Antioxidative responses of three oak species under ozone and water stress conditions

Elisa Pellegrini[1], Yasutomo Hoshika[2], Nicolas Dusart[3], Lorenzo Cotrozzi[1], Joëlle Gérard[3], Cristina Nali[1*], Marie-Noëlle Vaultier[3], Yves Jolivet[3], Giacomo Lorenzini[1], Elena Paoletti[2]

[(1)] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, Pisa, 56124, Italy.

[(2)] Institute for Sustainable Plant Protection, National Research Council, Via Madonna del Piano 10, Sesto Fiorentino, Florence, 50019, Italy.

[(3)] Université de Lorraine, AgroParisTech, INRA, UMR Silva, 54000 Nancy, France.

[1] E-mail address: cristina.nali@unipi.it (C. Nali)

[*] Corresponding author. Tel.: +39 0502210552.

24 **ABSTRACT**

25 Plants are frequently exposed to adverse environmental conditions such as drought and ozone (O_3).
26 Under these conditions, plants can survive due to their ability to adjust their metabolism. The aim of
27 the present study was to compare the detoxification mechanisms of three oak species showing
28 different O_3 sensitivity and water use strategy. Two-year-old seedlings of *Quercus ilex*, *Q.*
29 *pubescens* and *Q. robur* were grown under the combination of three levels of O_3 (1.0, 1.2 and 1.4
30 times the ambient O_3 concentration) and three levels of water availability (on average 100, 80 and
31 42% of field capacity in well-watered, moderate drought and severe drought, respectively) in an O_3
32 Free Air Controlled Exposure facility. Ozone and drought induced the accumulation of reactive
33 oxygen species (ROS) and this phenomenon was species-specific. Sometimes, ROS accumulation
34 was not associated with membrane injury suggesting that several antioxidative defense mechanisms
35 inhibited or alleviated the oxidative damage. Both O_3 and drought increased total carotenoids that
36 were able to prevent the peroxidation action by free radicals in *Q. ilex*, as confirmed by unchanged
37 malondialdehyde by-product values. The concomitant decrease of total flavonoids may be related to
38 the consumption of these compounds by the cell to inhibit the accumulation of hydrogen peroxide.
39 Unchanged total phenols confirmed that *Q. ilex* has a superior ability to counteract oxidative
40 conditions. Similar responses were found in *Q. pubescens*, although the negative impact of both
41 factors was less efficiently faced than in the sympatric *Q. ilex*. In *Q. robur*, high O_3 concentrations
42 and severe drought induced a partial rearrangement of the phenylpropanoid pathways. These
43 antioxidative mechanisms were not able to protect the cell structure (as confirmed by ROS
44 accumulation) suggesting that *Q. robur* showed a lower degree of tolerance than the other two
45 species.

46 **Keywords:** climate change, oxidative damage, reactive oxygen species, detoxification,
47 phenylpropanoids, Halliwell-Asada cycle.

48 **1. Introduction**

49 Evidence for changing climate, associated with higher atmospheric concentrations of greenhouse
50 gases, continues to increase. The years 2014 and 2015 are currently considered the warmest years in
51 Europe since instrumental records began, *i.e.* more than 1.1 °C warmer than the pre-industrial level
52 (EEA, 2017). For these years, the exceptional heat covered the whole summertime with mean
53 precipitation (June to August) significantly decreased by up to 20 mm per decade. The series of
54 summer heatwaves affecting Europe since 2003 has also contributed to several intense tropospheric
55 ozone (O_3) episodes. In 2015, 18 of the 28 states of the European Union (EU) and four other
56 European countries outside the EU registered concentrations above the EU O_3 target value for the
57 protection of human health (EEA, 2017).

