

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 (O₃) 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 O₃ 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 × O₃ (on average -59 versus -70%, respectively); (ii) the
17 steeper decline of photosynthesis found in *Q. pubescens* under drought (-87 vs -81%) and drought ×
18 O₃ (-69 vs -59%, respectively); (iii) the increments of malondialdehyde (MDA) by-products found
19 only in drought-stressed *Q. pubescens*; (iv) the impact of O₃, 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 O₃ stress. Also, an antagonistic effect was found once drought and O₃ 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.

26 **Keywords**

27 Climate change, drought, oxidative stress, physiological adjustments, *Quercus ilex*, *Quercus*
28 *pubescens*, tropospheric ozone, urban greening.

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35

36 **Introduction**

37 The Mediterranean area is recognized as a global biodiversity hotspot (Combourieu-Nebout et al.
38 2015), but this feature is threatened by the exacerbation of several abiotic stressors due to Global
39 Change (GC), which is expected to be here more extreme than in other areas worldwide (IPCC 2012).
40 So, an increase of temperature and/or frequency and severity of drought events has been predicted to
41 occur in the near future, especially in the Mediterranean basin (Bussotti et al. 2014). Furthermore,
42 these climatic conditions (high sunlight and high temperatures), usually co-occurring during a typical
43 Mediterranean summer, favour tropospheric ozone (O₃) photochemical production (Butkovic et al.
44 1990; Pellegrini 2014), the most relevant and pervasive pollutant which currently affects natural
45 vegetation, crops and trees species (Alonso et al., 2014). Its concentration in the air of Mediterranean
46 cities already frequently exceeds the European limit values for the protection of human health and
47 vegetation (EEA 2016) and O₃ levels are also expected to increase in the next years (Lorenzini et al.
48 2014). Specifically, projections for 2050 predict a +23% of ground-level O₃ in western Europe,
49 midwestern and eastern USA and eastern China (Morgan et al. 2006) and in 2100, a rise up to 80 ppb
50 as annual average is predicted in some parts of the world with peaks occasionally exceeding 200 ppb
51 (IPCC 2013). In the face of these changes, plants should adjust or adapt to novel and complex
52 combinations of a plethora of environmental constraints (Matesanz and Valladares 2014).

53 The challenge of Mediterranean plants to cope with the GC will be even more difficult in the
54 urban environment, which affects *per se* their longevity and vitality (Ugolini et al. 2012; Savi et al.
55 2015) due to already existing limiting factors (e.g. soil compaction, low air humidity, heat, vehicular
56 emissions, etc.). Trees offer several ecosystemic services to urban environment, not only in relation
57 to their aesthetic and social values, but also for their positive effects on air quality. They sequester
58 atmospheric CO₂ and other air pollutants, and provide a natural cooling mechanism (through
59 evapotranspiration and shade) able to reduce air-conditioning energy needs and to avoid contaminant
60 emissions (Pellegrini 2014). These abilities are essential to respond to GC but, at the same time, their
61 effectiveness might be compromised by the future climatic conditions (Sjöman and Nielsen 2010).

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

64 cannot be appropriately evaluated simply by combining unifactorial responses. So that, we need to
65 deserve more attention to interactions between O₃ 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 O₃
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 O₃ on trees (Witting et al., 2009; Pollastrini et al. 2010) shows that results
72 are sometimes contradictory: if usually drought stress seems to counteract O₃ impact through its
73 influence on reducing stomata opening, some outcomes reveal that drought does not preserve trees
74 from O₃ damage but further exacerbate O₃-triggered effects, showing that drought/O₃ 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 O₃ 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) O₃
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*)

98 (Reich et al. 1992). Due to their conservative resource-use strategy (Valladares et al. 2000), evergreen
99 species are commonly found in the most resource-limiting environments (Small 1972; Chapin 1980),
100 where competition with fast-growing and highly-demanding species is less severe. Curiously, the
101 occurrence of evergreen leaves in a hot, dry climate seems a sort of an ecological mismatch because
102 dropping the leaves during drought could preserve potentially the plants water loss: the main reason
103 for leaf maintenance is that plants can resume promptly their metabolic activities as soon as
104 environmental factors are no longer limiting (Cherubini et al. 2003). The higher degree of drought
105 tolerance reported for *Q. ilex* than *Q. pubescens* (Cotrozzi et al. 2016) might be related to their
106 difference in terms of water use strategies (isohydric vs anisohydric), which can significantly vary
107 between ring-porous (*Q. pubescens*) and diffuse-porous wood species (*Q. ilex*) (Corcuera et al. 2004)
108 and the consequent biochemical adjustments aimed to preserve the vitality of their long-living leaves.
109 In addition, the different water use strategy adopted by the two species (especially stomata regulation)
110 can influence the effect of O₃ when concomitantly applied with drought. Validation of this hypothesis
111 is essential to develop new criteria for the selection of urban trees in the era of GC, where, among
112 others, harshness of drought and O₃ is predicted to increase, posing serious problems for the new
113 generation of green urban “citizens”.

114

115 **Materials and Methods**

116 **Plant material and experimental design**

117 At the beginning of spring 2014, one hundred homogeneous 3-year-old saplings of *Q. pubescens* and
118 as many of *Q. ilex* were moved (in 3-L pots where they were grown) from a forest nursery of Gubbio,
119 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,
120 Italy (43°40'48"N, 10°20'46"E, 2 m a.s.l.), where the experimental activities were conducted. Here,
121 plants were transferred into 6.5-L pots containing a growing medium composed by standard soil
122 (Einhetserde Topfsubstrat ED 63 T grob; peat and clay, 34% organic C, 0.2% organic N and pH of
123 5.8-6.8) and sand (3.5:1 in volume), placed into a greenhouse for 60 days, well-irrigated and exposed
124 to charcoal filtered air (O₃ concentration was below 5 ppb, as determined by a photometric O₃
125 analyzer (mod. 8810, Monitor Labs, San Diego, CA, USA).

