Variations in physiological and biochemical traits of oak seedlings grown under drought and ozone stress

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Despite the huge biodiversity characterizing the Mediterranean environment, environmental constraints, such as high sunlight and high temperatures alongside with dry periods, makes plant survival hard. In addition, high irradiance leads to increasing ozone (O_3) concentrations in ambient air. In this era of global warming, it is necessary to understand the mechanisms that allow native species to tolerate these environmental constraints and how such mechanisms interact. Three Mediterranean oak species (*Quercus ilex*, *Q. pubescens* and *Q. cerris*) with different features (drought tolerant, evergreen or deciduous species) were selected to assess their biometrical, physiological and biochemical responses under drought and/or O_3 stress (80–100 nl l^{-1} of O_3 for 5 h d^{-1} for 77 consecutive days). Leaf visible injury appeared only under drought stress (alone or combined with O_3) in all three species. Drought × O_3 induced strong reductions in leaf dry weight in *Q. pubescens* and *Q. cerris* (–70 and –75%, respectively). Alterations in physiological (i.e. decrease in maximum carboxylation rate) and biochemical parameters (i.e. increase in proline content and build-up of malondialdehyde by-products) occurred in all the three species, although drought represented the major determinant. *Q. ilex* and *Q. pubescens,* which co-occur in dry environments, were more tolerant to drought and drought × O_3. *Quercus ilex* was the species in which oxidative stress occurred only when drought was applied with O_3. High plasticity at a biochemical level (i.e. proline content) and evergreen habitus are likely on the basis of the higher tolerance of *Q. ilex*.

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Abbreviations – Δ_{mean}, the deviation from the mean values; Φ_{CO2}, quantum efficiency for CO_2 assimilation; Φ_{PSII}, maximal photochemical efficiency in light adapted leaves; A, net CO_2 assimilation; A_{380}, CO_2 assimilation at light saturation level and 380 µl l^{-1} CO_2; ANOVA, analysis of variance; Chl, chlorophyll; Chl_{TOT}, total chlorophyll; C_i, intercellular CO_2 concentration; DW, dry weight; F_0, minimal fluorescence yield in dark-adapted leaves; F_m and F_m', maximum fluorescence yield in dark- and light-adapted leaves; F_s, fluorescence yield in steady-state conditions; F_v, variable fluorescence yield; F_v/F_m = potential PSII photochemical efficiency; g_s, stomatal conductance; LSD, least significant difference; MDA, malondialdehyde; NPQ, non-photochemical quenching; PAR, photosynthetic active radiation; $PD\Psi_w$, leaf water potential determined at pre-dawn; PI, plasticity index; PSII, photosystem II; Rubisco, ribulose-1,5-*bis*phosphate carboxylase/oxygenase; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; VAZ, violaxanthin+anteraxanthin+zeaxanthin; V_{cmax}, apparent maximum rate of carboxylation activity by Rubisco; WUE_i, intrinsic water use efficiency.

Introduction

An increase in the frequency and severity of drought events in several regions, especially in the Mediterranean basin, has been predicted to occur in the near future (Bussotti et al. 2014a and b). Low water availability usually occurs concurrently with high sunlight and high temperatures during the Mediterranean summer, in climatic conditions that favor ozone (O3) photochemical production (Butkovic et al. 1990).

Under drought, plants usually suffer from the impairment of many physiological processes at the whole-plant level and in the structure and ultra-structure of cell organelles (Ahuja et al. 2010, Harb et al. 2010). Drought results in (i) a decrease in photosynthesis and growth (Hu et al. 2010, Pinheiro and Chaves 2011); (ii) stomatal closure (Hoshika et al. 2013a); (iii) cell dehydration (Manes et al. 2006); (iv) excess excitation energy (Fini et al. 2012); (v) massive production of reactive oxygen species (ROS, Jubani-Marí et al. 2010); and, finally, (vi) necrosis (Fini et al. 2013). Similar effects have also been attributed to O_3 pollution (Hoshika et al. 2012 and 2013b, Dumont at al. 2013, Pellegrini et al. 2013, Gottardini et al. 2014, Vahisalu et al. 2010).

Much progress has been made in understanding the effects of a single stress, including drought and O_3, on tree performance. However, the effects of interacting stresses cannot be adequately assessed from the combination of unifactorial responses. This is because drought/O_3 interactions are highly variable in their antagonistic, additive, or synergistic effects on trees' biochemical and ecophysiological processes (Matyssek et al. 2005). Some investigations have focused on the interactions of drought and O_3 to determine putative mechanisms through which low water availability affects O_3 damage (Desotgiu et al. 2012, Hoshika et al. 2013a, Pollastrini et al. 2013 and 2014, Gerosa et al. 2014, Li et al. 2015).

However, few studies have focused on understanding the drought/O_3 interactions on biochemical

responses of trees. Conifers have been investigated (e.g. Kronfuss et al. 1998, Alonso et al. 2001, Haberer et al. 2008, Nikolova et al. 2010) and even more rarely in oak species of the Mediterranean basin (e.g. Kurz et al. 1998, Vitale et al. 2008, Calderòn Guerrero et al. 2013, Alonso et al. 2014). Unlike for biochemical findings, there have been many studies on the ecophysiological effects of drought/O_3 on trees, although the results being sometimes contradictory. It is believed that drought stress might counteract adverse O_3 effects through its influence on reducing stomata aperture (Grünehage and Jäger 2003). For example, in saplings of *Quercus ilex* exposed to acute O_3 in growth chambers and previously drought-stressed, Vitale et al. (2008) showed that there were lower O_3 uptake fluxes for drought-stressed plants compared to their well-watered counterparts. Pollastrini et al. (2013) reported a similar antagonistic interaction in O_3-sensitive poplar clones exposed to O_3 in open-top chambers and drought-stressed.

In contrast, other results suggest that drought does not protect trees from O_3 effects, but further exacerbates O_3 damage. In fact, in seedlings of two subspecies of *Q. ilex* (ssp. *ilex* and ssp. *ballota*) exposed continuously to several O_3 treatments in open-top chambers and drought, Alonso et al. (2014) reported that the combination of O_3 and drought caused further decreases in accumulated aboveground biomass, although no additive effects were observed in terms of gas exchange and root biomass. Li et al. (2015) reported a similar interaction in seedlings of *Acer truncatum* exposed continuously to O_3 in open-top chambers and drought. In *Fagus sylvatica* plants exposed to O_3 treatment in large-scale fumigation chambers and subsequently drought-stressed (the plants were deprived of water for a period of time), Pearson and Mansfield (1993) reported that the combination of O_3 and drought failed to induce the increase in stomatal resistance observed in O_3-treated, well-watered plants. . In these cases, the effects observed depended on (i) timing, (ii) intensity, and (iii) order of exposure to the stressors.