58 Due to favorable meteorological conditions, both drought and O_3 are very likely to occur
59 simultaneously. As a general rule, seasonal drought is typically associated with high insolation, and
60 such conditions are conducive to the photo-oxidative formation of high O_3 levels (Butkovic et al.,
61 1990). In addition, a rise in temperature significantly increases the emission rates of most biogenic
62 volatile organic compounds that can contribute to O_3 production (Avery, 2006). Under drought,
63 plants usually suffer from the impairment of many physiological and biochemical processes, such as
64 (i) alteration of photosynthetic performance, (ii) cell dehydration, (iii) high production of reaction
65 oxygen species (ROS) and, finally, (iv) early senescence and/or leaf necrosis (Chaves et al., 2003).
66 Similar effects have also been attributed to O_3 (Cotrozzi et al., 2017a; Jolivet et al., 2016). A
67 combination of drought and O_3 can induce responses considerably different from those observed
68 when each stressor is applied independently (Bohler et al., 2015). Interestingly, the effects of
69 drought and O_3 can be antagonistic, so that a simultaneous occurrence may be partially beneficial to
70 plants. The most common combined response, in fact, is that drought mitigates the negative effects
71 of O_3, basically by closing stomata and thus O_3 uptake into the plant (Pollastrini et al., 2013; Gao et
72 al., 2017). However, other results suggest that drought can exacerbate O_3 damage: Alonso et al.
73 (2014) reported that the combination of both stressors caused further decreases in accumulated
74 aboveground biomass in two subspecies of *Quercus ilex*. It appears that the combination of drought

75 and O_3 is highly dependent on (i) the severity and length of occurrence of both stress factors and (ii)
76 the balance between stomatal O_3 uptake (*i.e.*, Phytotoxic O_3 Dose, POD) and detoxification capacity
77 of foliar cells (Dizengremel et al., 2013; Bohler et al., 2015).

78 Some studies have investigated the effects of combined drought and O_3 exposure on plant
79 metabolism, especially in trees (see also Pollastrini et al., 2013; Cotrozzi et al., 2016; Yuan et al.,
80 2016; Gao et al., 2017; Cotrozzi et al., 2017b). However, none of them investigated antioxidant
81 molecules and/or physiological mechanisms. Whereas O_3 itself induces production of ROS and
82 leads to a strong ROS accumulation, physiological responses to drought mostly use ROS as
83 internally produced signalling molecules (Reddy et al., 2004), and severe drought may lead to
84 photo-oxidative stress (Czarnocka and Karpińsky, 2018). Consequently, accumulation of ROS is
85 likely to be considerably higher during O_3 stress, and more closely located to chloroplasts under
86 drought stress. Recently, Cotrozzi et al. (2017b) documented that hydrogen peroxide (H_2O_2) and
87 superoxide anion (O_2^-) were directly involved in the O_3-oxidative burst induced by an intense
88 episode of O_3 exposure (200 ppb for 5 h) in three-year-old *Q. ilex* saplings. By contrast, H_2O_2
89 content did not change in plants subjected to drought (20% of the effective daily evapotranspiration,
90 for 15 days). Such differences in ROS extent dynamics in relation to the stress factor suggested a
91 complex network of events in signal transduction, involving other molecules (*e.g.*, salicylic and
92 jasmonic acid) and processes (*e.g.*, proline biosynthesis).

93 Oaks (belonging to the genus *Quercus*) are widely distributed trees within the Mediterranean
94 area and are able to cope with several environmental stressors due to the high plasticity of their
95 phenotypic and physiological traits (Cotrozzi et al., 2016). Holm oak (*Q. ilex*) is likely the most
96 widely studied Mediterranean evergreen tree species and has been defined as "drought avoidant"
97 (Bussotti et al., 2002) and "O_3-tolerant" (Cotrozzi et al., 2018a; Hoshika et al., 2018). Downy oak
98 (*Q. pubescens*) is a typical Mediterranean deciduous tree distributed in Southern Europe and has
99 been defined as "drought-" (Curtu et al., 2011) and "O_3-tolerant" (Cotrozzi et al., 2018a; Hoshika et
100 al., 2018). Pedunculate oak (*Q. robur*) is one of the basic species in deciduous broadleaved forests

101 of Europe and has been defined as "moderately drought-tolerant" (Vranckx et al., 2014) and "O_3-
102 sensitive" (Hoshika et al., 2018).