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

130 uniformly sized plants were selected and transferred into four fumigation chambers [further details
131 are in Nali et al. (2004)] and treated for four consecutive weeks as follows: 20 plants were daily well-
132 watered and exposed to charcoal filtered air (controls, WW/O₃-); 20 plants were drought-stressed as
133 described above and exposed to charcoal filtered air (drought, WS/O₃-); 20 plants were regularly
134 irrigated and O₃ fumigated (80±13 ppb of O₃ for 5 h d⁻¹, in the form of a square wave between 10:00
135 and 15:00; ozone, WW/O₃+); 20 plants were water stressed and fumigated (drought × ozone,
136 WS/O₃+). With the purpose to simulate a future climate scenario, the O₃ level was established by
137 doubling the average concentration recorded by 14 monitoring stations owned by ARPAT (Regional
138 Agency for the Environment of Tuscany, Florence) from April to September 2012 (Table S1), a
139 period where high O₃ concentrations occurred as a result of favorable climatic conditions for the
140 production of the pollutant (high irradiance and temperature) (Pellegrini et al. 2007, Lorenzini et al.
141 2014). Midday photosynthetic active radiation (PAR) registered daily during the whole experiment
142 was, as average, 1644 μmol quanta m⁻² s⁻¹; minimum and maximum air temperatures and relative
143 humidity were 19.7 and 33.5 °C, and 67%, respectively, as reported by Tinytag Ultra 2 data loggers
144 (Gemini Dataloggers, Chichester, West Sussex, UK).

145 The onset of visible symptoms was checked every day on each plant. Throughout the exposure,
146 leaf gas exchange and chlorophyll (Chl) fluorescence measurements were conducted once a week at
147 11:00 whereas at the last day of exposure (28th) these analyses were performed every 2 h from 6:00
148 to 18:00. At the end of the exposure, also predawn leaf water potential (PDΨ_w) and relative water
149 content (RWC) were determined, and for each replicate five fully-developed mature leaves per plant
150 per treatment were mixed, divided into aliquots and stored at -20 °C for osmotic potential (Ψ_π) and
151 biochemical analyses. Aliquots for the assessment of Ψ_π included leaves previously used for PDΨ_w
152 measurements and were kept fresh and frozen until they were analyzed, whereas those for
153 biochemical analyses were later lyophilized.

154 **Plant biomass**

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

157 **Leaf water status**

158 According to Turner and Long (1980), the PDΨ_w was determined on one fully-expanded leaf per
159 plant by a Scholander pressure chamber (model 600, PMS Instrument, Albany, OR, USA). To
160 measure Ψ_π, aliquots of four frozen leaves with the major veins removed were thawed for 30 s, and
161 10 μl of sap were squeezed out for the determination of solute concentration with a Vapor Pressure

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

165 **Gas exchange and chlorophyll fluorescence measurements**

166 Leaf gas exchanges and Chl fluorescence measurements were conducted on two fully expanded
167 leaves per plant, on three plants per treatment. Net CO₂ assimilation rate (A), stomatal conductance
168 (g_s), WUE, intercellular CO₂ concentration (C_i) and evapotranspiration (E) were determined using a
169 LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) equipped with a 2 × 3 cm
170 chamber and a 6400-02B LED light source, as described by Cotrozzi et al. (2016) (390 ppm ambient
171 CO₂ concentration, saturating light conditions and ~1,200 μmol quanta m⁻² s⁻¹ of PAR).

172 Chl fluorescence was measured with a modulated PAM-2000 fluorometer (Walz, Effeltrich,
173 Germany) on the same leaves used for gas exchange after dark-adapting for 40 min. Maximum
174 fluorescence (F_m) and ground fluorescence (F₀), used for the calculation of the maximum quantum
175 yield of PSII [$F_v/F_m = (F_m - F_0)/F_m$], were determined as reported by Landi et al. (2013). The
176 saturation pulse method was used for analyzing the quenching components, as described by Schreiber
177 et al. (1986): photochemical efficiency of PSII [$\Phi_{PSII} = (F_m' - F_s) / F_m'$], where F_{m'} is the maximal
178 fluorescence in the light adapted state and F_s is Chl fluorescence emission in steady-state conditions]
179 and non-photochemical quenching [$q_{NP} = (F_m - F_m') / (F_m - F_0)$].

180 **Lipid peroxidation**

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

185 **Proline, abscisic acid and hexoses determination**

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

189 To determine abscisic acid (ABA) content, 80 mg of lyophilized leaves were extracted overnight
190 at 4 °C in 0.8 ml of 100% HPLC-grade water. Then, HPLC (P680 HPLC Pump, UVD170U Uv-Vis
191 detector, Dionex, Sunnyvale, CA, USA) was used according to the original method of Perata et al.
192 (1997), with some modifications as reported by Pellegrini et al. (2015).

193 To determine glucose and fructose levels, 60 mg of leaves were homogenized in 1 ml 100%
194 HPLC-grade water and heated for 60 min in a water bath at 60 °C. The extracts were analyzed by
195 HPLC (with the same pumps used for ABA) equipped with a BioRad column (Aminex HPX-87H,
196 300 × 7.8 mm, Richmond, CA, USA) at 50 °C, according to Pellegrini et al. (2015), with some minor
197 modifications.

198 **Photosynthetic and accessories pigment analysis**

199 Photosynthetic and accessories pigment were assessed according to Döring et al. (2014), with some
200 minor modifications. Briefly, 50 mg of lyophilized leaves were homogenized in 1 ml of 100% HPLC-
201 grade methanol and incubated overnight at 4 °C in the dark. Samples were centrifuged for 15 min at
202 16 000g at 5 °C and the supernatant was filtered through 0.2 µm Minisart® SRT 15 aseptic filters and
203 immediately analyzed. HPLC (P680 HPLC Pump, UVD170U Uv-Vis detector, Dionex, Sunnyvale,
204 CA, USA) separation was performed at room temperature with a Dionex column (Acclaim 120, C18,
205 5 µm particle size, 4.6 mm internal diameter x 150 mm length). The pigments were eluted using 100%
206 solvent A (acetonitrile/methanol, 75/25, v/v) for the first 14 min to elute all xanthophylls, also the
207 separation of lutein from zeaxanthin, followed by a 1.5 min linear gradient to 100% solvent B
208 (methanol/ethylacetate, 68/32, v/v), 15 min with 100% solvent B, which was pumped for 14.5 min to
209 elute chl b and chl a and β-carotene, followed by 2 min linear gradient to 100% solvent A. The flow-
210 rate was 1 ml min⁻¹. The column was allowed to re-equilibrate in 100% solvent A for 10 min before
211 the next injection. The pigments were detected by their absorbance at 445 nm. To quantify the
212 pigment content, known amounts of pure standard were injected into the HPLC system and an
213 equation, correlating peak area to pigment concentration, was formulated. The data were evaluated
214 by Dionex Chromeleon software.