The effects of stress interaction on Mediterranean vegetation are of particular interest given that these species are genetically equipped to withstand severe oxidative stress (Bussotti et al. 2014a) and to respond plastically to environmental change (Matesanz and Valladares 2014). Bussotti et al. (2015) reviewed the literature concerning the identification and the quantification of functional traits associated with drought resistance on the main tree species. Oaks (belonging to the genus *Quercus* which includes evergreen but also deciduous species) are considered as species that are well acclimated to cope with several environmental stressors (Corcobado et al. 2014) due to their phenotypic plasticity, despite a species-specific degree of tolerance. Holm oak (*Q. ilex*) is the most widely studied evergreen broadleaved species in terms of provenance trials and has been defined as "drought avoidant" and "water saving" with regard to its ecophysiological behavior (Bussotti et al. 2002). However, negative effects of drought have also been reported in *Q. ilex* (Pesoli et al. 2003, Gimeno et al. 2008). Downy oak (*Q. pubescens*) is a drought tolerant, winter deciduous species that occurs alongside *Q. ilex* in the Mediterranean basin (Damesin and Rambal 1995), however negative effects of drought have been reported regarding its photosynthetic, hydric and biometric parameters

(Arend et al. 2011, 2013). Another winter deciduous tree, Turkey oak (*Q. cerris*), is present over a wide range of environments (south-east Europe and Asia Minor) as a result of its fast-growing ability and less drought tolerant nature (Manes et al. 2006). Of the oak species, *Q. ilex* is the only one that has been studied in depth in response to O_3 and the negative effects of the pollutant in terms of visible injury as well as biometric and physiological patterns (Manes et al. 1998, Inclán et al. 1999, Ribas et al. 2005a,b, Calatayud et al. 2011, Alonso et al. 2014).

In this study we assessed various biometric, physiological and biochemical features in three oak species (*Q. ilex*, evergreen and drought tolerant; *Q. pubescens*, deciduous and drought tolerant, *Q. cerris*, deciduous, fast growing, but less drought tolerant) exposed to drought and chronic O_3. Our aim was to answer the following three questions: (i) do drought and O_3 interact with each other? (ii) is the effect of unifactorial and/or combined stressors similar in all three oaks? (iii) can phenotypic plasticity, with particular regard to physiological traits, lead to species-specific differences in the ability to counteract the deleterious effect of drought × O_3?

Materials and methods

Plant growth, and ozone and drought treatments

Experimental activities were conducted in the field-station of San Piero a Grado, Pisa (43°40′N, 10°21′E), Italy. Three hundred 3-year-old saplings of three *Quercus* species (*Q. ilex*, *Q. pubescens* and *Q. cerris*) were potted (6.5-l pots) in a growing medium containing a mixture of standard soil Einhetserde Topfsubstrat ED 63 grob (peat and clay, 34% organic C, 0.2% organic N and pH of 3.8–6.8) and sand (3.5:1 in volume). The pots were placed in a greenhouse under controlled irrigation for two months in air filtered through active charcoal [O_3 concentration was negligible, below 5 nl l^{-1}, as measured by an O_3 analyzer (Monitor Labs, mod. 8810, San Diego, CA, USA)]. Two weeks before the beginning of fumigation, half of the plants received 30% of the effective evapotranspiration daily, whereas the other half received 100% of the evapotranspiration.

On 7 June 2013 uniformly sized plants were divided into four sets: 20 plants were catalogued as the control set and regularly irrigated to a maximum soil water holding capacity and exposed to charcoal filtered air (WW/O_3–); 20 plants were water stressed as reported above and exposed to charcoal filtered air (WS/O_3–); 20 plants were regularly irrigated and O_3 fumigated (WW/O_3+); and 20 plants were water stressed and O_3 fumigated (WS/O_3+). Plants were transferred into four controlled environment fumigation facilities which were ventilated with charcoal filtered air (2 boxes of WW/O_3– and of WS/O_3–, respectively) or treated with O_3 (2 boxes of WW/O_3+ and of WS/O_3+, respectively). The water-stress treatment was applied to the WS/O_3– and WS/O_3+ sets until the end of the exposure, whereas the WW/O_3– and WW/O_3+ sets were kept at field water capacity. The entire methodology of O_3 exposure was performed according to Nali et al. (2004). In order to simulate a possible future seasonal trend of O_3, plants were exposed to increasing concentrations (80–100±13 nl l^{-1}) of pollutant for 77 consecutive days (5 h d^{-1}, in form of a square wave between 10:00 and 15:00

GMT). At the end of the fumigation period, photosynthetic (on the young fully expanded leaves) and hydric parameters were measured. Finally, leaves of five plants per species and per treatment were mixed, divided into aliquots, stored at –20°C and subsequently lyophilized for chemical analyses.

Ecophysiological measurements

Water potential (Ψ_w) was measured on one leaf per plant before dawn (PD) by a Scholander-type pressure chamber (PMS model 600, PMS Instrument Company, Albany, OR, USA), using the precautions of Turner and Long (1980).

Net CO_2 assimilation rate (A), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were determined using a LI-6400 portable photosynthesis system equipped with a 2 × 3 cm chamber and 6400-02B LED light source (Li-Cor Inc., Lincoln, NE, USA), operating at 380 µl l^{-1} ambient CO_2 concentration and saturating light conditions (photosynthetic active radiation, PAR about 1200 µmol photons m^{-2} s^{-1}). When the leaves did not cover completely the LI-6400 photosynthesis chamber, used sections were removed to estimate their exact area. Areas were calculated from scaled pictures, taken immediately after being removed, using the image analysis software ImageJ. Light response curves for A were determined between 0 and 1800 µmol m^{-2} s^{-1} and were used to determine the light saturation level and the quantum efficiency for CO_2 assimilation (Φ_{CO2}). Diurnal variations in A and g_s were recorded *in situ* from 06:00 to 18:00 under ambient light and CO_2 concentration. The responses of A to variations in internal CO_2 concentration (A/C_i curves) were performed as in Sharkey (1985). Measurements were taken with an infrared gas analyzer (CIRAS-2, PP-System International, Amesbury, MA, USA). The apparent maximum rate of carboxylation by ribulose-1,5-*bis*posphate carboxylase/oxygenase (Rubisco), V_{cmax}, was then estimated by the analysis of A/C_i curves.

