSII primary photochemistry measured on dark-adapted samples. } F_v/F_m \text{ expresses the probability that an absorbed photon will be trapped by the PSII reaction centre.}
\]

\[
J\text{-Phase: } \Psi_{Eo} = ET_o/TR_o = 1 - V_j = 1 - (F_{2ms} - F_0)/(F_m - F_0), \text{ expresses the probability that the energy of a trapped excitation is used for electron transport beyond } Q_A. \ V_j \text{ is the relative variable fluorescence at } 3 \text{ ms, calculated as } V_j = (F_j - F_0)/(F_m - F_0).
\]

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

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

\section*{RESULTS AND DISCUSSION}

\subsection*{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 (22$^{nd}$ of May) in correspondence with the end of active phenological phase. The highest measured LAI was 5.29 ± 0.28 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 ± 0.18 mg cm$^{-2}$ for QC and FO, respectively), coherently to what reported by Castro-Diez et al. (1997) along an ecological gradient with increasing drought stress conditions.

\subsection*{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, $\Psi_{pd}$ 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 $\Psi_{pd}$ values than FO as drought progressed during summer: at the beginning of July, $\Psi_{pd}$ was -0.48 ± 0.04 MPa in QC and -2.70 ± 0.36 MPa in FO, and, on DOI 231, $\Psi_{pd}$ 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

\section*{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.
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 Ψpd 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 (Ψpd, MPa) measured during summer 2005 on Quercus cerris (QC) and Fraxinus ornus (FO). Mean ± S.E., n = 4 leaves for Ψpd; 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 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \). 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 during the gas exchanges measurements, average PAR (Fig 2, a), QC was able to maintain a certain degree of CO2 assimilation, with Pn and gs values of 2.15 \( \pm 0.26 \) \( \mu \text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1} \) and 27.06 \( \pm 2.75 \) \( \text{mmol H}_2\text{O m}^{-2} \text{s}^{-1} \), 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 \( \pm 0.61 \) \( \text{mmol H}_2\text{O m}^{-2} \text{s}^{-1} \)), which lowered Pn values close to the CO2 compensation point (0.07 \( \pm 0.08 \) \( \mu \text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1} \)) (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 \( \pm 0.04 \), 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), CO2 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).

**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 (\( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)). Mean ± S.E., 60 ≤ n ≤ 100. For each parameter, different letters indicate statistically significant differences between day of measurements.

**Figure 4.** Chl a-fluorescence parameters measured during the gas exchanges measurements. a) stomatal conductance (gs); b) transpiration rate (ET) c) ratio between intercellular and ambient CO2 concentration (Ci/Ca); d) ratio between intercellular pressure and ambient pressure (cp/Ca); e) quantum yield of primary photochemistry (Fv/Fm); f) maximum quantum yield of PSII primary photochemistry (ΦPSII); g) photochemical quenching (qP); h) maximum photochemical quenching (Qm).
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 Pitot 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 pitot values rather constant during the early response to drought stress (DOY 186 and 198), in FO Pitot 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, Pitot 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.

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