103 The aim of the present study was to assess the combined effects of drought and O_3 exposure
104 on the antioxidant metabolism of three oak species showing different water use strategies and O_3
105 sensitivities, exposed for one growing season to three levels of water availability and three levels of
106 O_3 in an O_3 Free Air Controlled Exposure (FACE) facility. Specifically, we asked the following
107 questions: (i) How much ROS are induced by realistic O_3 and water stress levels? (ii) Which
108 antioxidant mechanisms are activated in response to individual stresses and to the combination of
109 the stressors at different intensities? (iii) Are metabolic responses markedly species-specific? (iv)
110 Are antioxidative metabolism and stomatal uptake of O_3 correlated? We postulated a protective
111 effect of drought against O_3 and that the interactive effects of both factors may depend on plant
112 species. In particular, we hypothesized that the evergreen tree species (which usually inhabits
113 limiting environments) will have a greater tolerance to drought and O_3 exposure than the deciduous
114 ones (characterized by shorter leaf lifespan), due to its stronger need to protect its long-lived leaves
115 from different environmental cues. In a previous work, Cotrozzi et al. (2016) demonstrated that *Q.*
116 *ilex* was able to successfully cope with several stressors due to the high plasticity of morpho-
117 anatomical, physiological and biochemical traits.

118 **2. Materials and methods**

119 *2.1. Plant material and experimental design*

120 At the beginning of autumn 2014, two-year-old saplings of *Q. ilex*, *Q. pubescens* and *Q. robur* were
121 transferred from nearby nurseries to the O_3-FACE facility of Sesto Fiorentino, Florence, Italy
122 (43°48'59"N, 11°12'01"E, 55 m a.s.l.), where the experimental activities were conducted. The
123 plants were established into 10-L pots containing peat:sand:nursery soil (1:1:1 in volume) and
124 maintained under field conditions until the beginning of the treatment. Uniform-sized plants were
125 selected and grown under the combination of three levels of O_3 (1.0, 1.2 and 1.4 times the ambient

126 air concentration, denoted as AA, 1.2 × AA and 1.4 × AA, respectively) and three levels of water
127 irrigation [100, 80 and 42% of field capacity on average, denoted as WW (well watered), MD
128 (moderate drought) and SD (severe drought), respectively] from 1st June to 15th October 2015 (4.5
129 months). A detailed description of the O_3 exposure methodology is available in Paoletti et al.
130 (2017). The maximum hourly ozone concentrations were 93 ppb in AA, 111 ppb in 1.2 × AA and
131 123 ppb in 1.4 × AA, respectively, throughout the experimental period. AOT40 (Accumulated
132 exposure Over Threshold of 40 ppb) values during the experimental period were 17.8 ppm h, 29.7
133 ppm h and 40.3 ppm h in AA, 1.2 × AA and 1.4 × AA, respectively. Biomass results from this
134 experiment were used for assessing O_3 risk in a previous paper (Hoshika et al., 2018), where further
135 details on O_3 metrics are also available. The amount of irrigation was related to the soil field
136 capacity, *i.e.* the maximum volume of water that was retained into the soil of the pots [volumetric
137 soil water content was measured in the root layer by EC-5 soil moisture sensors equipped with an
138 EM5b data logger, (Decagon Devices, Pullman, WA, USA), Hoshika et al. (2018)]. Three
139 replicated plots (5 × 5 × 2 m) were assigned to each O_3 treatment, with three plants per each
140 combination of species, O_3 level and water irrigation. At the end of the experiment, the first mature
141 (fully expanded) top leaves of all three plants per plot (one leaf with 5th to 8th order per plant) in
142 each O_3 × irrigation treatment were gathered, divided into aliquots (obtained from each combination
143 of species, O_3 level and water irrigation per plot), immediately frozen in liquid nitrogen and stored
144 at -80 °C until biochemical analyses were done. Sampling was performed from 11:00 am to 1:00
145 pm.

146 *2.2. Oxidative damage and H_2O_2 content*

147 Oxidative damage to membranes was estimated in terms of lipid peroxidation by determining the
148 malondialdehyde (MDA) by-product accumulation, according to the method of Guidi et al. (2017).
149 Samples (40 mg fresh weight, FW) were extracted with 1 mL of 0.1% (w/v) trichloroacetic acid.
150 The determination was performed with a spectrophotometer (6505 UV-Vis, Jenway, UK) at 532
151 and 600 nm.

152 H_2O_2 content was measured fluorometrically using the Amplex Red Hydrogen
153 Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA), according to
154 Cotrozzi et al. (2017b). Samples (30 mg FW) were extracted with 800 µL of 20 mM potassium-
155 phosphate (K-P) buffer (pH 6.5). The determination was performed with a fluorescence/absorbance
156 microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA) at 530 and
157 590 nm (excitation and emission of resorufin fluorescence, respectively).