Chlorophyll (Chl) fluorescence imaging was obtained using an Imaging-PAM chlorophyll fluorometer (Walz, Effeltrich, Germany) on the leaf area. A charge-coupled device camera with a resolution of 640 × 480 pixels collected the fluorescence signal emitted by dark-adapted leaves over a 30-minute period. The maximum efficiency of photosystem II (PSII) photochemistry was calculated as $F_v/F_m = (F_m – F_0) / F_m$, where F_v is the variable fluorescence, F_m is the maximum fluorescence of dark-adapted leaves, and F_0 is the minimal fluorescence yield in dark-adapted leaves. The F_0 values were recorded with a weak measuring beam (0.1 µmol m^{-2} s^{-1}). The maximum fluorescence yield F_m was determined with a saturating pulse of 8000 µmol m^{-2} s^{-1} PPFD for 1–2 s. Quenching analysis was carried out at about 600 µmol m^{-2} s^{-1}. Chl fluorescence images taken from illuminated leaves were used to calculate the operating photochemical efficiency of PSII [$\Phi_{PSII} = (F_m' – F_s) / F_m'$), where F_m' is the maximal fluorescence in the light adapted state and F_s is Chl fluorescence emission in steady-state conditions] (Genty et al. 1989). The values of steady-state Chl fluorescence (F_s) were normalized to dark-adapted basal rates (F_0) to take into account any difference between plants due to different leaf structure, Chl concentrations, etc. (Flexas et al. 2002). Non-photochemical quenching (NPQ) was calculated as $NPQ = (F_m – F_m') / F_m$ (Bilger and Björkman 1990).

Biochemical analyses

Proline content was determined following Bates et al. (1973), with some minor modifications. A hundred mg of lyophilized material was suspended in 1.5 ml of 3% sulfosalicylic acid. The extraction was conducted by sonication of the samples at 70°C for 10 min three-times and samples were constantly shaken. The homogenates were centrifuged for 20 min at 16 000 *g* at 20°C. The supernatant was filtered through 0.2 µm Minisart® SRT 15 aseptic filters and 0.8 ml of the filtrate was mixed with equal volumes of glacial acetic acid (0.8 ml) and 0.8 ml of ninhydrin reagent (1.25 g ninhydrin, 30 ml of glacial acetic acid, 20 ml of 6 M H_3PO_4) and incubated for 1 h at 100°C. The reaction was stopped by placing the test tubes in ice-cold water. The samples were vigorously mixed with 1.6 ml toluene. After 20 s, the light absorption of the toluene phase was estimated at 520 nm, using toluene as a blank. The proline concentration was determined using a standard curve.

Peroxidation was determined by TBARS (thiobarbituric acid reactive substances) (Döring et al. 2014). Lyophilized leaf samples (100 mg) were suspended in 1 ml of 0.1% trichloroacetic acid (TCA) and the extraction was conducted by sonicating the samples at 70°C for 10 min three-times, keeping the samples shaken and centrifuging for 20 min at 16 000 *g* at 20°C. The supernatant was collected and 300 µl was mixed with 1200 µl of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was maintained at 90°C for 30 min, quickly cooled and centrifuged for 10 min at 10 000 *g* at 4°C. The supernatant was used to determine malondialdehyde (MDA) concentration at 532 nm, corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Leaf pigments were determined by HPLC according to Döring et al. (2014), with some minor modifications. Fifty mg of lyophilized leaves were homogenized in 1 ml of 100% HPLC-grade methanol and incubated overnight at 4°C in the dark. Samples were centrifuged for 15 min at 16 000 *g* at 5°C and the supernatant was filtered through 0.2 µm Minisart® SRT 15 aseptic filters and immediately analyzed. HPLC separation was performed at room temperature with a Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter x 150 mm length). The pigments were eluted using 100% solvent A (acetonitrile/methanol, 75/25, v/v) for the first 14 min to elute all xanthophylls, and to separate lutein from zeaxanthin, followed by a 1.5 min linear gradient to 100% solvent B (methanol/ethylacetate, 68/32, v/v), 15 min with 100% solvent B, which was pumped for 14.5 min to elute Chl *b* and Chl *a* and β-carotene, followed by 2 min linear gradient to 100% solvent A. The flow-rate was 1 ml min^{-1}. The column was allowed to re-equilibrate in 100% solvent A for 10 min before the next injection. The pigments were detected by their absorbance at 445 nm. To quantify the pigment content, known amounts of pure standard were injected into the HPLC system. An equation correlating the peak area to pigment concentration was formulated. The data were processed using Dionex Chromeleon software.

Plant biomass

Above- and below-ground plant biomass production of five plants per treatment was harvested at the end of the experiment for each species. Dry plant material was obtained after drying the material in an oven at 70°C for 72 h.

Statistical analysis

The experiment was set up following a randomized design and the experimental plot consisted of one plant per container. Measurements were carried out on three replicates for each treatment and species (unless specified otherwise).

Following the Shapiro-Wilk W test, the effects of drought, O_3 and their combination were determined by two-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) test, with a significance level of $P = 0.05$ (GraphPad Software, San Diego, CA USA). In the case of measurements carried out for more than two time-points, data were analyzed using repeated three-way ANOVA measurements.

An index of phenotypic plasticity ranging from 0 to 1 was calculated for each variable and species as the difference between the minimum and the maximum mean values among treatments divided by the maximum mean value (Valladares et al. 2000). This index was calculated for each species so that changes in variables expressed in different units and with contrasting variation, ranges could be compared. A two-way ANOVA was applied between groups of variables (biometric, biochemical and leaf physiology as grouped in Table S7) and oak species.

Results

Visible symptoms and growth response

At the end of the exposure, no visible leaf injury was observed in $WW/O_3–$ and WW/O_3+ plants across the three species. Under drought (alone or in combination with O_3), plants of *Q. ilex*, *Q. pubescens* and *Q. cerris*, showed lateral and tip yellow-brown necrosis in the youngest fully expanded leaves and the onset was 47, 28 and 33 days from the beginning of the treatment, respectively (Fig. 1). In addition, at the end of the experiment, all plants, with the exception of $WW/O_3–$, showed a marked phylloptosis. The reduction in leaf number was –25, –60 and –51% in WS/O_3+ plants of *Q. ilex*, *Q. pubescens* and *Q. cerris*, respectively compared to $WW/O_3–$ plants (*data not shown*).

Biometric parameters are reported in Table S1. According to the two-way ANOVA analysis, the interaction between drought and O_3 was significant for all parameters only in *Q. pubescens*: total dry weight, shoot to root ratio and leaf dry weight decreased in $WS/O_3–$ (–54, –44 and –70%, respectively, compared to controls), in WW/O_3+ (–24, –19 and –20%) and in WS/O_3+ plants (–64, –31 and –70%). In *Q. cerris*, shoot to root ratio and leaf dry weight significantly decreased due to drought (–29 and –50%, respectively) and O_3 (–43 and –38%). Combined factors reduced only leaf dry weight (–75%) in *Q. cerris*, but did not significantly change any parameters in *Q. ilex*. In the latter species, drought

alone induced a different partitioning in biomass allocation (as the shoot/root ratio significantly decreased) and a decrease in total and leaf dry weight. O_3 alone was significant only for the shoot/root ratio and leaf dry weight.

Water status, proline and MDA-by product content

Drought (alone and combined with O_3) appeared to be the main determinant in reducing $PD\Psi_w$ in all the species under investigation (Table S2). Only in *Q. pubescens*, did O_3 induce a slight but significant effect on water status. Fig. 2a shows that WS/O_3+ saplings of the three species had lower values of $PD\Psi_w$ than $WW/O_3–$ saplings (from 2- to 3-fold).

