1 **Living in a Mediterranean city in 2050: broadleaf or evergreen "citizens"?**

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7 **Abstract**

8 The predicted effects of Global Change (GC) will be exacerbated in the more densely populated cities

9 of the future, especially in the Mediterranean basin where some environmental cues, such as drought

10 and tropospheric ozone (O_3) pollution, already mine seriously plant survival. Physiological and

11 biochemical responses of a Mediterranean, evergreen, isohydric plant species (*Quercus ilex*) were

12 compared to those of a sympatric, deciduous, anisohydric species (*Q. pubescens*) under severe

13 drought (20% of the effective daily evapotranspiration) and/or chronic O_3 exposure (80 ppb for 5 h

14 d^{-1} for 28 consecutive days) to test which one was more successful in those highly-limiting conditions.

15 Results show that (i) the lower reduction of total leaf biomass of *Q. ilex* as compared to *Q. pubescens*

16 when subjected to drought and drought $\times$ O_3 (on average -59 *versus* -70%, respectively); (ii) the

17 steeper decline of photosynthesis found in *Q. pubescens* under drought (-87 *vs* -81%) and drought $\times$

18 O_3 (-69 *vs* -59%, respectively); (iii) the increments of malondialdehyde (MDA) by-products found

19 only in drought-stressed *Q. pubescens*; (iv) the impact of O_3, found only in *Q. pubescens* leaves and

20 MDA, can be considered the best probes of the superiority of *Q. ilex* to counteract the effect of mild-

21 severe drought and O_3 stress. Also, an antagonistic effect was found once drought and O_3 occurred

22 simultaneously, as usually happens during typical Mediterranean summers. Our dataset suggests that

23 on future the urban greening should be wisely pondered on the ability of trees to cope the most

24 impacting factors of GC, and in particular their simultaneity.

25

26 **Keywords**

27 Climate change, drought, oxidative stress, physiological adjustments, *Quercus ilex*, *Quercus*

28 *pubescens*, tropospheric ozone, urban greening.

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30 **Acknowledgments**

Introduction

The Mediterranean area is recognized as a global biodiversity hotspot (Combourieu-Nebout et al. 2015), but this feature is threatened by the exacerbation of several abiotic stressors due to Global Change (GC), which is expected to be here more extreme than in other areas worldwide (IPCC 2012). So, an increase of temperature and/or frequency and severity of drought events has been predicted to occur in the near future, especially in the Mediterranean basin (Bussotti et al. 2014). Furthermore, these climatic conditions (high sunlight and high temperatures), usually co-occurring during a typical Mediterranean summer, favour tropospheric ozone (O_3) photochemical production (Butkovic et al. 1990; Pellegrini 2014), the most relevant and pervasive pollutant which currently affects natural vegetation, crops and trees species (Alonso et al., 2014). Its concentration in the air of Mediterranean cities already frequently exceeds the European limit values for the protection of human health and vegetation (EEA 2016) and O_3 levels are also expected to increase in the next years (Lorenzini et al. 2014). Specifically, projections for 2050 predict a +23% of ground-level O_3 in western Europe, midwestern and eastern USA and eastern China (Morgan et al. 2006) and in 2100, a rise up to 80 ppb as annual average is predicted in some parts of the world with peaks occasionally exceeding 200 ppb (IPCC 2013). In the face of these changes, plants should adjust or adapt to novel and complex combinations of a plethora of environmental constraints (Matesanz and Valladares 2014).

The challenge of Mediterranean plants to cope with the GC will be even more difficult in the urban environment, which affects *per se* their longevity and vitality (Ugolini et al. 2012; Savi et al. 2015) due to already existing limiting factors (e.g. soil compaction, low air humidity, heat, vehicular emissions, etc.). Trees offer several ecosystemic services to urban environment, not only in relation to their aesthetic and social values, but also for their positive effects on air quality. They sequester atmospheric CO_2 and other air pollutants, and provide a natural cooling mechanism (through evapotranspiration and shade) able to reduce air-conditioning energy needs and to avoid contaminant emissions (Pellegrini 2014). These abilities are essential to respond to GC but, at the same time, their effectiveness might be compromised by the future climatic conditions (Sjöman and Nielsen 2010).

Adverse effects of GC to urban Mediterranean plants should be better evaluated, in order to choose wisely species for urban greening, especially focusing on the multifactorial stress effects that

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64   cannot be appropriately evaluated simply by combining unifactorial responses. So that, we need to

65   deserve more attention to interactions between O_3 and other concurrently GC factors such as drought,

66   to more accurately assess impacts of current and future climates on plants health (e.g. Wilkinson and

67   Davies, 2010) and especially on tree species (Wittig et al. 2009). For instance, both drought and O_3

68   (applied singularly or in combination) potentially can (i) affect photosynthesis and growth, (ii) induce

69   stomatal closure (iii) dehydrate cells, (iv) lead to heavy generation of reactive oxygen species (ROS),

70   and (v) bring to necrosis (Bohler et al. 2015). However, the recent literature about the effects of the

71   interactions of drought and O_3 on trees (Witting et al., 2009; Pollastrini et al. 2010) shows that results

72   are sometimes contradictory: if usually drought stress seems to counteract O_3 impact through its

73   influence on reducing stomata opening, some outcomes reveal that drought does not preserve trees

74   from O_3 damage but further exacerbate O_3-triggered effects, showing that drought/O_3 interactions

75   could be antagonistic, additive, or synergistic (Matyssek et al. 2005; Bohler et al. 2015).

76   Widely distributed along with the Mediterranean area, oaks (belonging to the genus *Quercus*),

77   due to the high plasticity of their phenotypic and physiological traits, are able to cope several

78   environmental stressors (Corcobado et al. 2014), even though with species-specific degree of

79   tolerance (Gimeno et al. 2008; Cotrozzi et al. 2016). The responses of these species to the interaction

80   of drought and O_3 have yet to be extensively investigated as shown by the scarce information available

81   in literature (e.g. Kurz et al. 1998; Vitale et al. 2008; Calderòn Guerrero et al. 2013; Alonso et al.

82   2014; Cotrozzi et al. 2016). Downy oak (*Q. pubescens*) is a typical Mediterranean deciduous tree

83   defined as drought-tolerant (Cotrozzi et al. 2016), although negative effects in water-limited

84   conditions have been reported (Arend et al. 2011, 2013). Holm oak (*Q. ilex*), sympatric of *Q.*

85   *pubescens*, is likely the most widely studied Mediterranean evergreen tree species and has been

86   defined as 'drought avoidant' and 'water saver' with regard to its ecophysiological behavior (Bussotti

87   et al. 2002) and also the most tolerant species to realistic (but not predicted for future) O_3

88   concentrations among several other *Quercus* species (Calatayud et al. 2011). However, depending on

89   the severity of the imposed water withholding, adverse impacts of drought have also been observed

90   in this species (Pesoli et al. 2003; Gimeno et al. 2008).

91   Compiling data from existing literature done by Medrano et al. (2009) unveils that tree species

92   in the Mediterranean environment have higher values of water use efficiency (WUE) as compared to

93   shrubs and herbaceous species sharing the same habitat under no limiting conditions of water

94   availability. In addition, deciduous trees have slightly greater increment of WUE under drought when

95   compared to evergreens. In addition to the far longer leaf lifespan, peculiarity of most evergreen

96   species (including *Q. ilex*) is also to have slower photosynthetic and growth rate and lower leaf

97   nitrogen content if compared to broadleaved trees living at the same latitude (i.e. *Q. pubescens*)

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(Reich et al. 1992). Due to their conservative resource-use strategy (Valladares et al. 2000), evergreen species are commonly found in the most resource-limiting environments (Small 1972; Chapin 1980), where competition with fast-growing and highly-demanding species is less severe. Curiously, the occurrence of evergreen leaves in a hot, dry climate seems a sort of an ecological mismatch because dropping the leaves during drought could preserve potentially the plants water loss: the main reason for leaf maintenance is that plants can resume promptly their metabolic activities as soon as environmental factors are no longer limiting (Cherubini et al. 2003). The higher degree of drought tolerance reported for *Q. ilex* than *Q. pubescens* (Cotrozzi et al. 2016) might be related to their difference in terms of water use strategies (isohydric *vs* anisohydric), which can significantly vary between ring-porous (*Q. pubescens*) and diffuse-porous wood species (*Q. ilex*) (Corcuera et al. 2004) and the consequent biochemical adjustments aimed to preserve the vitality of their long-living leaves. In addition, the different water use strategy adopted by the two species (especially stomata regulation) can influence the effect of O_3 when concomitantly applied with drought. Validation of this hypothesis is essential to develop new criteria for the selection of urban trees in the era of GC, where, among others, harshness of drought and O_3 is predicted to increase, posing serious problems for the new generation of green urban "citizens".