215 **Statistical analyses**

216 The experiment was a completely randomized design and the experimental plot consisted of one plant
217 per container. Measurements were carried out on three replicates for each treatment and species. Since
218 no significant differences were detected in the environmental parameters and O₃ exposure among
219 fumigation chambers, individual plants were considered as the unit of replication. Recorded data were
220 preliminarily tested with the Shapiro-Wilk *W* test for normality and homogeneity of variance. Leaf
221 gas exchange and Chl fluorescence data were analyzed using two-way repeated measures analysis of
222 variance (ANOVA). All the other data were analyzed by two-way ANOVA and comparison among
223 means was determined by Fisher LSD post-test. Constitutive levels between the species were
224 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

228 **Results**

229 **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 O₃) showed visible foliar injury in form of marginal yellow-brown necrosis in
232 the adaxial surface of fully expanded leaves. No injury attributable to O₃ was observed.

233 **Leaf water status and MDA content**

234 Results of leaf PDΨ_w, Ψ_π, RWC, and MDA levels are detailed in Table 1. A drastic reduction of leaf
235 PDΨ_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 O₃. However, drought-stressed *Q. pubescens*
237 plants had ~2-fold lower values of leaf PDΨ_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/O₃⁻ and WS/O₃⁺ plants) in comparison with WW/O₃⁻. No reduction of
240 these parameters was found in *Q. ilex* to be attributable to water withholding (with or without O₃).
241 Moreover, *Q. pubescens* was the only species in which MDA levels increased significantly as a
242 consequence of each treatment (including O₃ alone).

243 **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/O₃⁻ plants of *Q.*
246 *pubescens*, steep increments of Pro (+211%), glucose (+39%) and fructose (+18%) were found,
247 whereas the simultaneous presence of O₃ (WS/O₃⁺ plants) induced marked changes of osmolytes
248 accumulation. In particular, the huge increment of Pro (+626%, in comparison to WW/O₃⁻) paralleled
249 the lower accumulation of glucose and fructose, whose values were similar or lower to those of
250 WW/O₃⁻ plants. Levels of ABA were ~2-fold higher in drought-stressed leaves (average of WS/O₃⁻
251 and WS/O₃⁺) as compared to WW/O₃⁻ counterparts, and this parameter was also affected by O₃
252 singularly (+40%). In leaves of *Q. ilex* only a drought-dependent increment of glucose (+54% average
253 of WS/O₃⁻ and WS/O₃⁺) and ~2-fold higher levels of Pro in WS/O₃⁺ plants were found. Noteworthy,
254 accumulation of ABA was pronounced in WS/O₃⁺ plants (0.15 vs 0.07 μmol g⁻¹ DW in controls) but

255 not in WS/O₃⁻ plants. On the other side, the strongest accumulation of ABA was recorded in leaves
256 of WW/O₃⁺ saplings (0.26 μmol g⁻¹ DW).

257 **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 CO₂ 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 CO₂ 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 CO₂ m⁻² s⁻¹ in both the species; *t*-test, *p*>0.05) and drought × O₃ (2.6 vs 2.9 μmol CO₂ 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 CO₂
270 observed only in WS/O₃⁻ 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/O₃⁺ and WS/O₃⁻ 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 × O₃ 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/O₃⁻ plants (~ 48.9 vs
276 63.7 μmol CO₂/mol H₂O on average on daily bases, respectively; *t*-test; *P*≤0.05) and for both an
277 increment of this ratio in WS/O₃⁻ 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 × O₃ showed increased values of WUE (80.6 μmol CO₂/mol
280 H₂O 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/O₃⁻
283 and WS/O₃⁺ saplings (~0.71 on average) and occurred only at midday. Differently, in WS/O₃⁻ and
284 WS/O₃⁺ 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/O₃⁺ plants at 14:00
287 were close to those found for *Q. pubescens* at midday (*t*-test, *P*>0.05), while a more severe

288 photoinhibition was found in WS/O₃- plants (0.59). A consistent and constant reduction of Φ_{PSII} was
289 found in drought-stressed plants of both the species during the whole day (Fig. 2b,e). The amplitude
290 of Φ_{PSII} decline was reasonably comparable for WS/O₃- and WS/O₃+ plants of both species (*Q.*
291 *pubescens*: 0.56 vs 0.36, WW/O₃- vs WS/O₃- and WS/O₃+ average; *Q. ilex*: 0.53 vs 0.29). No negative
292 effects were induced by O₃ alone, nor to the maximum or effective efficiency of PSII. The decline of
293 Φ_{PSII} observed in drought-stressed plants paralleled with the enhancement of qNP, whose increment
294 started progressively after 8:00 h with the same amplitude in WS/O₃- and WS/O₃+ plants of both
295 species. A slight enhancement of qNP was also promoted by O₃ independently of the species.

296 **Chlorophyll and carotenoid content**

297 In *Q. pubescens* leaves, drought treatment negatively affected Chl_{TOT}, β -carotene and depoxidation
298 state of xanthophylls (DEPS) with the same amplitude if applied singularly or in combination with
299 O₃ (-10.1, -8.3 and +8.0% on average between WS/O₃- and WS/O₃+ plants, respectively) (Tab. 3).
300 Zeaxanthin (Zea) increased under drought (independently of the presence of O₃), whereas lutein (Lut)
301 increased under drought alone (+25.0%) and even more if also O₃ was concomitantly applied
302 (+65.2%). Values of violaxanthin+antheraxanthin+zeaxanthin (VAZ) ratio were not statistically
303 affected by any treatment. The O₃ treatment induced only a slight reduction of DEPS (-4.3%).

304 Differently, Chl_{TOT} and β -carotene decreased in WS/O₃- *Q. ilex* plants (-7.5 and -13.5%,
305 respectively) and even more in WS/O₃+ ones (-28.2 and -34.1%), and VAZ showed alterations
306 especially in plants exposed only to drought (-56.8 and -11.6% in WS/O₃- and WS/O₃+ plants,
307 respectively). Lut increased slightly, but significantly, in drought-stressed plants (+6.9% as the
308 average of WS/O₃- and WS/O₃+ vs that of WW/O₃- and WW/O₃+). Fairly higher values of Zea were
309 found in drought stressed individuals (as compared to controls), but remarkably the highest values
310 were found in WS/O₃- plants (+118.6%), while only a +36.2% was found in WS/O₃+ plants. DEPS
311 showed in *Q. ilex* similar responses to those found in *Q. pubescens*, increasing similarly in both
312 WS/O₃- and WS/O₃+ saplings (+28.8%, on average) and decreasing in WW/O₃+ ones (-25.6%). In
313 *Q. ilex* leaves, O₃ also induced a decrease of VAZ ratio (-22.1%).