Proline content significantly increased in $WS/O_3–$ plants, especially in *Q. pubescens* (3 fold higher compared with $WW/O_3–$ individuals) (Fig. 2b). Following O_3 exposure, proline content significantly increased in *Q. ilex* (+117%, in comparison to $WW/O_3–$), however, the strongest accumulation of proline was observed in WS/O_3+ plants of *Q. ilex* and *Q. cerris* (about 7-and 2-fold compared to controls, respectively) (Fig. 2b).

In *Q. pubescens* and *Q. cerris*, a significant increase in MDA by-products was observed in $WS/O_3–$ (+10 and +25%, respectively), WW/O_3+ (+11 and +17%) and more in WS/O_3+ plants (about +30% in both species) (Fig. 2c). In *Q. pubescens* the increase observed in $WS/O_3–$ and WW/O_3+ plants was significantly lower than that recorded in WS/O_3+. In *Q. ilex*, the level of MDA by-products significantly increased only in WS/O_3+ plants (+23% compared to the controls).

Gas exchanges and chlorophyll *a* fluorescence

In $WW/O_3–$ plants of *Q. ilex* and *Q. pubescens*, g_s peaked at 10:00 a.m. while in *Q. cerris* g_s peaked at 12:00 (Figs. 3 a, c and e). Diurnal trends of A in $WW/O_3–$ plants followed those of g_s (Figs. 3 d and f) even though in *Q. ilex* A peaked in the range of 10:00 a.m. to 14:00 p.m. (Fig. 3 b). Notably, the daily reduction in A and g_s in all the species was markedly pronounced in $WS/O_3–$ and WS/O_3+ plants, while plants treated with O_3 alone were less affected as compared to the other treatments (Figs. 3a–f). In all species, drought and O_3 (alone or in combination) highly affected the diurnal pattern of A (Figs. 3b, d and f; Table S3).

Drought and/or O_3 also induced negative effects on the CO_2 assimilation rate at light saturation (A_{380}) (Fig. 4a and Table S4). In $WS/O_3–$, WW/O_3+ and WS/O_3+ plants, significant reductions were recorded in *Q. ilex* (–86, –37 and –83% compared to controls) and in *Q. cerris* (–65, –39 and –63%), but not significantly in *Q. pubescens* (only drought induced a statistically significant effect), in which a high variability was found. According to the two-way ANOVA analysis, the interaction between drought and O_3 was significant for g_s in all species except for *Q. pubescens*. $WS/O_3–$ plants had a significant reduction in g_s in all of the species ($P<0.001$, $P<0.01$ and $P<0.01$ for *Q. ilex, Q. pubescens* and *Q. cerris*, respectively) (Fig. 4b and Table S4) whereas g_s decreased only in *Q. cerris* WW/O_3+ plants ($P<0.01$). The interaction between drought and ozone was significant only in *Q. ilex* and *Q.*

cerris (Table S4). According to the two-way ANOVA analysis, the interaction between drought and O_3 was significant for the intrinsic water use efficiency (WUE_i) only in *Q. pubescens*. WUE_i decreased steeply in WS/O_3+ plants (–22% compared to controls) and increased strongly in WS/O_3– plants. By contrast, it remained unchanged or even decreased (–42%) in *Q. cerris* and *Q. ilex*, respectively (Fig. 4c). Drought and/or O_3 also decreased apparent V_{cmax} in all the species (Fig. 4d; Table S4). No significant changes in C_i were observed in any of the species regardless of the treatment (*data not shown*).

In the dark-adapted leaves of all control plants, the mean maximal photochemical efficiency (F_v/F_m ratio) was 0.83±0.009 (Fig. 5a), which lies in the range ($0.80 \leq F_v/F_m \leq 0.86$) reported by Björkman and Demmig (1987) for healthy plants. In *Q. pubescens*, water shortage combined with O_3 slowly reduced the ratio that remained in the range reported above (Fig. 5a). In WS/O_3+ *Q. cerris* plants, a slight reduction in this ratio (–7%) was observed, whereas there was a reduction in *Q. ilex* as well, although it was not significant because the two stressors contributed similarly in decreasing F_v/F_m (Table S5). A reduction in Φ_{PSII} in WS/O_3+ plants of *Q. ilex* and *Q. pubescens* was observed, while in *Q. cerris* it was not significant (Fig. 5b; (Table S5).

In all species drought significantly reduced Φ_{PSII}, while the effect of O_3 was significant only in *Q. pubescens* (Fig. 5b and Table S5). The two-way ANOVA analysis revealed that the interaction between drought and O_3 was significant for NPQ in all species except in *Q. cerris*. Dynamic photoinhibition induced by the combination of both stresses led to an increase in thermal dissipation of excess excitation energy in *Q. ilex*, while a decrease was observed in *Q. pubescens* (Fig. 5c; Table S5). The highest value of NPQ was found in WS/O_3– plants of *Q. ilex* (2.6±0.63 *vs* 1.5±0.58; Fig. 5c).

In WS/O_3–, WW/O_3+ and WS/O_3+ plants of *Q. ilex*, the F_s/F_0 ratio significantly decreased. The latter parameter did not change in *Q. cerris* plants and significantly increased in WS/O_3– (+39%), WW/O_3+ (+68%) and WS/O_3+ (+28%) plants of *Q. pubescens* (Fig. 5d; Table S5). The degree of partitioning of the reductive power between CO_2 assimilation and non-assimilative processes (revealed by Φ_{PSII}/Φ_{CO2} ratio) increased following the combination of the two stresses in *Q. ilex* and *Q. pubescens* (+69 and +268%, respectively). In the latter species, a strong and significant increase was also observed under drought (+447%). No changes were observed in WS/O_3+ plants of *Q. cerris* (Fig. 5e; Table S5).

Pigment content

The two-way ANOVA analysis revealed that the interaction between drought and O_3 was significant for total chlorophyll (Chl_{TOT}) in all species except in *Q. pubescens*. In WS/O_3– and WS/O_3+ plants of *Q. ilex*, Chl_{TOT} content decreased significantly (–13 and –20%, respectively), while no differences were observed in WW/O_3+ plants (Table S6). A strong increase of Chl_{TOT} was found in WS/O_3–, WW/O_3+ and WS/O_3+ plants of *Q. cerris* (+75, +38 and +81% respectively). The ratio Chl *a*/*b* and VAZ content remained unchanged after all the treatments in all the species with few exceptions (Table

S6). In *Q. cerris*, drought was statistically significant in relation to Chl *a*/*b*, and in *Q. pubescens*, drought and ozone were statistically significant in relation to VAZ.