158 *2.3. Pigments*

159 Pigments were assessed according to Cotrozzi et al. (2017b), with minor modifications. Samples
160 (30 mg FW) were homogenized in 1 mL of 100% HPLC-grade methanol and incubated overnight at
161 4 °C in the dark. High Performance Liquid Chromatography (HPLC; P680 Pump, UVD170U UV-
162 VIS detector, Dionex, Sunnyvale, CA, USA) separation was performed at room temperature with a
163 reverse-phase Dionex column [Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter
164 (i.d.) 150 mm length]. A detailed description of analytical conditions is available in Cotrozzi et al.
165 (2017b).

166 *2.4. Metabolites involved in the Halliwell-Asada cycle*

167 Contents of ascorbate and glutathione were assessed according to Davey et al. (2003), with minor
168 modifications. Samples (100 mg FW) were extracted in 1 mL of chilled extraction buffer [6%
169 metaphosphoric acid in 65% HPO_3 (w/v), pH 1.5] containing 2 mM EDTA and 1%
170 polyvinylpolypyrrolidone (w/w). The supernatant was divided in order to determine the reduced
171 form of ascorbate and glutathione (*i.e.* AsA and GSH, respectively) and the total pool of each
172 component (total ascorbate and total glutathione). To determine total pools, extracts were mixed
173 with 200 mM dithiothreitol in 2 M Tris base to reach a pH between 6 and 6.8. HPLC separation was
174 performed on a Prominence Shimadzu system (LC-20AT pump, SPD-M20A diode array detector,
175 Shimadzu, Tokyo, Japan) at 25 °C with a reverse-phase column (Kinetex EVO C18, 2.6 µm
176 spherical particle size, 4.6 mm i.d., 100 mm length).

177 *2.5. Metabolites involved in the phenylpropanoid pathway*

178 Contents of phenylpropanoids were assessed according to Cotrozzi et al. (2018b), with some
179 modifications. Samples (30 mg FW) were homogenized in 500 µL of 80% HPLC-grade methanol
180 [in water (v/v)]. The supernatant was diluted five-fold with an aqueous solution of 0.2% formic
181 acid. UHPLC-ESI-MS/MS analyses were performed on an Agilent 1290 Infinity II LC system
182 coupled to a 6495 Triple Quadrupole mass spectrometer equipped with a Jet Stream electrospray
183 (ESI) ionization source (Agilent Technologies, Santa Clara, CA, USA). The separation was
184 achieved at 35 °C using a reverse-phase Agilent column (Zorbax Eclipse Plus, C18, 1.8 µm particle
185 size, 2.1 mm i.d., 50 mm length). A detailed description of analytical conditions is available in
186 Assumpção et al. (2018). In the present study, phenylpropanoid metabolites are grouped and
187 presented as total free phenolic acids (Tot Phen) and flavonoids (Tot flav) on the basis of their
188 chemical structure.

189 *2.6. Relationship of oxidative metabolism and stomatal ozone uptake (POD)*

190 The O_3 dose during the experiment was calculated as the phytotoxic ozone dose (POD_Y) above an
191 hourly stomatal uptake threshold of 0 nmol m^{-2} s^{-1} (POD_0) determined for each oak species in our
192 previous work (Hoshika et al. 2018), in order to assess relationships between the observed
193 parameters of oxidative metabolism and stomatal O_3 uptake. POD_0 was calculated by considering
194 species-specific stomatal responses to environmental stimuli according to the Manual on
195 Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution
196 Effects, Risks and Trends (CLRTAP, 2017). The details were described in Hoshika et al. (2018).