314

315 **Discussion**

316 A further massive inflow of people to the urban areas is expected in a near future because of the
317 attractiveness of city's lifestyle. But are we sure that also trees will enjoy living in our cities under
318 the predicted environmental conditions, especially in the Mediterranean basin where some
319 environmental cues, such as drought and O₃, already seriously mine plant survival and will be more

320 exacerbated by GC? Here, we discuss the water relations, physiological and biochemical mechanisms
321 adopted by the sympatric Mediterranean tree species *Q. pubescens* and *Q. ilex* in the attempt to
322 counteract mild-severe drought and/or O₃ exposure, trying also to take a step forward in solving the
323 long-standing question of Bohler et al. (2015): “Interactive effects between drought and O₃: sorrow
324 or joy?”. Data reported in the present study offer clear evidences of the higher ability of *Q. ilex*, an
325 evergreen, diffuse-porous wood species with an isohydric behavior, to tolerate the concomitance of
326 O₃ and mild-severe drought as compared to *Q. pubescens*, a broadleaved, ring-porous wood species
327 with an anisohydric behavior.

328 Firstly, the two species responded differently to water withholding, which resulted the most
329 impacting factor in this experiment. Whereas in *Q. pubescens* the effect of drought significantly
330 depressed the values of leaf RWC, in *Q. ilex* seedlings foliar water content remained unchanged
331 independently of the treatment, thus suggesting a wiser ability to preserve their leaves from
332 dehydration. According to data showed in Table 1, *Q. ilex* can be classified as an isohydric species
333 (minimum changes of PDΨ_w in relation to soil moisture), whilst *Q. pubescens* showed an anisohydric
334 behavior (strong decline of PDΨ_w in relation to soil moisture) on the bases of the distinction proposed
335 by Tardieu and Simonneau (1998). For the sack of truth, anisohydric species should also exhibit
336 higher values of g_s when compared to isohydric ones (Aguadé et al. 2015), whereas in our experiment
337 daily values of g_s were similar on daily bases in both the species under drought (alone or combined
338 with O₃). Moreover, the g_s depression in *Q. pubescens* WS/O₃⁻ and WS/O₃⁺ plants was even higher
339 than that of *Q. ilex* ones when compared to their relative controls (WW/O₃⁻ plants). However, it has
340 been already observed that this dichotomy (isohydric/anisohydric) seems an oversimplified view of
341 stomatal behavior with increasing water demand and that in many cases anisohydric species can
342 switch to an isohydric behavior under highly negative soil water potential (Zhang et al. 2012), and as
343 a function of stressful environmental conditions (Aguadé et al., 2015) which can play a pivotal role
344 in the urban environment.

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

354 plants) and can sustain longer periods of mild dry conditions than isohydric species do (McDowell
355 2011). Conversely, under severe drought stress conditions this strategy may cause anisohydric species
356 to be more prone to xylem dysfunction and thus to hydraulic failure (Hoffmann et al. 2011, McDowell
357 2011). There are some evidences concerning the reduction of g_s in concomitance to an increase in
358 cavitation events in anisohydric grape genotypes under severe drought (Zufferey et al. 2011), thus
359 giving a putative explanation for the strong (unexpected) reduction of g_s we found in *Q. pubescens*.
360 The failure of the anisohydric strategy under drought as imposed in this experiment is confirmed by
361 the significant reduction of RWC observed in *Q. pubescens* (but not in *Q. ilex*) under these conditions.

362 The maintenance of an appropriate leaf water status during water deficit is essential for plant
363 growth and, beyond stomatal regulation, the orchestrated responses against the reduction of leaf water
364 status include the accumulation of compatible solute (Sofa et al. 2004, Ashraf and Foolad 2007). The
365 strong accumulation of osmolytes found in WS/O₃⁻ (proline and hexoses) and in WS/O₃⁺ (proline)
366 *Q. pubescens* leaves, reducing in turn Ψ_π and consequently PD Ψ_w , can be considered as an attempt
367 to contrast cell water loss and the inevitable leaf dehydration. Furthermore, also the raise of ABA
368 observed in drought-stressed *Q. pubescens* individuals might have been crucial in drought response,
369 as this ubiquitous hormone and signaling molecule is known to induce accumulation of osmotically
370 active compounds (Jarzyniak and Jasiński 2014). It is worth to be noted how compatible solute
371 profiles in *Q. pubescens* varied between WS/O₃⁻ and WS/O₃⁺ plants (at least those contemplated and
372 how the combined treatments induced a huge (~ +600%) accumulation of proline but not of glucose
373 and fructose (as in WS/O₃⁻ plants). We do not have enough data to postulate a clear hypothesis about
374 that, but this shift of osmolytes accumulation might be a direct effect of O₃ in view of the well-known
375 ROS-triggered effect of this pollutant, and the additional role as antioxidant (and/or antioxidant
376 defenses activator) demonstrated in many cases by proline under osmotic stress (Szabados and
377 Savouré, 2010). This hypothesis would also be strengthened by the marked proline accumulation found
378 only in WS/O₃⁺ leaves of holm oak, the species for which foliar osmolyte accumulation was less
379 relevant (Ψ_π did not change and only glucose accumulated slightly in drought-stressed plants
380 regardless the presence or absence of O₃). It is also worthy to note that in both species the effect of
381 O₃ (alone or in combination) did not have any negative significant consequence in terms of water
382 status and compatible solute accumulation. This is likely attributable to a moderate (but significant)
383 effect of O₃ on g_s when compared to the strong depression induced by water stress *per se*.