Phenotypic plasticity

In all the species biometric parameters PI values were significantly higher that physiological and biochemical respectively (Tables S7 and S8). The phenotypic response to stress in terms of deviation from the mean PI values (Δ_{mean}) was lower in *Q. cerris* (–0.05 compared to +0.03 both in *Q. ilex* and *Q. pubescens*), especially for leaf physiological features (–0.11) compared to the other species (+0.05 and +0.09 for *Q. ilex* and *Q. pubescens*, respectively) (Table S7). In the same way, Δ_{mean} for leaf water potential was lower (–0.09) in *Q. cerris* than in the other oaks (+0.03 and +0.07 for *Q. ilex* and *Q. pubescens*, respectively) (Table S7). Within all the biochemical parameters, PI and Δ_{mean} for proline in *Q. ilex* were 0.85 and +0.19 respectively, and strongly contributed to increasing the mean values of all the biochemical parameters (Table S7).

Discussion

Drought and O_3, which are two stressors typically experienced by plants in the Mediterranean basin during the warm season, may restrict CO_2 photoassimilation and plant growth (Vitale et al. 2008, Pollastrini et al. 2013, Alonso et al. 2014). Their effects change however according to the genotype and environmental conditions, such as the duration of the stress, its intensity, and the concurrence of other constrained conditions. Research on the interaction between drought and O_3 has led to contrasting results. In some cases, drought-induced stomatal closure limited the O_3 flux to leaves and the consequent O_3-induced damage (Vitale et al. 2008). In other cases, a water deficit did not cause a similar ameliorative effect (Ribas et al. 2005b). The model that predicts that drought-induced stomatal closure limits the entry of O_3 into the leaf, preserving and/or reducing damage, appears too simplistic and not universally applicable. Consequently, the first question is "Do drought and ozone interact with each other?"

We found that the combination of drought and O_3 had strong negative effects on most of the biometric and physiological parameters of the three oak species. However, the reduction in plant growth following the combination of the two stresses was attributable principally to drought, given that in all three species the plant biomass reduction is reasonably comparable in both WS/O_3– and WS/O_3+ plants. However, the impact of O_3 alone on growth seems to have been species-specific in view of the reduction of total dry weight in *Q. pubescens*. Gas exchange parameters also highlighted that oaks facing a combination of drought × O_3 had a significantly reduced CO_2 assimilation rate, and to a similar extent to plants only experiencing water deficit. In all the oak species tested in this work, the limitations in photoassimilation were attributable to both the reduction in stomatal conductance and mesophyllic (biochemical and diffusional) alterations, i.e. unchanged C_i and reduction in apparent

V_{cmax}. Plants submitted to drought and drought × O_3 endured a decrease in daily A and g_s from control plants.

The O_3 concentration applied did not give rise to the same dramatic effects induced by drought stress. However, alterations in daily assimilation rate and gas exchange parameters were observed in all three oaks species. Thus under the combination of drought and O_3, despite the effects of drought is predominant, the effect of O_3 might be not negligible. Besides similar effects on biomass and biomass partitioning, our results seem to indicate that drought has a less deleterious impact than drought × O_3 on some physiological parameters (i.e., photochemical PSII efficiency and intrinsic water use efficiency) in all the three species. Further biochemical responses as well as physiological adjustments are activated when O_3 is added as a treatment for drought. In the light of the above, the second question is: "How differently did the three oak species respond to drought and drought × O_3?"

Following withholding water, all three species exhibited a greater decrease in PDΨw. *Q. pubescens* showed a strong increase in WUE_i, compared to its control. Conversely, WUE_i had a significant decrease in the evergreen *Q. ilex*. In all three oaks, drought induced a different partitioning in biomass allocation observed through the significant reduction in the shoot/root ratio, particularly in *Q. pubescens*. The greater carbon partitioning to roots found in *Q. pubescens* is in agreement with previous findings (Nardini and Pitt 1999), which highlight that this species is able to tolerate severe water stress conditions by compensating water loss with an equal amount of water uptake. This drought avoidance strategy showed by *Q. pubescens* is made possible by the high hydraulic efficiency of the stem and roots under water stress (Nardini and Pitt 1999). However, when drought is combined with O_3, *Q. pubescens* showed the strongest biomass reduction among all the species, and the putative ability to counteract water withholding was lost. This is confirmed by the significant reduction in WUE_i which remained unchanged in the other two species in comparison to the drought-stressed individuals. It is probable that the pronounced phylloptosis observed in *Q. pubescens* contributed to the decrease in CO_2 assimilation, hence reducing the ability to maintain an efficient development of the root system.

A different picture emerged with regard to the evergreen *Q. ilex,* in which the drought × O_3 combination further increased the amount of carbon allocated to the root compared to that accumulated in the shoot (from 2.4 in WS/O_3– to 1.6 in WS/O_3+ plants). In addition, in this species phyllotopsis was not as pronounced as in the other two deciduous oaks (*data not shown*). Finally, *Q. cerris* showed a similar behavior to that recorded in *Q. ilex,* but when concomitantly subjected to drought × O_3, root and shoot biomass partitioning was similar to that of the control plants. Thus, in terms of biomass accumulation and loss of WUE_i, *Q. pubescens* was the species in which the effect of drought × O_3 had the most deleterious impact. Besides the effects of these stressors on the biometric and WUE_i traits of the three oak species, other physiological mechanisms that underline the behaviour of the different oak species under drought and drought × O_3 are evidenced. In WS/O_3– and WS/O_3+ plants of *Q. ilex*, Φ_{PSII} significantly decreased and the excess of excitation energy was effectively

dissipated as non-photochemical quenching, i.e., an increase in NPQ. Notably, holm oak was the only species in which the oxidative stress did not increase under drought (unchanged MDA by-product values). However, in WS/O_3+ plants of *Q. ilex,* these mechanisms were not sufficient to prevent PSII photoinhibition and a significant decrease in F_v/F_m was recorded, as already reported in this species under contrasting climatic conditions (xeric, continental and mesic sites) (Camarero et al. 2012). Consequently, an increase in oxidative stress was evident, as revealed by a significant build-up of MDA by-products. The strong increase in proline content (+600% compared to the controls) could have preserved *Q. ilex* plants from further oxidative damage. In fact proline not only facilitates water uptake under drought conditions (Ashraf and Foolad 2007), but also protects plant cells against reactive oxygen species accumulation under stress conditions (Filippou et al. 2014). The Φ_{PSII}/Φ_{CO2} ratio strongly increased following the combination drought × O_3 in *Q. ilex* plants, which indicates the activation of non-reductive processes that dissipate excitation energy, such as photorespiration which is high in this species (Tsonev et al. 2014). Such a pathway would be energetically favorable because the pathway assists in re-oxidation of the photosystems. α-ketoglutaric acid, which is another metabolite connected to photorespiration, can be channeled *via* glutamate, into the biosynthesis of the osmolyte proline. This consequently highlights the relation between the response shown by *Q. ilex* and confirming the recorded increase in proline content.