197 *2.7. Statistical analyses*

198 Statistical analyses were performed with Microsoft Office Excel 2010 (Microsoft, Redmond, WA,
199 USA) and JMP 11.0 (SAS Institute, Cary, NC, USA). The statistical unit was the single plot, *i.e.* all
200 leaves from the three plants per species in each plot were merged in a single sample per O_3 ×
201 irrigation replicate (N = 3 plots). Data for all biochemical parameters were tested with the Shapiro-

202 Wilk *W* test for normality and with the Levene test for homogeneity of variance. All data were
203 normally distributed and thus were analyzed by three-way ANOVA with O_3, drought and species as
204 fixed factors. Comparisons among means were determined by the Tukey HSD post-test. MDA data
205 were analyzed using one-way ANOVA followed by the Tukey HSD post-test. To determine
206 whether O_3 and drought exerted additive, synergistic or antagonistic impacts on the traits, the
207 observed (Obs) effects were compared with the expected (Exp) additive ones for each oak species
208 exposed to both stressors, according to Bansal et al. (2013). When the difference between Obs and
209 Exp was positive and the lower 95% confidence limit was greater than zero, the impact from the
210 combined stressors was classified as "synergistic". When the difference between Obs and Exp was
211 negative and the upper 95% confidence limit was less than zero, the impact from the combined
212 impact was classified as "antagonistic". When the 95% confidence interval crossed the zero line, the
213 impact was classified as "additive". The relationships between oxidative metabolism and POD_0 of
214 the three oak species were tested by a simple linear regression analysis, and species differences in
215 statistically significant regressions were evaluated by analysis of covariance (ANCOVA).

216 **3. Results**

217 *3.1. Oxidative damage and H_2O_2 content*

218 For MDA, the interaction among O_3, drought and species was not significant (Table 1, Fig. 1S). The
219 effects of single factors and their interactions in all binary combinations ($O_3 \times$ drought; $O_3 \times$ species
220 and drought × species) were significant. Ozone *per se* had a significant impact on MDA only in the
221 deciduous species. In *Q. pubescens*, O_3 markedly increased the values of MDA, without significant
222 differences between the two higher O_3 concentrations (one-way ANOVA with O_3 as factor:
223 $P \leq 0.001$). Only moderate O_3 concentrations induced an accumulation of MDA in *Q. robur* (one-
224 way ANOVA with O_3 as factor: $P \leq 0.05$). Drought *per se* had a significant impact on MDA only in
225 the deciduous species. In *Q. pubescens*, drought markedly increased MDA, without significant
226 differences between reduced water availability (one-way ANOVA with drought as factor: $P \leq 0.001$).
227 Only MD induced an accumulation of MDA in *Q. robur* (one-way ANOVA with drought as factor:

228 $P \leq 0.05$). *Q. ilex* displayed MDA values about 2-fold higher than the other species (one-way
229 ANOVA with plant species as factor: $P \leq 0.001$) and no effects of O_3 and drought were observed.

230 The effects of all combinations of O_3, drought and species were significant for H_2O_2 (Table
231 1, Fig. 1). O_3 *per se* increased the content of H_2O_2 in *Q. ilex* WW plants, without significant
232 differences between the two higher O_3 concentrations (Fig. 1). Drought *per se* markedly increased
233 H_2O_2 in *Q. ilex* under AA conditions, with significant differences between reduced water
234 availability (+127 and +74% under MD and SD conditions, compared to WW). *Q. pubescens*
235 exhibited a SD-induced accumulation of H_2O_2 (about 2-fold higher than WW). Similarly, *Q. robur*
236 exhibited a MD-induced accumulation of H_2O_2 (+89% compared to WW). Under AA-WW
237 conditions, *Q. ilex* displayed H_2O_2 values about 5-fold lower than the other species. Under
238 combined conditions (O_3 and drought), H_2O_2 contents remained unaltered in *Q. ilex* exposed to
239 increasing O_3 levels, independently of the watering regimes, except for $1.2 \times$ AA combined with
240 SD (+44% in comparison to AA-SD).

241 Ozone and drought in combination had synergistic effects on H_2O_2 in *Q. ilex*, except when
242 the higher O_3 concentrations and SD treatments were combined as in this case they acted
243 antagonistically (Fig. 2A). The two stressors generally had antagonistic effects on H_2O_2 content also
244 in deciduous species (Fig. 2A-C). Weak additive effects, however, were found in *Q. pubescens*
245 when moderate levels of O_3 were combined with SD, and in *Q. robur* exposed to the highest
246 intensity of both stressors (Fig. 2B-C).