384 Secondly, in view of the inherent higher photosynthetic rate of *Q. pubescens* control plants, that
385 the values of A were comparable in WS/O₃⁻ and WS/O₃⁺ plants independently of the species is an
386 evident probe of how the effect of drought translated into a smaller depression of A and g_s in *Q. ilex*
387 than in *Q. pubescens*. This superior ability was observed both on daily bases and at midday, when

388 high irradiance can strongly exceed the photosystem requirement of stressed leaves. In view of the
389 conservative use strategy usually adopted by evergreen species which are characterized by longer leaf
390 lifespan, a key prerogative in resource-limiting environments (Reich et al. 1992; Valladares et al.
391 2000), it is not surprising that *Q. pubescens* plants exhibited a higher decline of photosynthetic rate
392 in such harsh limiting conditions; the lower ability to sustain the carboxylation process of WS/O₃-
393 plants of *Q. pubescens* is also corroborated by the steep intercellular accumulation of CO₂ observed
394 at midday. Such higher values of C_i were not found in WS/O₃- plants of *Q. ilex*. Conversely, what is
395 surprising is the decline of A found in *Q. ilex* (but not in *Q. pubescens*) under O₃ in daily central hours
396 and the lower reduction of carbon gain on daily bases (-27.5% of A in *Q. ilex* vs -12% in *Q.*
397 *pubescens*).

398 The ability of plants to modulate A and g_s (A/g_s, namely WUE), and in particular to increase
399 WUE in response to moderate-to-severe drought events, is a typical multifactorial-driven trait of
400 Mediterranean species, especially of shrubs and trees (Valladares et al. 2005; Medrano et al. 2009).
401 In this study, pooling together data of A and g_s collected once a week, independently of the treatments
402 received by plants, resulted in highly significant linear correlations for both the species (Fig. S1).
403 This confirms the interdependence of those parameters, as already reported for *Quercus* species, even
404 under stress (Ogaya and Peñuelas 2003; Quero et al. 2006; Medrano et al. 2009). In accordance with
405 the meta-analyses performed by Medrano et al. (2009), we found higher values of WUE in well-
406 watered plants of the evergreen species (*Q. ilex*) when compared to the deciduous counterpart (*Q.*
407 *pubescens*), incremented values of WUE in both species when subjected to water stress alone, and a
408 fairly higher buildup of this ratio in the deciduous species. Notably, an increment of WUE was found
409 only in *Q. pubescens* WS/O₃+ plants. Increments of WUE may be achieved through the controlled
410 regulation of water losses and/or by presenting photosynthetic machinery more protected under water
411 stress conditions. On one hand, the strong accumulation of ABA found in WS/O₃+ plants of both the
412 species might have driven the considerable stomatal closure aim to control leaf water loss; on the
413 other side, it is presumable that in both the species the photosynthetic efficiency may be augmented
414 *via* different mechanisms, such as an increment of transcript coding for Rubisco, reduction of stomata
415 to chloroplast CO₂ diffusion, and/or higher carboxylation efficiency per unit of Rubisco (Chaves et
416 al. 2009). Increment of CO₂ assimilation in relation to g_s (leading to higher WUE) was likely to be
417 more necessary for *Q. pubescens* to increase net leaf carbon gain in view of the dramatic stomatal
418 closure found in this species under drought (in both WS/O₃- and WS/O₃+ plants) which was severely
419 greater than that found in WS/O₃- *Q. ilex* saplings.

420 Thirdly, in addition to stomatal limitations, chlorophyll fluorescence kinetics highlight that
421 WS/O₃- and WS/O₃+ *Q. ilex* plants responded more promptly than *Q. pubescens* ones in terms of

422 dynamic photoinhibition (stronger reduction of F_v/F_m in daily central hours) with a complete recovery
423 in late evening. Dynamic photoinhibition of PSII was associated with high non-radiative energy
424 dissipation, q_{NP} , and the drought-promoted enhancement of q_{NP} found in both the species, paralleled
425 the increase of DEPS, suggesting a higher contribution of the quenching component associated with
426 the xanthophyll cycle activity (namely, q_E). Xanthophylls are associated with the pigment-protein
427 complexes of photosynthetic membranes, where they play a photoprotective and light-harvesting
428 function (Bassi et al. 1993). According to Jahns and Holzwarth (2012), two different xanthophyll
429 cycles have been described for land plants: the violaxanthin cycle (violaxanthin is reversibly
430 converted to zeaxanthin and antheraxanthin) and the lutein epoxide cycle (lutein epoxide is reversibly
431 converted into lutein). Even though no reports have studied specifically the lutein epoxide cycle in
432 *Q. pubescens*, García-Plazaola et al. (2002) described as this cycle was helpful to support the VAZ
433 cycle under photoinhibitory conditions in other eight *Quercus* species. Thereby, it seems presumable
434 that the marked increase of lutein found in WS/O₃⁻ and even more in WS/O₃⁺ *Q. pubescens* can be
435 the result of a deactivation of excited triplet Chl operated by lutein epoxide (García-Plazaola et al.
436 2007). By contrast, drought-stressed saplings of *Q. ilex* showed a significant rise of zeaxanthin levels
437 and only a slight increment of lutein, which highlights the scarce necessity to activate the lutein
438 epoxide cycle in support the VAZ cycle (García-Plazaola et al. 2007). It is interesting to note that *Q.*
439 *pubescens* leaves had about 2-fold higher ratio of VAZ as compared to those of *Q. ilex*, and this might
440 explain why in *Q. ilex* we observed a steep increment of this ratio while no increment was found in
441 *Q. pubescens* leaves. The severe reduction of β -carotene found in both the species under drought (in
442 *Q. ilex* it was even more marked in WS/O₃⁺ plants than in WS/O₃⁻ ones) suggests that this compound
443 represents both the substrate for a *de novo* biosynthesis of xanthophylls and/or acts directly as a
444 thylakoid and chloroplastic membrane-bound antioxidant able to prevent the formation of singlet
445 oxygen by intercepting the chlorophyll triplet states and by scavenging any additional singlet oxygen
446 present (Havaux et al. 2000). The increment of DEPS paralleled with the reduction of β -carotene is
447 in agreement with previous researches conducted in plants suffering for water withholding, in
448 particular at midday when the need of photo-protection is higher (Munné-Bosch et al. 2001).

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

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

461 Finally, the following observations: (i) *Q. ilex* showed less reduction of total leaf biomass as
462 compared to *Q. pubescens* when subjected to drought and drought \times O₃ (on average -59 vs -70%,
463 respectively for both treatments; *data not shown*); (ii) the incremented levels of MDA were found
464 only in *Q. pubescens* leaves; (iii) O₃ impacted *Q. pubescens* leaf biomass (*data not shown*) and MDA
465 and not those of *Q. ilex*; this can be considered the best probes of the higher ability of holm oak to
466 counteract better than downy oak the effect of severe drought and O₃, at least with the severity of
467 factors applied in this experiment.