Compared to the other oak species, *Q. cerris* had the lowest reduction in stomatal conductance and A_{380} in response to drought and drought × O_3. In addition, in WS/O_3– and WS/O_3+ plants of *Q. cerris*, there was a strong reduction in Φ_{PSII} which was not detected in WW/O_3+ plants. A response to drought and drought × O_3 shown by *Q. cerris* was the unchanged values of NPQ, which indicates that non-photochemical mechanisms aimed at preserving the photosynthetic apparatus against damage induced by high excitation energy, were not activated.

These features do not indicate that *Q. cerris* has a higher degree of tolerance compared to the other two species. Indeed, the unvaried WUE_i and thus the non-controlled stomatal closure related to CO_2 assimilation, could have contributed to an increase in MDA levels which were similarly (and with a similar extent) strongly increased both under drought, O_3 and in combination. In addition, in WS/O_3+ of this species, the F_v/F_m ratio decrease (compared to that of healthy control plants) was the highest among all the three species, suggesting a deep impairment of PSII efficiency. WS/O_3– and WS/O_3+ leaves of *Q. cerris* showed a marked increase in the amount of chlorophyll content, probably as an attempt to increase the proportion of functional PSII reaction centers.

Finally, *Q. pubescens* plants, in which phylloptosis markedly pronounced as was the biomass reduction, showed a strong reduction in CO_2 assimilation in both WS/O_3– and WS/O_3+ plants. The decrease in Φ_{PSII} in WS/O_3+ plants (compared to drought alone) indicates that besides the photochemical processes, other electron sinks from PSII, such as photorespiration or the Mehler reaction, might not efficiently sustain the high electron transport rate when O_3 was combined with

drought. *Quercus pubescens* subjected to the experimental constraints in this work showed photoinhibition because of a lower capacity to develop an efficient NPQ pathway. In this species, the oxidative stress increased under all the treatments, similarly to *Q. cerris*. However, while in *Q. cerris*, the increment in MDA was strong under each treatment, for *Q. pubescens* the increment was only slight in WS/O_3- and WW/O_3+, and became strong only in WS/O_3+ plants.

Plant plasticity is dependent on the environments that the plant inhabits (Bussotti et al. 2014a). *Q. ilex* and *Q. pubescens* appear to be the most plastic species in terms of phenotypic traits (Fig. 6). Conversely, *Q. cerris*, the species that is least adapted to drought because of the inherent differences of its natural habitat, exhibited a significantly reduced plasticity (Fig. 6).

As already reported (Valladares et al. 2002, Gratani et al. 2013), all these species are characterized by high values of PI for biometric traits. However, physiological and biochemical features are also likely to be involved in plant responses to environmental factors. *Q. ilex* and *Q. pubescens* appear to be the most plastic in terms of physiological parameters (Δ_{mean}= +0.05 and +0.09, respectively) compared to *Q. cerris* (–0.11). Consequently, *Q. cerris,* which is usually found in habitats where drought is less frequent, exhibited less conservative water-use characteristics (Δ_{mean}= –0.09 for $PD\Psi_w$) probably in an attempt to combat the negative effect of drought with a pronounced phylloptosis.

These results are in agreement with those found by Tognetti et al. (2007), who reported that *Q. cerris* exhibited relatively high stomatal conductance, low WUE_i, and low soil-to-leaf hydraulic conductance. In addition, *Q. cerris* showed a stress-induced damage to photosynthetic apparatus and an increased oxidative stress following drought and/or O_3, as attested by a very strong level of MDA by-products, even when unifactorial treatments were applied. The increase in Chl biosynthesis was the only alternative mechanism to a "wiser" water control in this species. Conversely, the other two species (usually considered more drought tolerant) maintained higher $PD\Psi_w$ than *Q. cerris*. Thus, due to their plasticity of physiological traits (in terms of Δ_{mean} values), they can adjust their physiology to harsher variations in water ability better than *Q. cerris*. *Q. pubescens* is in fact a thermophilous, xerophilous species and typically grows on dry, lime-rich soils in the sub-Mediterranean region, which is characterized by hot dry summers and mild dry winters (Damesin and Rambal 1995). Baldocchi et al. (2010) reported that *Q. pubescens* followed a similar drought-avoidance strategy to *Q. ilex*, but maximized gas exchange during a shorter growing season which thus induced a high transpiration rate throughout summer.

Physiological mechanisms do not protect *Q. ilex* or *Q. pubescens* against oxidative stress once drought occurs concomitantly with O_3. However, the higher plasticity of biochemical traits found in *Q. ilex* compared to *Q. pubescens*, which is above all due to a high ability to modulate proline content under stress conditions, enabled *Q. ilex* to prevent oxidative stress under drought or O_3 alone. However, this mechanism was less efficiently modulated in *Q. pubescens*, with MDA by-products

strongly enhanced after the exposure to each stress alone.

Our dataset supports the hypothesis that *Q. ilex* was the most tolerant species to drought and O_3 when applied as single factors. Its photosynthetic apparatus seems to be well adapted to withstand several environmental adverse conditions, as already reported by Garcia-Plazaola et al. (1999), which could explain its wider ecological distribution (Gratani et al. 2000, Crescente et al. 2002, Niinemets and Keenan 2014).

Given that global change may induce plastic responses in co-occurring Mediterranean species (Valladares et al. 2007), morpho-anatomical traits twinned with physiological plasticity could assist plant species in counteracting the negative effects of several stresses as in these species, despite not always completely preventing the occurrence of the oxidative load, as in *Q. pubescens* and *Q. cerris*. In this context, also the biochemical traits (i.e. content of chlorophyll, antioxidant enzymes, carotenoids, flavonoids), which depend on both the species and the environmental conditions (i.e. sunlight irradiance) at which leaves developed, are crucial for avoiding and countering oxidative damage during stress conditions (Munné-Bosch and Alegre 2000, Valladares and Pearcy 2002, Guidi et al. 2011, Bussotti et al. 2014a).

Consequently the third question is: "Is the slight difference of plasticity in terms of biochemical features found in *Q. ilex* (compared to *Q. pubescens*) enough to explain its superior ability (at least in terms of oxidative stress, i.e., MDA by-products) to counteract the deleterious effect of drought and O_3 over *Q. pubescens*?"

The inherent differences between *Q. ilex* and *Q. pubescens* (evergreen and deciduous, respectively) further differentiate their plant responses. To fully exploit its long-lived leaves, it is essential for sclerophyllous holm oak to maintain its functionality during stress, also in an attempt to reduce the level of phylloptosis, which can be particularly onerous for an evergreen species. In fact, holm oak is the main sclerophyllous evergreen species in the Mediterranean area and is characterized by a xeromorphic leaf structure and an efficient stomatal control, which ensure tolerance to summer drought (Camarero et al. 2012, Calderòn Guerrero et al. 2013).