Materials and Methods

Plant material and experimental design

At the beginning of spring 2014, one hundred homogeneous 3-year-old saplings of *Q. pubescens* and as many of *Q. ilex* were moved (in 3-L pots where they were grown) from a forest nursery of Gubbio, Perugia, Italy (43°19′43″N, 12°33′10″E, 431 m a.s.l.), to the field-station of San Piero a Grado, Pisa, Italy (43°40′48″N, 10°20′46″E, 2 m a.s.l.), where the experimental activities were conducted. Here, plants were transferred into 6.5-L pots containing a growing medium composed by standard soil (Einhetserde Topfsubstrat ED 63 T grob; peat and clay, 34% organic C, 0.2% organic N and pH of 5.8-6.8) and sand (3.5:1 in volume), placed into a greenhouse for 60 days, well-irrigated and exposed to charcoal filtered air (O_3 concentration was below 5 ppb, as determined by a photometric O_3 analyzer (mod. 8810, Monitor Labs, San Diego, CA, USA).

Starting 14 days before the beginning of the fumigation, two groups of plants were established: half of the saplings were irrigated daily with a volume of water equal to the 20% of their effective evapotranspiration (estimated by the average of daily weight loss of five plants initially watered to field capacity) whereas the other half were well-watered. Then, on the beginning of June 2014, 80

uniformly sized plants were selected and transferred into four fumigation chambers [further details are in Nali et al. (2004)] and treated for four consecutive weeks as follows: 20 plants were daily well-watered and exposed to charcoal filtered air (controls, WW/O_3-); 20 plants were drought-stressed as described above and exposed to charcoal filtered air (drought, WS/O_3-); 20 plants were regularly irrigated and O_3 fumigated (80±13 ppb of O_3 for 5 h d^{-1}, in the form of a square wave between 10:00 and 15:00; ozone, WW/O_3+); 20 plants were water stressed and fumigated (drought × ozone, WS/O_3+). With the purpose to simulate a future climate scenario, the O_3 level was established by doubling the average concentration recorded by 14 monitoring stations owned by ARPAT (Regional Agency for the Environment of Tuscany, Florence) from April to September 2012 (Table S1), a period where high O_3 concentrations occurred as a result of favorable climatic conditions for the production of the pollutant (high irradiance and temperature) (Pellegrini et al. 2007, Lorenzini et al. 2014). Midday photosynthetic active radiation (PAR) registered daily during the whole experiment was, as average, 1644 μmol quanta m^{-2} s^{-1}; minimum and maximum air temperatures and relative humidity were 19.7 and 33.5 °C, and 67%, respectively, as reported by Tinytag Ultra 2 data loggers (Gemini Dataloggers, Chichester, West Sussex, UK).

The onset of visible symptoms was checked every day on each plant. Throughout the exposure, leaf gas exchange and chlorophyll (Chl) fluorescence measurements were conducted once a week at 11:00 whereas at the last day of exposure (28th) these analyses were performed every 2 h from 6:00 to 18:00. At the end of the exposure, also predawn leaf water potential (PDΨ_w) and relative water content (RWC) were determined, and for each replicate five fully-developed mature leaves per plant per treatment were mixed, divided into aliquots and stored at -20 °C for osmotic potential (Ψ_π) and biochemical analyses. Aliquots for the assessment of Ψ_π included leaves previously used for PDΨ_w measurements and were kept fresh and frozen until they were analyzed, whereas those for biochemical analyses were later lyophilized.

Plant biomass

At the end of the experiment, plant biomass production of five plants per treatment was determined. Dry plant material was obtained after drying the material in an oven at 70 °C for 72 h.

Leaf water status

According to Turner and Long (1980), the PDΨ_w was determined on one fully-expanded leaf per plant by a Scholander pressure chamber (model 600, PMS Instrument, Albany, OR, USA). To measure Ψ_π, aliquots of four frozen leaves with the major veins removed were thawed for 30 s, and 10 μl of sap were squeezed out for the determination of solute concentration with a Vapor Pressure

Osmometer (Wescor 5500, Midland, ON, Canada). Each aliquot was measured in triplicate and three replications were taken for each treatment. Following standard procedures (Nali et al. 2005), the RWC was estimated on the same leaves previously analysed for gas exchange and Chl fluorescence.

Gas exchange and chlorophyll fluorescence measurements

Leaf gas exchanges and Chl fluorescence measurements were conducted on two fully expanded leaves per plant, on three plants per treatment. Net CO_2 assimilation rate (A), stomatal conductance (g_s), WUE, intercellular CO_2 concentration (C_i) and evapotranspiration (E) were determined using a LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) equipped with a 2×3 cm chamber and a 6400-02B LED light source, as described by Cotrozzi et al. (2016) (390 ppm ambient CO_2 concentration, saturating light conditions and ~1,200 μmol quanta m^{-2} s^{-1} of PAR).

Chl fluorescence was measured with a modulated PAM-2000 fluorometer (Walz, Effeltrich, Germany) on the same leaves used for gas exchange after dark-adapting for 40 min. Maximum fluorescence (F_m) and ground fluorescence (F_0), used for the calculation of the maximum quantum yield of PSII [$F_v/F_m = (F_m - F_0)/F_m$], were determined as reported by Landi et al. (2013). The saturation pulse method was used for analyzing the quenching components, as described by Schreiber et al. (1986): 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] and non-photochemical quenching [qNP = $(F_m-F_m') / (F_m-F_0)$].

Lipid peroxidation

Lipid peroxidation was evaluated spectrophotometrically by determining the malondialdehyde (MDA) by-product accumulation as reported by Penella et al. (2016). This assay takes into account the possible influence of interfering metabolites (such as phenols) for the thiobarbituric acid reactive substances.

Proline, abscisic acid and hexoses determination

The protocol of Bates et al. (1973) with minor modifications (Cotrozzi et al. 2016) was adopted to determine the leaf proline (Pro) content. The levels of proline were calculated spectrophotometrically on a dry weight (DW) basis by means of a standard curve.

To determine abscisic acid (ABA) content, 80 mg of lyophilized leaves were extracted overnight at 4 °C in 0.8 ml of 100% HPLC-grade water. Then, HPLC (P680 HPLC Pump, UVD170U Uv-Vis detector, Dionex, Sunnyvale, CA, USA) was used according to the original method of Perata et al. (1997), with some modifications as reported by Pellegrini et al. (2015).

To determine glucose and fructose levels, 60 mg of leaves were homogenized in 1 ml 100% HPLC-grade water and heated for 60 min in a water bath at 60 °C. The extracts were analyzed by HPLC (with the same pumps used for ABA) equipped with a BioRad column (Aminex HPX-87H, 300×7.8 mm, Richmond, CA, USA) at 50 °C, according to Pellegrini et al. (2015), with some minor modifications.

Photosynthetic and accessories pigment analysis

Photosynthetic and accessories pigment were assessed according to Döring et al. (2014), with some minor modifications. Briefly, 50 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 000g at 5 °C and the supernatant was filtered through 0.2 μm Minisart® SRT 15 aseptic filters and immediately analyzed. HPLC (P680 HPLC Pump, UVD170U Uv-Vis detector, Dionex, Sunnyvale, CA, USA) 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, also the separation of 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 and an equation, correlating peak area to pigment concentration, was formulated. The data were evaluated by Dionex Chromeleon software.