247 *3.2. Total carotenoids*

248 The effects of all combinations of O_3, drought and species on total carotenoids (Tot Car) were
249 significant (Table 1, Fig. 3). O_3 *per se* induced a slight accumulation of Tot Car in *Q. ilex* WW
250 plants in comparison to AA ones, without significant differences between the higher O_3
251 concentrations. High O_3 concentrations decreased the content of Tot Car in *Q. pubescens* (-19% in
252 comparison to AA conditions) and even more in *Q. robur*, with significant differences between

253 increasing O_3 levels (-28 and -38% in 1.2 × AA and 1.4 × AA, respectively). Drought *per se* also
254 affected Tot Car in all species (except in *Q. ilex* under MD and in *Q. pubescens* under SD) in
255 comparison to WW conditions. An SD-induced accumulation of Tot Car content occurred in *Q. ilex*
256 (+45% compared to WW), while MD slightly decreased the levels of these metabolites in *Q.*
257 *pubescens* (-18% in comparison to WW). Drought *per se* decreased Tot Car of *Q. robur*, with
258 significant differences between drought regimes (-40 and -51% under MD and SD compared to
259 WW). Under combined conditions (O_3 and drought), Tot Car content was significantly affected in
260 all species exposed to the higher O_3 concentrations and subjected to reducing watering regimes in
261 comparison to AA (except for *Q. ilex* MD plants). In particular, O_3 induced a decrease of Tot Car in
262 *Q. ilex* and *Q. pubescens* SD plants, with significant differences between increasing O_3 levels (-23
263 and -82%, -26 and -12% in 1.2 × AA and 1.4 × AA, respectively). An opposite trend of Tot Car was
264 observed in *Q. pubescens* MD plants in response to increasing O_3 levels: -18 and +39% compared
265 to AA ones (in 1.2 × AA and 1.4 × AA, respectively). An O_3-induced accumulation of Tot Car was
266 also observed in *Q. robur* MD and SD plants, with significant differences between increasing O_3
267 levels (+27 and +81%, +157 and +55% in 1.2 × AA and 1.4 × AA, respectively).

268 Ozone and drought in combination generally had antagonistic effects on Tot Car in all
269 species (Fig. 2D-F). Additive effects were found in *Q. ilex* and in *Q. pubescens* when both stressors
270 were of severe intensity. Synergistic effects were found only in *Q. pubescens* when high O_3
271 concentrations and MD treatments were combined.

272 *3.3. Metabolites involved in the Halliwell-Asada cycle and in the phenylpropanoid pathway*

273 The three-way ANOVA test of total ascorbate (Tot AsA) and total glutathione (Tot Glu) content
274 revealed that the interaction among O_3, drought and species was not significant (Table 1, Fig. 2S
275 and 3S). The effects of single factors (except "drought" for Tot Glu) were significant. This was also
276 true for the binary interaction "drought × species" in the case of Tot AsA. O_3 *per se* had a
277 significant impact on Tot AsA in all the three species under AA conditions. Only moderate O_3
278 concentrations induced an accumulation of ToT AsA in *Q. ilex* (+21% in comparison to AA; one-

279 way ANOVA with O_3 as factor: $P \leq 0.01$). Drought *per se* had a significant impact on Tot AsA only
280 in *Q. robur*, with significant differences between reduced water availability (one-way ANOVA with
281 drought as factor: $P \leq 0.001$). *Q. pubescens* displayed Tot AsA values about 2-fold lower than the
282 other species (one-way ANOVA with plant species as factor: $P \leq 0.01$). Ozone *per se* had a slight
283 impact on Tot GSH only in *Q. pubescens* WW plants in comparison to AA ones: moderate O_3
284 concentrations significantly increased Tot GSH content (+76%). Under AA-WW conditions, *Q. ilex*
285 displayed Tot GSH values about 1.5-fold lower than the other species.