To conclude, climate change factors such as drought and O_3 have contrasting effects when considered separately or combined. The chronic O_3 concentration adopted in this experiment seemed to have a minor impact compared to drought on the responsiveness of the three oak species. This highlights that the plasticity of the plant species is dependent on the environment in which the plant inhabits. Plant species that inhabit environments characterized by seasonal variations in water availability with long periods of drought (i.e. *Q. ilex* and *Q. pubescens*), are usually more plastic under the same stress compared to those that rarely face the same stressor (*Q. cerris*).

Our dataset suggests that biochemical and physiological adjustments may reduce the impact of O_3 when combined with the effect of water stress. Sclerophyllous habitus can further increase the tolerance to environmental constraints in Mediterranean areas.

Author contributions

The work presented here was carried out in collaboration among all authors. GL and RM defined the research theme and obtained funding. LC, DR, EP and ML designed methods, carried out laboratory experiments, and analyzed the data. LG and CN co-designed experiments, discussed analyses, interpreted the results and wrote the paper. All authors have contributed to, seen and approved the manuscript.

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Figure legends

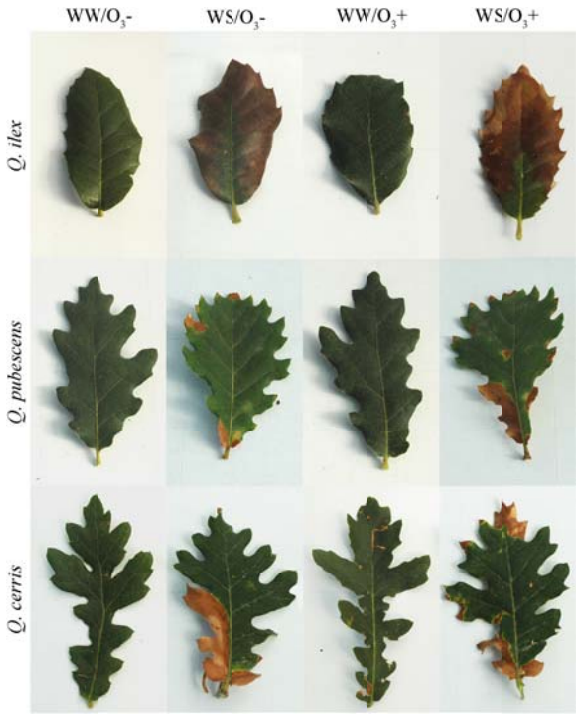

Fig. 1. Symptoms in leaves of *Quercus ilex, Q. pubescens* and *Q. cerris* exposed to drought (daily irrigation with 30% of effective evapotranspiration; $WS/O_3–$), ozone (80–100 nl l^{-1} for 77 consecutive days, 5 h day^{-1}; WW/O_3+) and drought × ozone (WS/O_3+). Controls were kept in charcoal-filtered air and were well watered ($WW/O_3–$).

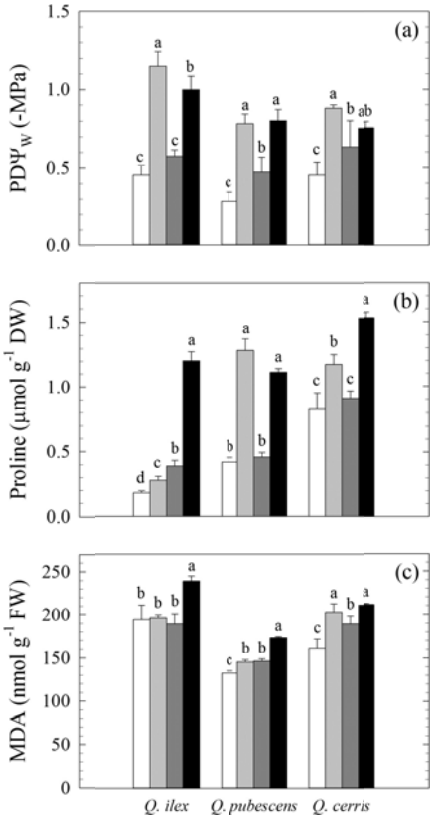

Fig. 2. Pre-dawn water potential ($PD\Psi_w$; a), leaf proline content (b) and malondialdehyde (MDA) by-products (c) estimated in *Quercus ilex, Q. pubescens* and *Q. cerris* plants exposed to drought (daily irrigation with 30% of effective evapotranspiration; grey bars), ozone (80–100 nl l^{-1} for 77 consecutive days, 5 h day^{-1}; dark grey bars) and drought × ozone (black bars). Controls were kept in charcoal-filtered air and were well watered (white bars). Data are shown as mean ± standard deviation (n = 3). For each plant species letters above bars indicate significant differences between drought and O_3 when $P<0.05$ (see Table S2). Abbreviations: DW, dry weight; FW, fresh weight.

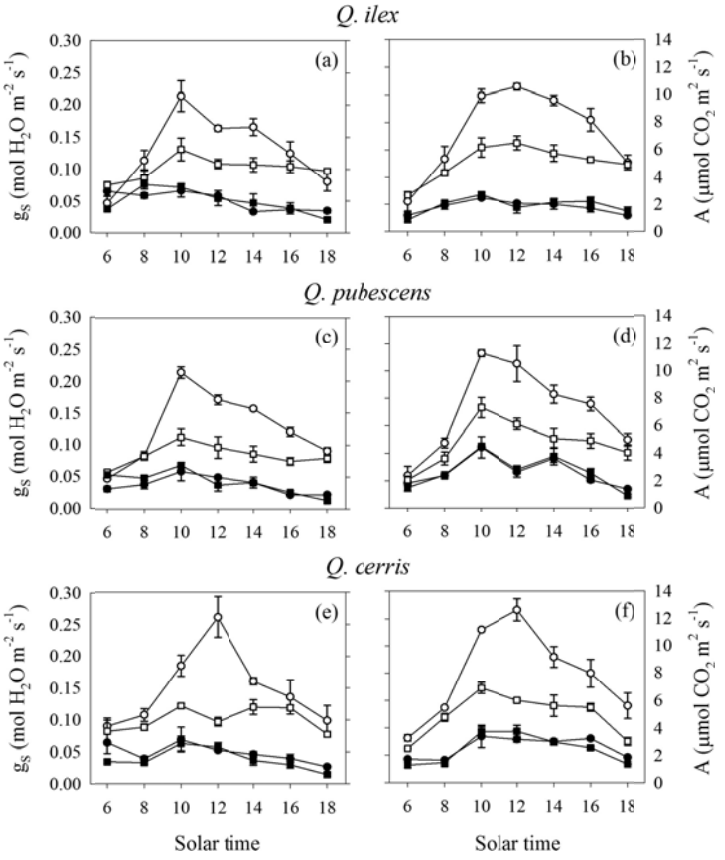

Fig. 3. Daily leaf photosynthesis (A) and stomatal conductance (g_s) in *Quercus ilex, Q. pubescens* and *Q. cerris* plants exposed to drought (daily irrigation with 30% effective evapotranspiration; closed circle), ozone (80–100 nl l^{-1} for 77 consecutive days, 5 h day^{-1}; open square) and drought × ozone (closed square). Controls were kept in charcoal-filtered air and were well watered (open circle). Data are shown as mean ± standard deviation (n = 3).