Statistical analyses

The experiment was a completely randomized design and the experimental plot consisted of one plant per container. Measurements were carried out on three replicates for each treatment and species. Since no significant differences were detected in the environmental parameters and O_3 exposure among fumigation chambers, individual plants were considered as the unit of replication. Recorded data were preliminarily tested with the Shapiro-Wilk *W* test for normality and homogeneity of variance. Leaf gas exchange and Chl fluorescence data were analyzed using two-way repeated measures analysis of variance (ANOVA). All the other data were analyzed by two-way ANOVA and comparison among means was determined by Fisher LSD post-test. Constitutive levels between the species were analyzed using Student's *t*-test with a confidence level of $P \leq 0.05$. Linear regressions were applied to

225 A *vs* g_s and A *vs* E. Analyses were performed by NCSS 2007 Statistical Analysis System Software

226 (NCSS, Kaysville, UT, USA).

227

Results

Visible injury

230 At the end of the exposure, only drought-stressed plants of *Q. pubescens* (regardless of the concurrent

231 presence or absence of O_3) showed visible foliar injury in form of marginal yellow-brown necrosis in

232 the adaxial surface of fully expanded leaves. No injury attributable to O_3 was observed.

Leaf water status and MDA content

234 Results of leaf $PD\Psi_W$, Ψ_π, RWC, and MDA levels are detailed in Table 1. A drastic reduction of leaf

235 $PD\Psi_W$ was found in both the species only as a consequence of water shortage, the amplitude of which

236 was not dependent upon the simultaneous presence of O_3. However, drought-stressed *Q. pubescens*

237 plants had ~2-fold lower values of leaf $PD\Psi_W$ than *Q. ilex* ones (*t*-test, *P*<0.001). Furthermore, only

238 *Q. pubescens* leaves from drought-stressed plants exhibited lower values of Ψ_π and RWC (-76 and -

239 7% on average between WS/O_3- and WS/O_3+ plants) in comparison with WW/O_3-. No reduction of

240 these parameters was found in *Q. ilex* to be attributable to water withholding (with or without O_3).

241 Moreover, *Q. pubescens* was the only species in which MDA levels increased significantly as a

242 consequence of each treatment (including O_3 alone).

Osmolytes and ABA

244 Variations of Pro, ABA, glucose and fructose are reported in Table 2. Accumulation of foliar

245 compatible solutes was more evident in *Q. pubescens* than in *Q. ilex*. In WS/O_3- plants of *Q.*

246 *pubescens*, steep increments of Pro (+211%), glucose (+39%) and fructose (+18%) were found,

247 whereas the simultaneous presence of O_3 (WS/O_3+ plants) induced marked changes of osmolytes

248 accumulation. In particular, the huge increment of Pro (+626%, in comparison to WW/O_3-) paralleled

249 the lower accumulation of glucose and fructose, whose values were similar or lower to those of

250 WW/O_3- plants. Levels of ABA were ~2-fold higher in drought-stressed leaves (average of WS/O_3-

251 and WS/O_3+) as compared to WW/O_3- counterparts, and this parameter was also affected by O_3

252 singularly (+40%). In leaves of *Q. ilex* only a drought-dependent increment of glucose (+54% average

253 of WS/O_3- and WS/O_3+) and ~2-fold higher levels of Pro in WS/O_3+ plants were found. Noteworthy,

254 accumulation of ABA was pronounced in WS/O_3+ plants (0.15 *vs* 0.07 µmol g^{-1} DW in controls) but

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255 not in WS/O_3- plants. On the other side, the strongest accumulation of ABA was recorded in leaves

256 of WW/O_3+ saplings (0.26 µmol g^{-1} DW).

Gas exchange and chlorophyll fluorescence parameters

258 Taking into account data collected throughout the four weeks of exposure, similar close relationships

259 between A and g_s were observed between the species, independently on the treatment (Fig. S1).

260 Differently, a strong relationship between A and E was observed only in *Q. pubescens* (Fig. S1).

261 Daily profiles of gas exchange and Chl fluorescence are reported in Figs. 1 and 2. Leaves of *Q.*

262 *pubescens* had constitutively higher A (Fig. 1a) and g_s (Fig. 1b) when compared to those of *Q. ilex*

263 (Fig. 1e,f) on a daily base: daily average of A was 9.2 *vs* 6.2 µmol CO_2 m^{-2} s^{-1} (*t*-test, *P*<0.01), daily

264 average of g_s was 0.19 *vs* 0.09 (*t*-test, *P*<0.001), respectively. The higher CO_2 assimilation of *Q.*

265 *pubescens* is evident overall during the morning (8:00-12:00) (Fig. 1a,e). However, photosynthetic

266 rates reached similar values in drought-stressed plants of both the species (daily average of A was 1.2

267 µmol CO_2 m^{-2} s^{-1} in both the species; *t*-test, p>0.05) and drought $\times$ O_3 (2.6 *vs* 2.9 µmol CO_2 m^{-2} s^{-1} in

268 *Q. ilex* and *pubescens*, respectively; *t*-test, *P*>0.05), denoting a higher decline of *Q. pubescens*

269 photosynthetic performances. This was confirmed by the steep accumulation of intercellular CO_2

270 observed only in WS/O_3- plants of this species at midday, whereas this parameter increased only

271 slightly in the afternoon in *Q. ilex* (Fig. 1d,h). Changes of A in WS/O_3+ and WS/O_3- paralleled the

272 steep decline found in g_s. It should be highlighted that for both the parameters the reduction was less

273 marked under drought than under the combination of drought $\times$ O_3 in both *Q. ilex* (-79.3% and -

274 55.9%, respectively) and Q. *pubescens* (-96.2% and -81.2%, respectively).

275 Values of intrinsic WUE were lower in *Q. pubescens* than in *Q. ilex* WW/O_3- plants (~ 48.9 vs

276 63.7 µmol CO_2/mol H_2O on average on daily bases, respectively; *t*-test; *P*≤0.05) and for both an

277 increment of this ratio in WS/O_3- plants was observed, which was fairly greater in *Q. pubescens* than

278 in *Q. ilex* (+75.3 and +10.7%, respectively; *t*-test; *P*<0.05) (Fig. 1c,g). Furthermore, only *Q.*

279 *pubescens* plants exposed to drought $\times$ O_3 showed increased values of WUE (80.6 µmol CO_2/mol

280 H_2O on average on daily bases).

281 Chl fluorescence analyses highlighted significant reductions of F_v/F_m in drought-stressed plants

282 belonging to both the species (Fig. 2a,b). In *Q. pubescens*, the reduction was moderate in both WS/O_3-

283 and WS/O_3+ saplings (~0.71 on average) and occurred only at midday. Differently, in WS/O_3- and

284 WS/O_3+ plants of holm oak a progressive reduction of F_v/F_m was shown, the latter starting from 10:00

285 h to 14:00 h (maximum photoinhibition) followed by a progressive recover until 18:00 h, when

286 stressed plants reached again control values. In *Q. ilex*, values of F_v/F_m of WS/O_3+ plants at 14:00

287 were close to those found for *Q. pubescens* at midday (*t*-test, *P*>0.05), while a more severe

photoinhibition was found in WS/O_3- plants (0.59). A consistent and constant reduction of Φ_{PSII} was found in drought-stressed plants of both the species during the whole day (Fig. 2b,e). The amplitude of Φ_{PSII} decline was reasonably comparable for WS/O_3- and WS/O_3+ plants of both species (*Q. pubescens*: 0.56 *vs* 0.36, WW/O_3- *vs* WS/O_3- and WS/O_3+ average; *Q. ilex*: 0.53 *vs* 0.29). No negative effects were induced by O_3 alone, nor to the maximum or effective efficiency of PSII. The decline of Φ_{PSII} observed in drought-stressed plants paralleled with the enhancement of qNP, whose increment started progressively after 8:00 h with the same amplitude in WS/O_3- and WS/O_3+ plants of both species. A slight enhancement of qNP was also promoted by O_3 independently of the species.