468 Beyond the superior ability of *Q. ilex* to counteract the effect of drought and drought \times O₃
469 simulating prediction conditions in Mediterranean environments, it is also worthy to note that in
470 WS/O₃+ plants of both the species an antagonistic effect in A and g_s reduction was found, suggesting
471 that drought stress can partially protect the leaves from O₃-triggered effects on gas exchanges once
472 the two stressors occur simultaneously, as usually happens during Mediterranean summer. This
473 outcome is in agreement with previous findings in oak species, including *Q. ilex* (Watanabe et al.
474 2005; Alonso et al. 2014). The effect was more consistent under severe than in moderate water
475 withholding and the authors attributed this ameliorative effect to the lower O₃ uptake flux of drought-
476 stressed plants. In other cases, the ameliorative effect can be attributable to the enhancement of the
477 antioxidant apparatus induced by O₃ (Watanabe et al. 2005). That drought protects from O₃ insult is
478 however a highly debated matter and there are other reports worthy to be mentioned in which drought
479 and O₃ acted synergically (Ribas et al. 2005a,b; Tausz et al. 2007; Pollastrini et al. 2014). The
480 explanations that have been proposed for the exacerbating effect of O₃ on drought-stressed plants
481 include: (i) massive production of reactive oxygen species with a consequent oxidative stress (Tausz
482 et al. 2007), and (ii) loss of stomatal control induced by O₃ (stomata close slower, or even remain
483 open, under drought \times O₃) (Bussotti 2008). Our dataset confirms that the antagonistic response seems
484 preponderant under severe water shortage; it is indeed remarkably how we did not find the same
485 ameliorative effect in a previous experiment where we imposed only a moderate water shortage, but
486 for a longer time (11 weeks), to the same tree species (Cotrozzi et al. 2016).

487

488 **Conclusive remarks**

489 Our dataset confirms the higher ability of evergreen species to counteract the effect of drought and
490 O₃ when compared to deciduous species in the Mediterranean environment, even under the increasing
491 harshness of those factors as predicted in a near future. From an ecological point of view, this ability
492 seems to be correlated with the stronger need of evergreens (which inhabits usually limiting
493 environment) for protecting their long-lived leaves from different environmental cues. This
494 peculiarity seems less relevant for highly-demanding, fast-growing deciduous species characterized
495 by shorter leaf lifespan, which have superior fitness than evergreens in non-limiting environment. As
496 a practical outcome, this work offers evidences that the recruitment of green urban “citizens” for
497 greening the city of future (a highly-limiting environment) should be wisely driven by a deep
498 knowledge of tree species behavior under the predicted conditions of GC, and in particular their
499 simultaneity.

500

501 **Conflict of interest**

502 None declared.

503

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701 and repair in petioles of grapevine (*Vitis vinifera* cv. Chasselas). *J Exp Bot* 62:3885-3894

702 Table 1. Predawn leaf water potential ($PD\Psi_w$), leaf osmotic potential (Ψ_π), relative water content
703 (RWC), and malondialdehyde (MDA)-by products estimated in *Quercus pubescens* and *Quercus ilex*
704 plants (i) regularly irrigated to maximum soil water holding capacity and exposed to charcoal filtered
705 air (WW/O₃-); (ii) water stressed and exposed to charcoal filtered air (WS/O₃-); (iii) regularly
706 irrigated and O₃ fumigated (WW/O₃+); and (iv) water stressed and O₃ fumigated (WS/O₃+ for 28
707 consecutive days. WS/O₃- and WS/O₃+ plants daily received 20% of effective evapotranspiration.
708 WW/O₃+ and WS/O₃+ plants were exposed to 80±13 ppb of O₃ for 5 h d⁻¹, in form of a square wave
709 between 10:00 and 15:00. Data are shown as mean ± standard deviation (n = 3). Following two-way
710 ANOVA, for each parameter (columns) different letters indicate significant differences: *** $P \leq$
711 0.001, ** $P \leq 0.01$, * $P \leq 0.05$, ns $P > 0.05$.

<i>Quercus pubescens</i>	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
<i>Drought</i>	78.63***	38.12***	16.75**	15.78**
<i>Ozone</i>	0.08ns	0.32ns	4.57ns	0.44ns
<i>Drought × Ozone</i>	2.63ns	4.46ns	0.14ns	5.69*
<i>Quercus ilex</i>	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
<i>Drought</i>	22.32**	18.75**	1.55ns	38.74***
<i>Ozone</i>	0.04ns	10.58*	0.30ns	11.54**
<i>Drought × Ozone</i>	1.75ns	7.00*	0.00ns	106.12***

712

713

714 Table 2. Proline (Pro), abscisic acid (ABA), glucose and fructose ($\mu\text{mol g}^{-1}$ 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/O₃-); (ii) water stressed and exposed to charcoal filtered air
717 (WS/O₃-); (iii) regularly irrigated and O₃ fumigated (WW/O₃+); and (iv) water stressed and O₃
718 fumigated (WS/O₃+ for 28 consecutive days. WS/O₃- and WS/O₃+ plants daily received 20% of
719 effective evapotranspiration. WW/O₃+ and WS/O₃+ plants were exposed to 80 ± 13 ppb of O₃ 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: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$, ns $P > 0.05$.