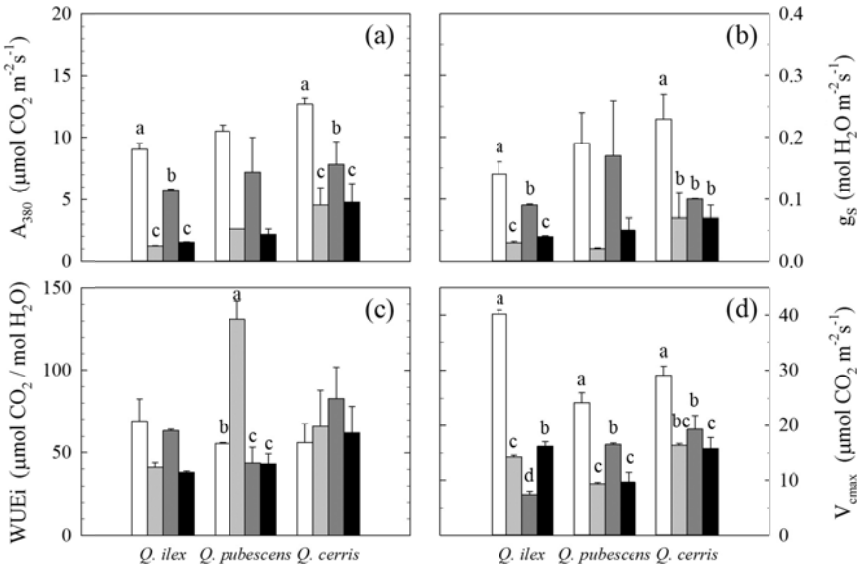

Fig. 4. Foliar gas exchange parameters in *Quercus ilex, Q. pubescens* and *Q. cerris* plants exposed to drought (daily irrigation with 30% of effective evapotranspiration; grey bars), ozone (80–100 nl l^{-1} for

77 consecutive days, 5 h day^{-1}; dark grey bars) and drought × ozone (black bars). Controls were kept in charcoal-filtered air and were well-watered (white bars). Data are shown as mean ± standard deviation (n=3). For each plant species letters above bars indicate significant differences between drought and O_3 when P<0.05 (see Table S2). Abbreviations: A_{380}, CO_2 assimilation rate at light saturation (a); g_s, stomatal conductance to water vapor (b); WUE_i, intrinsic water use efficiency (c); apparent V_{cmax}, maximum rate of carboxylation (d).

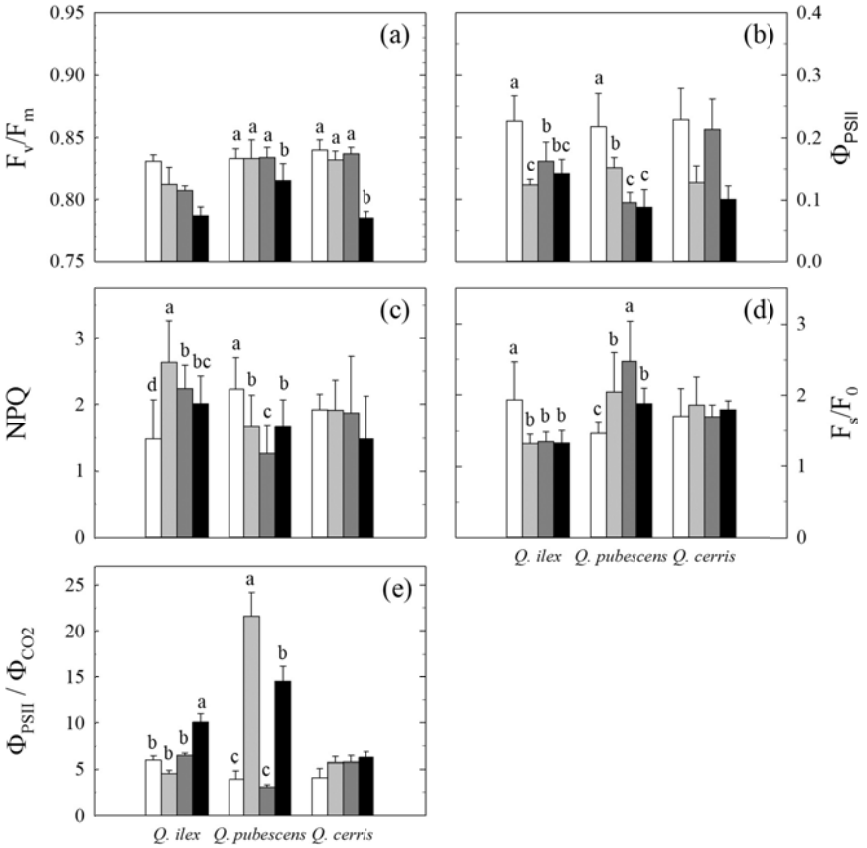

Fig. 5. Leaf chlorophyll *a* fluorescence parameters in *Quercus ilex*, *Q. pubescens* and *Q. cerris* plants exposed to (daily irrigation with 30% effective evapotranspiration; grey bars), ozone (80–100 nl l^{-1} for 77 consecutive days, 5 h day^{-1}; dark grey bars) and drought × ozone (black bars). Controls were kept in charcoal-filtered air and were well watered (white bars). Data are shown as mean ± standard deviation (n = 6). For each plant species letters above bars indicate significant differences between drought and O_3 when P<0.05 (see Table S2). Quenching analysis parameters are determined at a light intensity of about 600 µmol photon m^{-2} s^{-1}. Abbreviations: F_v/F_m, potential PSII photochemical activity (a); Φ_{PSII}, actual PSII photochemical activity (b); NPQ, non-photochemical quenching (c); F_s/F_0, steady-state fluorescence value normalized to minimal fluorescence (d); Φ_{PSII}/Φ_{CO2} ratio (e).

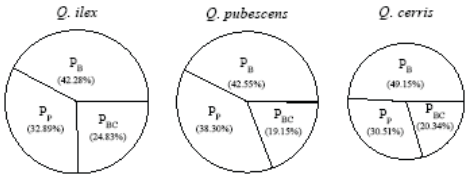

Fig. 6. *Quercus ilex*, *Q. pubescens* and *Q. cerris* ordered by mean phenotypic plasticity. For each species, the percentage contributions of biometric (P_B), biochemical (P_{BC}) and physiological (P_P) plasticity to the total phenotypic plasticity are reported.