Chlorophyll and carotenoid content

In *Q. pubescens* leaves, drought treatment negatively affected Chl$_{TOT}$, β-carotene and depoxidation state of xanthophylls (DEPS) with the same amplitude if applied singularly or in combination with O_3 (-10.1, -8.3 and +8.0% on average between WS/O_3- and WS/O_3+ plants, respectively) (Tab. 3). Zeaxanthin (Zea) increased under drought (independently of the presence of O_3), whereas lutein (Lut) increased under drought alone (+25.0%) and even more if also O_3 was concomitantly applied (+65.2%). Values of violaxanthin+antheraxanthin+zeaxanthin (VAZ) ratio were not statistically affected by any treatment. The O_3 treatment induced only a slight reduction of DEPS (-4.3%).

Differently, Chl$_{TOT}$ and β-carotene decreased in WS/O_3- *Q. ilex* plants (-7.5 and -13.5%, respectively) and even more in WS/O_3+ ones (-28.2 and -34.1%), and VAZ showed alterations especially in plants exposed only to drought (-56.8 and -11.6% in WS/O_3- and WS/O_3+ plants, respectively). Lut increased slightly, but significantly, in drought-stressed plants (+6.9% as the average of WS/O_3- and WS/O_3+ *vs* that of WW/O_3- and WW/O_3+). Fairly higher values of Zea were found in drought stressed individuals (as compared to controls), but remarkably the highest values were found in WS/O_3- plants (+118.6%), while only a +36.2% was found in WS/O_3+ plants. DEPS showed in *Q. ilex* similar responses to those found in *Q. pubescens*, increasing similarly in both WS/O_3- and WS/O_3+ saplings (+28.8%, on average) and decreasing in WW/O_3+ ones (-25.6%). In *Q. ilex* leaves, O_3 also induced a decrease of VAZ ratio (-22.1%).

Discussion

A further massive inflow of people to the urban areas is expected in a near future because of the attractiveness of city's lifestyle. But are we sure that also trees will enjoy living in our cities under the predicted environmental conditions, especially in the Mediterranean basin where some environmental cues, such as drought and O_3, already seriously mine plant survival and will be more

exacerbated by GC? Here, we discuss the water relations, physiological and biochemical mechanisms adopted by the sympatric Mediterranean tree species *Q. pubescens* and *Q. ilex* in the attempt to counteract mild-severe drought and/or O_3 exposure, trying also to take a step forward in solving the long-standing question of Bohler et al. (2015): "Interactive effects between drought and O_3: sorrow or joy?". Data reported in the present study offer clear evidences of the higher ability of *Q. ilex*, an evergreen, diffuse-porous wood species with an isohydric behavior, to tolerate the concomitance of O_3 and mild-severe drought as compared to *Q. pubescens,* a broadleaved, ring-porous wood species with an anisohydric behavior.

Firstly, the two species responded differently to water withholding, which resulted the most impacting factor in this experiment. Whereas in *Q. pubescens* the effect of drought significantly depressed the values of leaf RWC, in *Q. ilex* seedlings foliar water content remained unchanged independently of the treatment, thus suggesting a wiser ability to preserve their leaves from dehydration. According to data showed in Table 1, *Q. ilex* can be classified as an isohydric species (minimum changes of $PD\Psi_W$ in relation to soil moisture), whilst *Q. pubescens* showed an anisohydric behavior (strong decline of $PD\Psi_W$ in relation to soil moisture) on the bases of the distinction proposed by Tardieu and Simonneau (1998). For the sack of truth, anisohydric species should also exhibit higher values of g_s when compared to isohydric ones (Aguadé et al. 2015), whereas in our experiment daily values of g_s were similar on daily bases in both the species under drought (alone or combined with O_3). Moreover, the g_s depression in *Q. pubescens* WS/O_3- and WS/O_3+ plants was even higher than that of *Q. ilex* ones when compared to their relative controls (WW/O_3- plants). However, it has been already observed that this dichotomy (isohydric/anisohydric) seems an oversimplified view of stomatal behavior with increasing water demand and that in many cases anisohydric species can switch to an isohydric behavior under highly negative soil water potential (Zhang et al. 2012), and as a function of stressful environmental conditions (Aguadé et al., 2015) which can play a pivotal role in the urban environment.

If stomata dynamically adjust leaf transpiration, xylem properties provide a long-term control of water flux and plant's water use strategies vary sensibly between ring-porous (*Q. pubescens*) and diffuse-porous wood species (*Q. ilex*; Corcuera et al. 2004). The 2-fold more negative $PD\Psi_W$ found in *Q. pubescens* under drought is in agreement with the meta-analyses performed by Klein (2014), who reported that ring-porous species usually show a more severe decline of leaf water potential with reduced soil water availability, as compared to diffuse-porous species. Therefore, both stomatal regulation and xylem features correlate each other and anisohydric species are usually characterized by ring-porous wood, while isohydric species are associated to a diffuse-porous xylem structure. In view of the above, anisohydric species usually have higher A and g_s (as found here in *Q. pubescens*

plants) and can sustain longer periods of mild dry conditions than isohydric species do (McDowell 2011). Conversely, under severe drought stress conditions this strategy may cause anisohydric species to be more prone to xylem dysfunction and thus to hydraulic failure (Hoffmann et al. 2011, McDowell 2011). There are some evidences concerning the reduction of g_s in concomitance to an increase in cavitation events in anisohydric grape genotypes under severe drought (Zufferey et al. 2011), thus giving a putative explanation for the strong (unexpected) reduction of g_s we found in *Q. pubescens*. The failure of the anisohydric strategy under drought as imposed in this experiment is confirmed by the significant reduction of RWC observed in *Q. pubescens* (but not in *Q. ilex*) under these conditions.

The maintenance of an appropriate leaf water status during water deficit is essential for plant growth and, beyond stomatal regulation, the orchestrated responses against the reduction of leaf water status include the accumulation of compatible solute (Sofo et al. 2004, Ashraf and Foolad 2007). The strong accumulation of osmolytes found in WS/O_3- (proline and hexoses) and in WS/O_3+ (proline) *Q. pubescens* leaves, reducing in turn Ψ_π and consequently PDΨ_W, can be considered as an attempt to contrast cell water loss and the inevitable leaf dehydration. Furthermore, also the raise of ABA observed in drought-stressed *Q. pubescens* individuals might have been crucial in drought response, as this ubiquitous hormone and signaling molecule is known to induce accumulation of osmotically active compounds (Jarzyniak and Jasiński 2014). It is worth to be noted how compatible solute profiles in *Q. pubescens* varied between WS/O_3- and WS/O_3+ plants (at least those contemplated and how the combined treatments induced a huge (~ +600%) accumulation of proline but not of glucose and fructose (as in WS/O_3- plants). We do not have enough data to postulate a clear hypothesis about that, but this shift of osmolytes accumulation might be a direct effect of O_3 in view of the well-known ROS-triggered effect of this pollutant, and the additional role as antioxidant (and/or antioxidant defenses activator) demonstrated in many cases by proline under osmotic stress (Szabados and Savouré, 2010). This hypothesis would also be strengthen by the marked proline accumulation found only in WS/O_3+ leaves of holm oak, the species for which foliar osmolyte accumulation was less relevant (Ψ_π did not change and only glucose accumulated slightly in drought-stressed plants regardless the presence or absence of O_3). It is also worthy to note that in both species the effect of O_3 (alone or in combination) did not have any negative significant consequence in terms of water status and compatible solute accumulation. This is likely attributable to a moderate (but significant) effect of O_3 on g_s when compared to the strong depression induced by water stress *per se*.