<i>Quercus pubescens</i>	Pro	ABA	Glucose	Fructose
WW/O ₃ -	0.54 \pm 0.115 a	0.05 \pm 0.004	219.6 \pm 1.46 a	742.7 \pm 11.11 b
WS/O ₃ -	1.68 \pm 0.181 b	0.10 \pm 0.006	305.2 \pm 15.21 b	873.7 \pm 44.18 c
WW/O ₃ +	0.74 \pm 0.032 a	0.07 \pm 0.002	203.1 \pm 4.06 a	646.2 \pm 7.53 a
WS/O ₃ +	3.92 \pm 0.010 c	0.12 \pm 0.015	203.4 \pm 9.45 a	669.7 \pm 32.48 a
<i>Drought</i>	1192.09***	106.07***	65.43***	22.46**
<i>Ozone</i>	376.50***	15.19*	123.75***	84.98***
<i>Drought</i> \times <i>Ozone</i>	262.86***	0.12ns	64.35***	10.86*
<i>Quercus ilex</i>	Pro	ABA	Glucose	Fructose
WW/O ₃ -	1.42 \pm 0.146 c	0.07 \pm 0.006 a	108.9 \pm 1.73	425.1 \pm 9.07
WS/O ₃ -	0.60 \pm 0.067 a	0.07 \pm 0.002 a	168.3 \pm 5.41	421.7 \pm 4.6
WW/O ₃ +	0.95 \pm 0.065 b	0.26 \pm 0.008 c	101.2 \pm 2.98	457.1 \pm 15.58
WS/O ₃ +	2.37 \pm 0.156 d	0.15 \pm 0.002 b	166.7 \pm 6.95	443.6 \pm 8.29
<i>Drought</i>	20.38**	302.00***	522.93***	2.05ns
<i>Ozone</i>	92.38***	2108.62***	2.98ns	20.91**
<i>Drought</i> \times <i>Ozone</i>	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/O₃-); (ii) water stressed and exposed to charcoal filtered air (WS/O₃-); (iii) regularly irrigated and O₃ fumigated (WW/O₃+); (iv) water stressed and O₃ fumigated (WS/O₃+ for 28 consecutive days. WS/O₃- and WS/O₃+ plants daily received 20% of effective evapotranspiration. WW/O₃+ and WS/O₃+ plants were exposed to 80±13 ppb of O₃ 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 expressed on a Chl_{TOT} bases (mmol mol⁻¹ Chl_{TOT})

<i>Quercus pubescens</i>	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
<i>Drought</i>	8.51*	15.96**	881.92***	25.86***	65.17***	1.94ns
<i>Ozone</i>	1.06ns	0.00ns	131.84***	3.86ns	0.80ns	1.75ns
<i>Drought</i> × <i>Ozone</i>	0.72ns	0.67ns	199.83***	0.09ns	7.18*	1.11ns
<i>Quercus ilex</i>	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
<i>Drought</i>	83.90***	148.47***	31.17***	525.54***	246.20***	346.85***
<i>Ozone</i>	56.90***	28.52***	0.03ns	226.11***	79.69***	160.13***
<i>Drought</i> × <i>Ozone</i>	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/O₃⁻, open circle); (ii) water stressed and exposed to charcoal filtered air (WS/O₃⁻, open square); (iii) regularly irrigated and O₃ fumigated (WW/O₃⁺, closed circle); (iv) water stressed and O₃ fumigated (WS/O₃⁺, closed square) for 28 consecutive days. WS/O₃⁻ and WS/O₃⁺ plants daily received 20% of effective evapotranspiration. WW/O₃⁺ and WS/O₃⁺ plants were exposed to 80±13 ppb of O₃ 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/O₃⁻, open circle); (ii) water stressed and exposed to charcoal filtered air (WS/O₃⁻, open square); (iii) regularly irrigated and O₃ fumigated (WW/O₃⁺, closed circle); (iv) water stressed and O₃ fumigated (WS/O₃⁺, closed square) for 28 consecutive days. WS/O₃⁻ and WS/O₃⁺ plants daily received 20% of effective evapotranspiration. WW/O₃⁺ and WS/O₃⁺ plants were exposed to 80±13 ppb of O₃ 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