Secondly, in view of the inherent higher photosynthetic rate of *Q. pubescens* control plants, that the values of A were comparable in WS/O_3- and WS/O_3+ plants independently of the species is an evident probe of how the effect of drought translated into a smaller depression of A and g_s in *Q. ilex* than in *Q. pubescens*. This superior ability was observed both on daily bases and at midday, when

high irradiance can strongly exceed the photosystem requirement of stressed leaves. In view of the conservative use strategy usually adopted by evergreen species which are characterized by longer leaf lifespan, a key prerogative in resource-limiting environments (Reich et al. 1992; Valladares et al. 2000), it is not surprising that *Q. pubescens* plants exhibited a higher decline of photosynthetic rate in such harsh limiting conditions; the lower ability to sustain the carboxylation process of WS/O$_3$- plants of *Q. pubescens* is also corroborated by the steep intercellular accumulation of CO_2 observed at midday. Such higher values of C_i were not found in WS/O$_3$- plants of *Q. ilex*. Conversely, what is surprising is the decline of A found in *Q. ilex* (but not in *Q. pubescens*) under O$_3$ in daily central hours and the lower reduction of carbon gain on daily bases (-27.5% of A in *Q. ilex* *vs* -12% in *Q. pubescens*).

The ability of plants to modulate A and g_s (A/g_s, namely WUE), and in particular to increase WUE in response to moderate-to-severe drought events, is a typical multifactorial-driven trait of Mediterranean species, especially of shrubs and trees (Valladares et al. 2005; Medrano et al. 2009). In this study, pooling together data of A and g_s collected once a week, independently of the treatments received by plants, resulted in highly significant linear correlations for both the species (Fig. S1). This confirms the interdependence of those parameters, as already reported for *Quercus* species, even under stress (Ogaya and Peñuelas 2003; Quero et al. 2006; Medrano et al. 2009). In accordance with the meta-analyses performed by Medrano et al. (2009), we found higher values of WUE in well-watered plants of the evergreen species (*Q. ilex*) when compared to the deciduous counterpart (*Q. pubescens*), incremented values of WUE in both species when subjected to water stress alone, and a fairly higher buildup of this ratio in the deciduous species. Notably, an increment of WUE was found only in *Q. pubescens* WS/O$_3$+ plants. Increments of WUE may be achieved through the controlled regulation of water losses and/or by presenting photosynthetic machinery more protected under water stress conditions. On one hand, the strong accumulation of ABA found in WS/O$_3$+ plants of both the species might have driven the considerable stomatal closure aim to control leaf water loss; on the other side, it is presumable that in both the species the photosynthetic efficiency may be augmented *via* different mechanisms, such as an increment of transcript coding for Rubisco, reduction of stomata to chloroplast CO_2 diffusion, and/or higher carboxylation efficiency per unit of Rubisco (Chaves et al. 2009). Increment of CO_2 assimilation in relation to g_s (leading to higher WUE) was likely to be more necessary for *Q. pubescens* to increase net leaf carbon gain in view of the dramatic stomatal closure found in this species under drought (in both WS/O$_3$- and WS/O$_3$+ plants) which was severely greater than that found in WS/O$_3$- *Q. ilex* saplings.

Thirdly, in addition to stomatal limitations, chlorophyll fluorescence kinetics highlight that WS/O$_3$- and WS/O$_3$+ *Q. ilex* plants responded more promptly than *Q. pubescens* ones in terms of

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dynamic photoinhibition (stronger reduction of F_v/F_m in daily central hours) with a complete recovery in late evening. Dynamic photoinhibition of PSII was associated with high non-radiative energy dissipation, qNP, and the drought-promoted enhancement of qNP found in both the species, paralleled the increase of DEPS, suggesting a higher contribution of the quenching component associated with the xanthophyll cycle activity (namely, q_E). Xanthophylls are associated with the pigment-protein complexes of photosynthetic membranes, where they play a photoprotective and light-harvesting function (Bassi et al. 1993). According to Jahns and Holzwart (2012), two different xanthophyll cycles have been described for land plants: the violaxanthin cycle (violaxanthin is reversibly converted to zeaxanthin and antheraxathin) and the lutein epoxide cycle (lutein epoxide is reversibly converted into lutein). Even though no reports have studied specifically the lutein epoxide cycle in *Q. pubescens*, García-Plazaola et al. (2002) described as this cycle was helpful to support the VAZ cycle under photoinhibitory conditions in other eight *Quercus* species. Thereby, it seems presumable that the marked increase of lutein found in WS/O₃- and even more in WS/O₃+ *Q. pubescens* can be the result of a deactivation of excited triplet Chl operated by lutein epoxide (García-Plazaola et al. 2007). By contrast, drought-stressed saplings of *Q. ilex* showed a significant rise of zeaxanthin levels and only a slight increment of lutein, which highlights the scarce necessity to activate the lutein epoxide cycle in support the VAZ cycle (García-Plazaola et al. 2007). It is interesting to note that *Q. pubescens* leaves had about 2-fold higher ratio of VAZ as compared to those of *Q. ilex*, and this might explain why in *Q. ilex* we observed a steep increment of this ratio while no increment was found in *Q. pubescens* leaves. The severe reduction of *β*-carotene found in both the species under drought (in *Q. ilex* it was even more marked in WS/O₃+ plants than in WS/O₃- ones) suggests that this compound represents both the substrate for a *de novo* biosynthesis of xanthophylls and/or acts directly as a thylakoid and chloroplastic membrane-bound antioxidant able to prevent the formation of singlet oxygen by intercepting the chlorophyll triplet states and by scavenging any additional singlet oxygen present (Havaux et al. 2000). The increment of DEPS paralleled with the reduction of *β*-carotene is in agreement with previous researches conducted in plants suffering for water withholding, in particular at midday when the need of photo-protection is higher (Munné-Bosch et al. 2001).

In addition to the protective mechanisms offered by carotenoids leading to efficient energy dissipation, changes in chlorophyll profile have also been commonly observed in Mediterranean tree species during summer as a typical response of plants which experience prolonged drought. The drought-dependent decline of Chl$_{TOT}$ content found in both our species represents a protective adaptive mechanism of stressed plants to reduce the absorption of excitation energy, prevent over-excitation of photosystems and increase the capacity to dissipate excess excitation energy per intercepted PPFD (Munné- Bosch and Alegre 2000). Chl$_{TOT}$ decrease under combined stress was

more pronounced in *Q. ilex*, which is indicative of further biochemical adjustment undertaken by this species to preserve leaf functionality under stress. These observations strengthen the hypothesis for which the higher plasticity of *Q. ilex* against environmental constrains is necessary to induce promptly the physiological and biochemical responses aimed to preserve from damages their long-lived leaves (Cotrozzi et al. 2016).

Finally, the following observations: (i) *Q. ilex* showed less reduction of total leaf biomass as compared to *Q. pubescens* when subjected to drought and drought $\times$ O_3 (on average -59 *vs* -70%, respectively for both treatments; *data not shown*); (ii) the incremented levels of MDA were found only in *Q. pubescens* leaves; (iii) O_3 impacted *Q. pubescens* leaf biomass (*data not shown*) and MDA and not those of *Q. ilex*; this can be considered the best probes of the higher ability of holm oak to counteract better than downy oak the effect of severe drought and O_3, at least with the severity of factors applied in this experiment.

Beyond the superior ability of *Q. ilex* to counteract the effect of drought and drought $\times$ O_3 simulating prediction conditions in Mediterranean environments, it is also worthy to note that in WS/O_3+ plants of both the species an antagonistic effect in A and g_s reduction was found, suggesting that drought stress can partially protect the leaves from O_3-triggered effects on gas exchanges once the two stressors occur simultaneously, as usually happens during Mediterranean summer. This outcome is in agreement with previous findings in oak species, including *Q. ilex* (Watanabe et al. 2005; Alonso et al. 2014). The effect was more consistent under severe than in moderate water withholding and the authors attributed this ameliorative effect to the lower O_3 uptake flux of drought-stressed plants. In other cases, the ameliorative effect can be attributable to the enhancement of the antioxidant apparatus induced by O_3 (Watanabe et al. 2005). That drought protects from O_3 insult is however a highly debated matter and there are other reports worthy to be mentioned in which drought and O_3 acted sinergically (Ribas et al. 2005a,b; Tausz et al. 2007; Pollastrini et al. 2014). The explanations that have been proposed for the exacerbating effect of O_3 on drought-stressed plants include: (i) massive production of reactive oxygen species with a consequent oxidative stress (Tausz et al. 2007), and (ii) loss of stomatal control induced by O_3 (stomata close slower, or even remain open, under drought $\times$ O_3) (Bussotti 2008). Our dataset confirms that the antagonistic response seems preponderant under severe water shortage; it is indeed remarkably how we did not find the same ameliorative effect in a previous experiment where we imposed only a moderate water shortage, but for a longer time (11 weeks), to the same tree species (Cotrozzi et al. 2016).

Conclusive remarks

Our dataset confirms the higher ability of evergreen species to counteract the effect of drought and O$_3$ when compared to deciduous species in the Mediterranean environment, even under the increasing harshness of those factors as predicted in a near future. From an ecological point of view, this ability seems to be correlated with the stronger need of evergreens (which inhabits usually limiting environment) for protecting their long-lived leaves from different environmental cues. This peculiarity seems less relevant for highly-demanding, fast-growing deciduous species characterized by shorter leaf lifespan, which have superior fitness than evergreens in non-limiting environment. As a practical outcome, this work offers evidences that the recruitment of green urban "citizens" for greening the city of future (a highly-limiting environment) should be wisely driven by a deep knowledge of tree species behavior under the predicted conditions of GC, and in particular their simultaneity.

Conflict of interest

None declared.

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Table 1. Predawn leaf water potential (PDΨ_w), leaf osmotic potential (Ψ_π), relative water content (RWC), and malondialdehyde (MDA)-by products estimated in *Quercus pubescens* and *Quercus ilex* plants (i) regularly irrigated to maximum soil water holding capacity and exposed to charcoal filtered air (WW/O$_3$-); (ii) water stressed and exposed to charcoal filtered air (WS/O$_3$-); (iii) regularly irrigated and O$_3$ fumigated (WW/O$_3$+); and (iv) water stressed and O$_3$ fumigated (WS/O$_3$+) for 28 consecutive days. WS/O$_3$- and WS/O$_3$+ plants daily received 20% of effective evapotranspiration. WW/O$_3$+ and WS/O$_3$+ plants were exposed to 80±13 ppb of O$_3$ for 5 h d^{-1}, in form of a square wave between 10:00 and 15:00. Data are shown as mean ± standard deviation (n = 3). Following two-way ANOVA, for each parameter (columns) different letters indicate significant differences: *** $P \le$ 0.001, ** $P \le 0.01$, * $P \le 0.05$, ns $P > 0.05$.

Quercus pubescens	PDΨ_w (-MPa)	Ψ_π (-MPa)	RWC (%)	MDA (nmol g^{-1} DW)
WW/O$_3$-	0.41±0.134	2.1±0.22	81.5±3.12	93.6±1.68 a
WS/O$_3$-	2.27±0.381	4.0±0.55	76.8±0.09	143.8±10.66 c
WW/O$_3$+	0.64±0.058	2.5±0.43	79.3±2.61	112.4±0.05 b
WS/O$_3$+	1.93±0.459	3.4±0.28	73.6±1.39	132.5±15.59 c
Drought	78.63***	38.12***	16.75**	15.78**
Ozone	0.08ns	0.32ns	4.57ns	0.44ns
Drought × *Ozone*	2.63ns	4.46ns	0.14ns	5.69*
Quercus ilex	PDΨ_w (-MPa)	Ψ_π (-MPa)	RWC (%)	MDA (nmol g^{-1} DW)
WW/O$_3$-	0.49±0.057	2.9±0.27 b	86.3±9.52	190.3±11.21 c
WS/O$_3$-	1.08±0.212	3.1±0.34 b	81.9±2.73	169.5±3.55 b
WW/O$_3$+	0.68±0.063	2.1±0.09 a	88.0±6.33	120.2±3.74 a
WS/O$_3$+	1.02±0.208	3.1±0.17 b	83.9±1.14	204.8±12.70 c
Drought	22.32**	18.75**	1.55ns	38.74***
Ozone	0.04ns	10.58*	0.30ns	11.54**
Drought × *Ozone*	1.75ns	7.00*	0.00ns	106.12***

714 Table 2. Proline (Pro), abscisic acid (ABA), glucose and fructose (μmol g^{-1} DW) estimated in *Quercus*

715 *pubescens* and *Quercus ilex* plants (i) regularly irrigated to maximum soil water holding capacity and

716 exposed to charcoal filtered air (WW/O$_3$-); (ii) water stressed and exposed to charcoal filtered air

717 (WS/O$_3$-); (iii) regularly irrigated and O$_3$ fumigated (WW/O$_3$+); and (iv) water stressed and O$_3$

718 fumigated (WS/O$_3$+) for 28 consecutive days. WS/O$_3$- and WS/O$_3$+ plants daily received 20% of

719 effective evapotranspiration. WW/O$_3$+ and WS/O$_3$+ plants were exposed to 80$\pm$13 ppb of O$_3$ for 5 h

720 d^{-1}, in form of a square wave between 10:00 and 15:00. Data are shown as mean $\pm$ standard deviation

721 (n = 3). Following two-way ANOVA, for each parameter (columns), different letters indicate

722 significant differences: *** $P \le 0.001$, ** $P \le 0.01$, * $P \le 0.05$, ns $P > 0.05$.

Quercus pubescens	Pro	ABA	Glucose	Fructose
WW/O$_3$-	0.54±0.115 a	0.05±0.004	219.6±1.46 a	742.7±11.11 b
WS/O$_3$-	1.68±0.181 b	0.10±0.006	305.2±15.21 b	873.7±44.18 c
WW/O$_3$+	0.74±0.032 a	0.07±0.002	203.1±4.06 a	646.2±7.53 a
WS/O$_3$+	3.92±0.010 c	0.12±0.015	203.4±9.45 a	669.7±32.48 a
Drought	1192.09***	106.07***	65.43***	22.46**
Ozone	376.50***	15.19*	123.75***	84.98***
Drought × Ozone	262.86***	0.12ns	64.35***	10.86*
Quercus ilex	Pro	ABA	Glucose	Fructose
WW/O$_3$-	1.42±0.146 c	0.07±0.006 a	108.9±1.73	425.1±9.07
WS/O$_3$-	0.60±0.067 a	0.07±0.002 a	168.3±5.41	421.7±4.6
WW/O$_3$+	0.95±0.065 b	0.26±0.008 c	101.2±2.98	457.1±15.58
WS/O$_3$+	2.37±0.156 d	0.15±0.002 b	166.7±6.95	443.6±8.29
Drought	20.38**	302.00***	522.93***	2.05ns
Ozone	92.38***	2108.62***	2.98ns	20.91**
Drought × Ozone	280.19***	396.41***	1.26ns	0.74ns

723

Table 3. Leaf pigments content in *Quercus pubescens* and *Quercus ilex* plants (i) regularly irrigated to maximum soil water holding capacity and exposed to charcoal filtered air (WW/O_3-); (ii) water stressed and exposed to charcoal filtered air (WS/O_3-); (iii) regularly irrigated and O_3 fumigated (WW/O_3+); (iv) water stressed and O_3 fumigated (WS/O_3+) for 28 consecutive days. WS/O_3- and WS/O_3+ plants daily received 20% of effective evapotranspiration. WW/O_3+ and WS/O_3+ plants were exposed to 80±13 ppb of O_3 for 5 h d^{-1}, in form of a square wave between 10:00 and 15:00. Data are shown as mean ± standard deviation (n = 3). Following two-way ANOVA, for each parameter (columns) different letters indicate significant differences: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, ns $P > 0.05$. Abbreviations: β-car, β-carotene; Chl_{TOT}, chlorophyll *a* + chlorophyll *b*; DEPS, depoxidation state; DW, dry weight; Lut, Lutein; VAZ, Violaxanthin + Antheraxanthin + Zeaxanthin; Zea, Zeaxanthin. Values of Lut, Zea and VAZ are expresses on a Chl_{TOT} bases (mmol mol^{-1} Chl_{TOT})