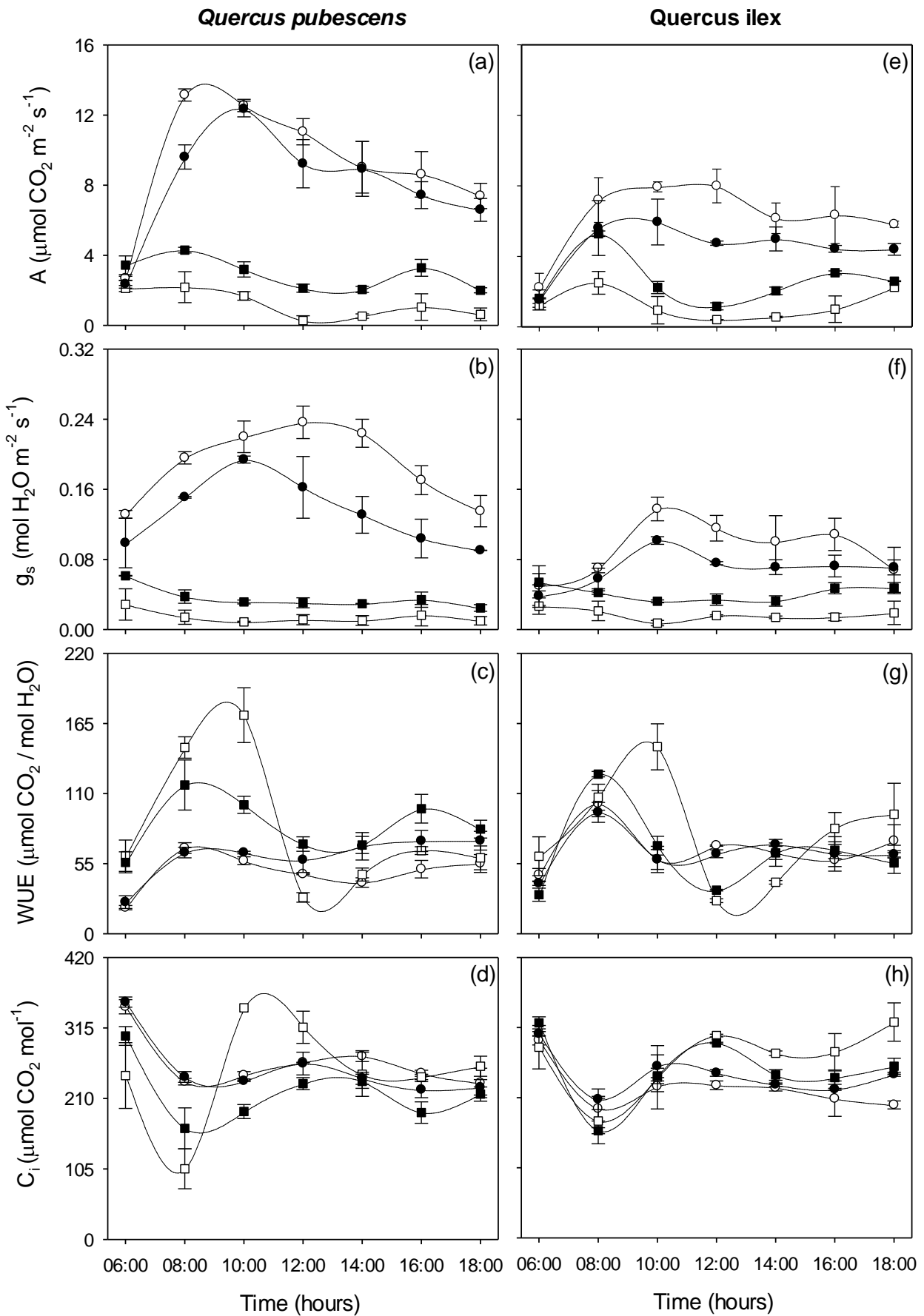


Figure 2

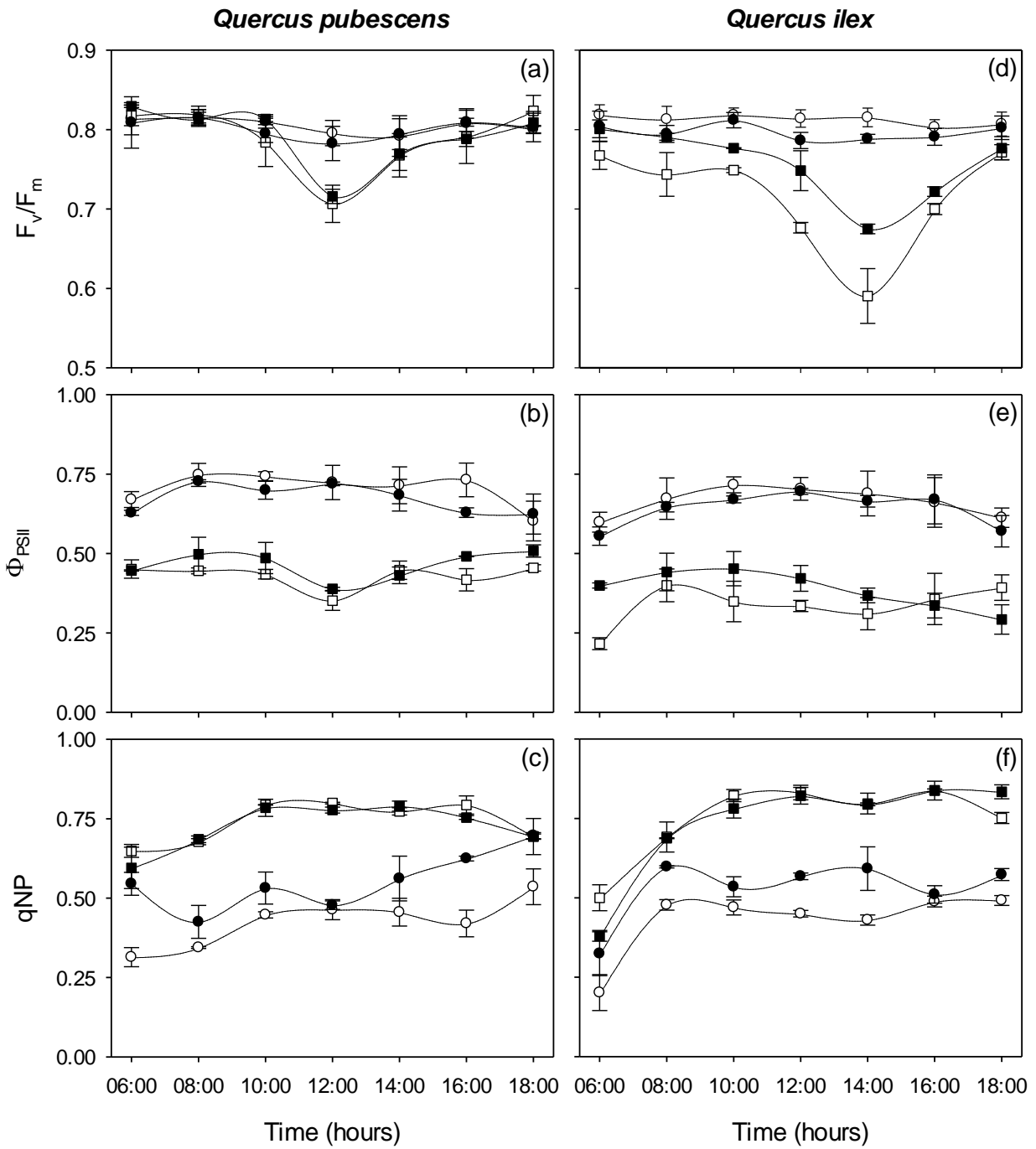


Table S1. *F* values of two-way repeated measures ANOVA of the effects of drought (daily irrigation with 20% of effective evapotranspiration) and ozone (80 ppb for 28 consecutive days, 5 h day⁻¹, in form of a square wave between 10:00 and 15:00 in time (06:00, 08:00, 10:00, 12:00, 14:00, 16:00 and 18:00 hours) on CO₂ assimilation rate (A), stomatal conductance to water vapor (g_s), water use efficiency (WUE), intercellular CO₂ 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* ≤ 0.001, ** *P* ≤ 0.01, * *P* ≤ 0.05, ns *P* > 0.05. d.f. represents the degrees of freedom.

Effects	d.f.	<i>Quercus pubescens</i>							<i>Quercus ilex</i>						
		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
<i>Drought</i>	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 ***
<i>Ozone</i>	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 ***
<i>Time</i>	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 ***
<i>Drought × Ozone</i>	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 ***
<i>Drought × Time</i>	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 ***
<i>Ozone × Time</i>	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 **
<i>Drought × Ozone × Time</i>	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. CO₂ 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/O₃⁻, open circle); (ii) water stressed and exposed to charcoal filtered air (WS/O₃⁻, close triangle); (iii) regularly irrigated and O₃ fumigated (WW/O₃⁺, open triangle); (iv) water stressed and O₃ fumigated (WS/O₃⁺, closed circle) for 28 consecutive days. WS/O₃⁻ and WS/O₃⁺ plants daily received 20% of effective evapotranspiration. WW/O₃⁺ and WS/O₃⁺ plants were exposed to 80±13 ppb of O₃ 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 (R²) are shown.