Quercus pubescens	Chl_{TOT} (μmol g^{-1} DW)	β-car (μmol g^{-1} DW)	Lut/Chl_{TOT} (mmol mol^{-1})	Zea/Chl_{TOT} (mmol mol^{-1})	DEPS	VAZ/Chl_{TOT} (mmol mol^{-1})
WW/O_3-	31.6±0.36	23.6±0.36	16.1±0.50 a	112.5±5.11	0.68±0.008 b	169.7±6.68
WS/O_3-	29.4±0.66	22.1±0.25	20.0±0.53 b	134.6±12.03	0.74±0.009 c	184.6±14.84
WW/O_3+	31.4±3.50	24.1±1.63	15.5±0.05 a	102.0±2.82	0.65±0.034 a	168.1±5.01
WS/O_3+	27.5±0.29	21.7±0.33	26.4±0.46 c	127.0±8.88	0.76±0.009 c	170.2±12.40
Drought	8.51*	15.96**	881.92***	25.86***	65.17***	1.94ns
Ozone	1.06ns	0.00ns	131.84***	3.86ns	0.80ns	1.75ns
Drought × Ozone	0.72ns	0.67ns	199.83***	0.09ns	7.18*	1.11ns
Quercus ilex	Chl_{TOT} (μmol g^{-1} DW)	β-car (μmol g^{-1} DW)	Lut/Chl_{TOT} (mmol mol^{-1})	Zea/Chl_{TOT} (mmol mol^{-1})	DEPS	VAZ/Chl_{TOT} (mmol mol^{-1})
WW/O_3-	37.5±1.82 c	23.1±0.73 c	19.0±0.18	49.0±2.17 b	0.61±0.021	91.8±3.098 b
WS/O_3-	34.9±0.98 b	20.1±0.34 b	20.6±0.17	112.1±4.56 d	0.85±0.020	136.8±1.98 d
WW/O_3+	35.9±0.54 bc	23.2±1.25 c	19.3±0.73	26.7 ± 5.72 a	0.46±0.046	74.9±5.68 a
WS/O_3+	26.9±0.45 a	15.1±0.49 a	20.4±0.30	66.7 ± 1.57 c	0.72±0.012	103.6±1.12 c
Drought	83.90***	148.47***	31.17***	525.54***	246.20***	346.85***
Ozone	56.90***	28.52***	0.03ns	226.11***	79.69***	160.13***
Drought × Ozone	26.02***	32.35***	1.51ns	26.23***	0.23ns	16.71**

Figure captions

Figure 1. Daily profiles of foliar gas exchange parameters in *Quercus pubescens* and *Quercus ilex* plants (i) regularly irrigated to maximum soil water holding capacity and exposed to charcoal filtered air (WW/O$_3$-, open circle); (ii) water stressed and exposed to charcoal filtered air (WS/O$_3$-, open square); (iii) regularly irrigated and O$_3$ fumigated (WW/O$_3$+, closed circle); (iv) water stressed and O$_3$ fumigated (WS/O$_3$+, closed square) for 28 consecutive days. WS/O$_3$- and WS/O$_3$+ plants daily received 20% of effective evapotranspiration. WW/O$_3$+ and WS/O$_3$+ plants were exposed to 80±13 ppb of O$_3$ for 5 h d^{-1}, in form of a square wave between 10:00 and 15:00. Data are shown as mean ± standard deviation (n = 3). Abbreviations: A, leaf photosynthesis (a, e); g$_s$, stomatal conductance (b, f); WUE, water use efficiency (c, g); C$_i$, and intercellular carbon dioxide (d, h).

Figure 2. Daily profiles of leaf chlorophyll *a* fluorescence parameters in *Quercus pubescens* and *Quercus ilex* plants (i) regularly irrigated to maximum soil water holding capacity and exposed to charcoal filtered air (WW/O$_3$-, open circle); (ii) water stressed and exposed to charcoal filtered air (WS/O$_3$-, open square); (iii) regularly irrigated and O$_3$ fumigated (WW/O$_3$+, closed circle); (iv) water stressed and O$_3$ fumigated (WS/O$_3$+, closed square) for 28 consecutive days. WS/O$_3$- and WS/O$_3$+ plants daily received 20% of effective evapotranspiration. WW/O$_3$+ and WS/O$_3$+ plants were exposed to 80±13 ppb of O$_3$ for 5 h d^{-1}, in form of a square wave between 10:00 and 15:00. Data are shown as mean ± standard deviation (n = 3). Abbreviations: F$_v$/F$_m$, potential PSII photochemical activity (a, d); Φ$_{PSII}$, actual PSII photochemical activity (b, e); qNP, non-photochemical quenching (c, f)
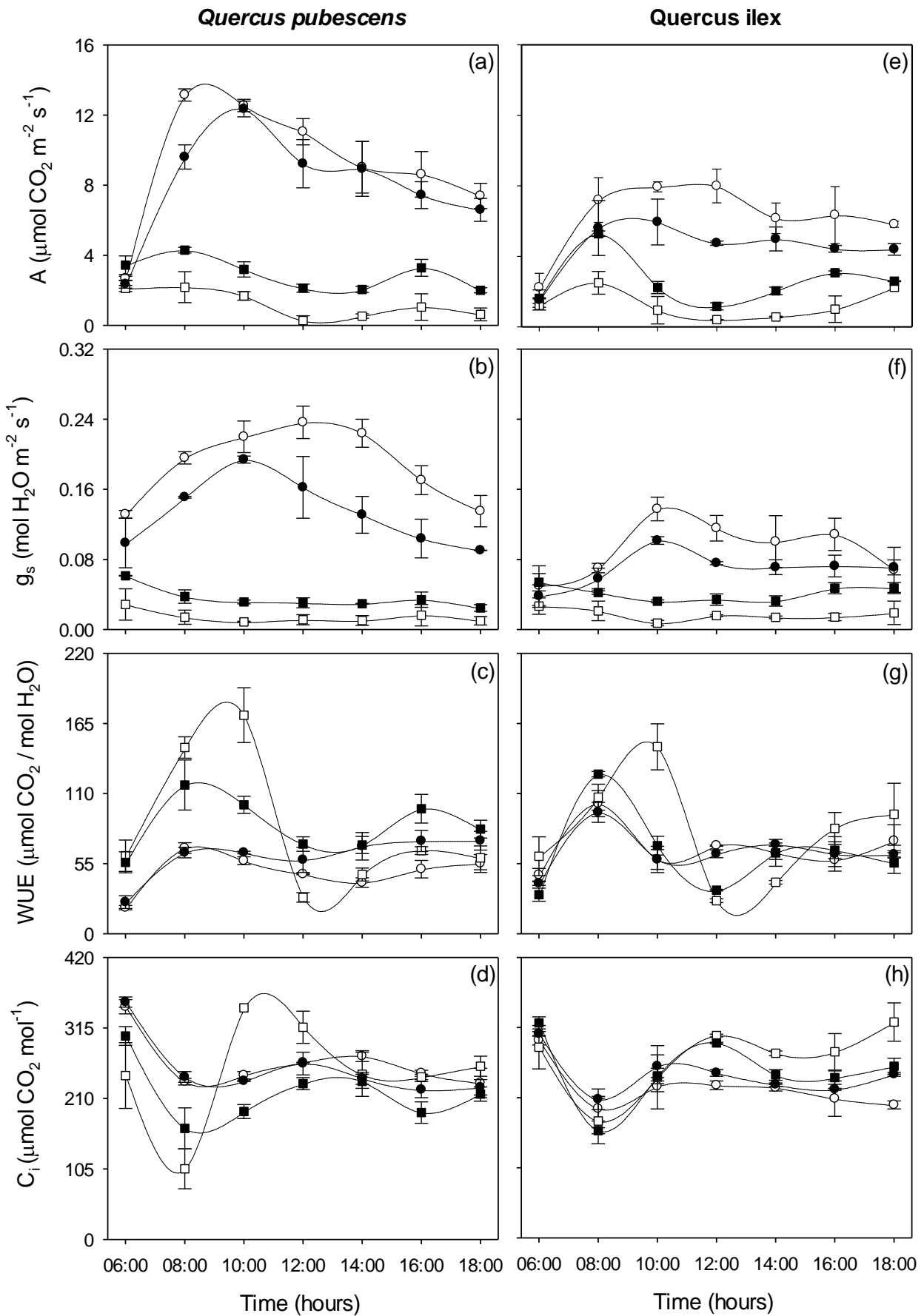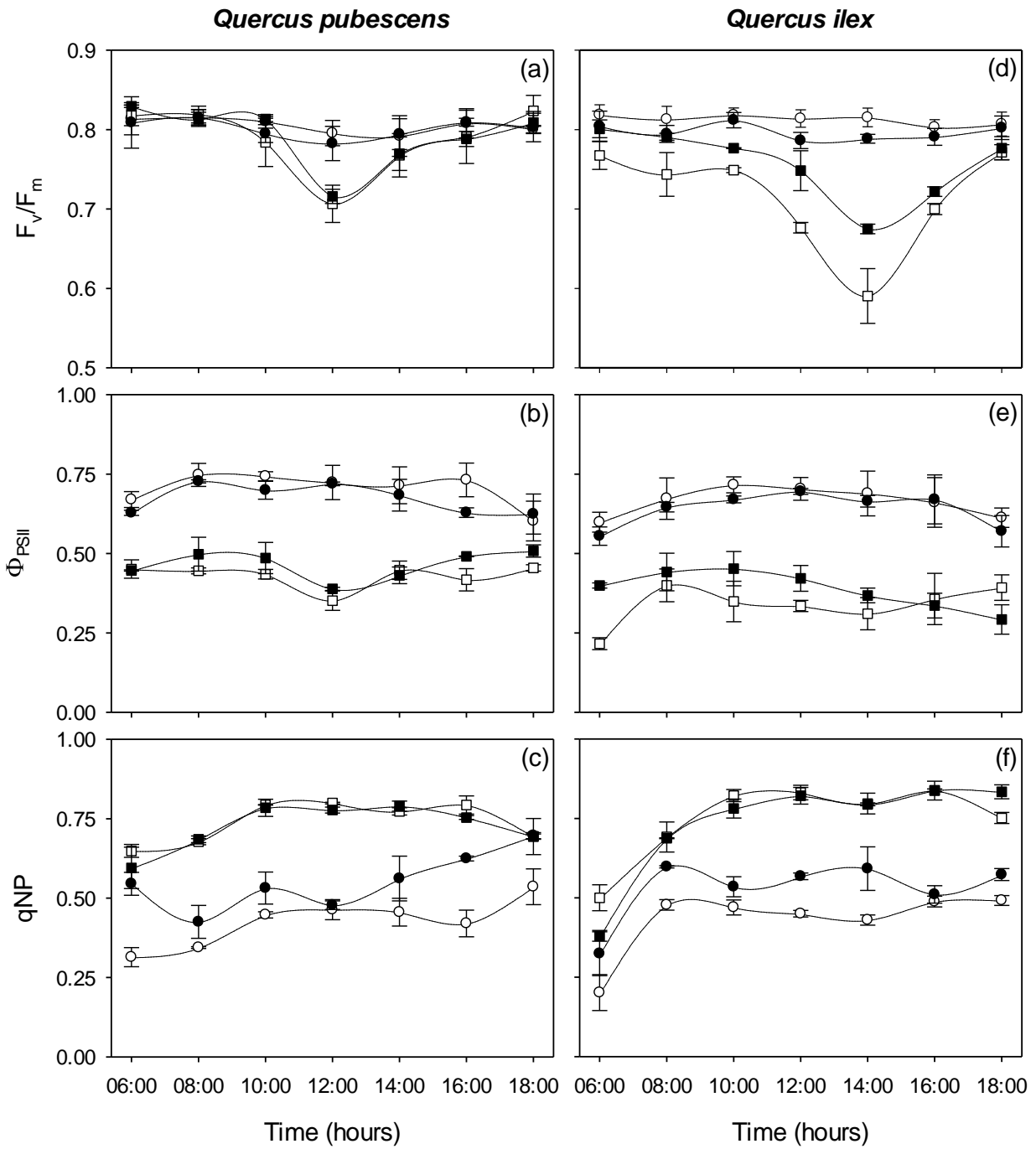
Figure 1

Figure 2

Table S1. *F* values of two-way repeated measures ANOVA of the effects of drought (daily irrigation with 20% of effective evapotranspiration) and ozone (80 ppb for 28 consecutive days, 5 h day^{-1}, in form of a square wave between 10:00 and 15:00 in time (06:00, 08:00, 10:00, 12:00, 14:00, 16:00 and 18:00 hours) on CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), water use efficiency (WUE), intercellular CO_2 concentration (C_i), potential PSII photochemical activity (F_v/F_m), actual PSII photochemical activity (Φ_{PSII}), and no photochemical quenching (qNP) in *Quercus pubescens* and *Quercus ilex* plants. Asterisks show the significance of factors/interaction: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, ns $P > 0.05$. d.f. represents the degrees of freedom.

Effects	d.f.	*Quercus pubescens*							*Quercus ilex*						
		A	g_s	WUE	C_i	F_v/F_m	Φ_{PSII}	qNP	A	g_s	WUE	C_i	F_v/F_m	Φ_{PSII}	qNP
Drought	1	1536.67 ***	1226.75 ***	79.41 ***	17.51 **	5.15 ns	413.67 ***	652.25 ***	166.51 ***	271.23 ***	43.33 ***	19.09 **	372.66 ***	348.89 ***	1222.55 ***
Ozone	1	2.28 ns	18.32 **	4.92 ns	12.36 **	0.00 ns	0.02 ns	34.09 ***	0.59 ns	0.04 ns	118.01 ***	0.02 ns	12.79 **	0.68 ns	32.44 ***
Time	9	72.67 ***	21.77 ***	122.94 ***	75.76 ***	26.90 ***	7.02 ***	53.92 ***	40.71 ***	7.32 ***	55.63 ***	64.50 ***	52.71 ***	11.07 ***	179.78 ***
Drought × Ozone	1	72.22 ***	96.98 ***	2.41 ns	4.09 ns	0.43 ns	7.89 *	39.19 ***	31.43 ***	56.22 ***	60.22 ***	10.73 *	62.67 ***	6.68 *	56.11 ***
Drought × Time	9	72.85 ***	33.53 ***	59.28 ***	29.56 ***	12.03 ***	15.34 ***	21.97 ***	29.07 ***	20.97 ***	21.18 ***	16.81 ***	40.40 ***	2.57 *	17.01 ***
Ozone × Time	9	1.65 ns	3.24 **	34.98 ***	21.91 ***	0.24 ns	1.82 ns	3.34 **	2.98 *	1.79 ns	10.31 ***	2.64 *	1.54 ns	3.40 **	3.86 **
Drought × Ozone × Time	9	3.56 **	2.11 ns	17.81 ***	17.06 ***	1.31 ns	2.59 *	6.71 ***	3.37 **	1.41 ns	9.43 ***	5.39 ***	5.42 ***	4.21 **	5.76 ***

Figure S1. CO_2 assimilation rate (A) in relation to stomatal conductance to water vapor (g_s) and evapotranspiration (E) in *Quercus pubescens* and *Quercus ilex* plants (i) regularly irrigated to maximum soil water holding capacity and exposed to charcoal filtered air (WW/O_3-, open circle); (ii) water stressed and exposed to charcoal filtered air (WS/O_3-, close triangle); (iii) regularly irrigated and O_3 fumigated (WW/O_3+, open triangle); (iv) water stressed and O_3 fumigated (WS/O_3+, closed circle) for 28 consecutive days. WS/O_3- and WS/O_3+ plants daily received 20% of effective evapotranspiration. WW/O_3+ and WS/O_3+ plants were exposed to 80±13 ppb of O_3 for 5 h d^{-1}, in form of a square wave between 10:00 and 15:00. Data were collected weekly during the experiment (28 days). Linear correlation lines with the coefficients of determination (R^2) are shown.