286 The three-way ANOVA test of Tot Phen and Tot Flav revealed that the interaction among
287 O_3, drought and species, the effects of each factor (except drought) and their binary interactions
288 were significant (Table 1). Ozone *per se* had a slight impact of Tot Phen only in the deciduous
289 species. Moderate O_3 concentrations negatively altered Tot Phen in *Q. pubescens* WW (-20%
290 compared to AA, Fig. 4). High O_3 concentrations significantly increased Tot Phen in *Q. robur* WW
291 plants (+61 compared to AA). Drought *per se* did not alter Tot Phen in all species (except SD in *Q.*
292 *robur*). Under AA-WW conditions, *Q. ilex* displayed Tot Phen values about 5-fold lower than the
293 other species, with *Q. robur* showing slightly higher levels than *Q. pubescens*. Under combined
294 conditions (O_3 and drought), Tot Phen content was significantly affected in all species exposed to
295 the higher O_3 concentrations and subjected to reducing watering regimes in comparison to AA. In
296 particular, *Q. ilex* exhibited an O_3-induced accumulation of Tot Phen under MD and SD conditions,
297 without significant differences between the higher O_3 concentrations. Ozone induced a decrease of
298 Tot Phen in *Q. pubescens* MD plants, without significant differences between the higher O_3
299 concentrations. An opposite trend of Tot Phen was observed in *Q. pubescens* SD and *Q. robur* MD
300 plants in response to increasing O_3 levels: -9 and +61%, +51 and -20% in 1.2 × AA and 1.4 × AA,
301 respectively. Increasing O_3 levels combined with SD induced a slight increase of Tot Phen in *Q.*
302 *robur*, without significant differences between the higher O_3 concentrations.

303 Ozone *per se* significantly affected the content of Tot Flav in all species; in particular,
304 decreased Tot Flav in *Q. ilex* and *Q. pubescens* WW, without significant differences between the

305 higher O_3 concentrations (Fig. 5). An O_3-induced accumulation of Tot Flav was observed in *Q. robur* WW, with significant differences between O_3 treatments (+43 and +16% in 1.2 × AA and 1.4 × AA, respectively). Drought *per se* significantly influenced Tot Flav in all species (except SD in *Q. pubescens*). Increasing severity of drought induced an evident decrease of Tot Flav in *Q. ilex* under AA, without significant differences between reducing water availability. An MD-induced reduction of Tot Flav was observed in *Q. pubescens* under AA conditions (-27% in comparison to WW). Drought *per se* induced a rise of Tot Flav in *Q. robur* under AA, without significant differences between O_3 levels. Under AA-WW conditions, *Q. pubescens* displayed lower Tot Flav values than the other species. Under combined conditions (O_3 and drought), Tot Flav were significantly affected in all species exposed to increasing O_3 concentrations, independently on the severity of drought (except in *Q. ilex* SD plants). *Q. ilex* MD plants exhibited an O_3-induced increase of Tot Flav, with significant differences between O_3 levels (+66 and +37% in 1.2 × AA and 1.4 × AA, respectively). Moderate O_3 concentrations markedly increased Tot Flav in *Q. pubescens* MD plants (+69% in comparison to AA ones). Increasing O_3 levels negatively affected Tot Flav in *Q. pubescens* SD plants, with significant differences between O_3 levels (-29 and -19% in 1.2 × AA and 1.4 × AA, respectively). An opposite trend of Tot Flav was observed in *Q. robur* MD plants exposed to increasing O_3 levels: +11 and -29% in 1.2 × AA and 1.4 × AA, respectively. High O_3 levels slightly altered Tot Flav in *Q. robur* SD plants (+16% compared to AA).

323 Ozone and drought in combination generally had antagonistic effects on Tot Phen content in all species (Fig. 2G-I). Additive effects were found in *Q. robur* when moderate and high O_3 concentrations were in combination with SD (Fig. 2I). By contrast, O_3 and drought in combination generally had synergistic effects on Tot Flav content in *Q. ilex* (Fig. 2J). Antagonistic effects were found in the deciduous species when moderate and high O_3 concentrations were in combination with MD and SD treatments in *Q. pubescens* and *Q. robur*, respectively (Fig. 2J-L). Weak additive effects were found for Tot Flav in *Q. robur*, when high O_3 concentrations and MD treatments were combined (Fig. 2L).

331 *3.4. Relationship of oxidative metabolism and stomatal ozone uptake (POD)*

332 Higher POD_0 was found in deciduous oaks (*Q. robur*: 10.9 to 20.4 mmol m^{-2}; *Q. pubescens*: 12.6 to
333 24.0 mmol m^{-2}) than evergreen oaks (*Q. ilex*: 8.8 to 15.4 mmol m^{-2}) (Fig. 6). Soil water deficit
334 limited POD_0 in all three oaks (25 to 30% limitation in SD treatments relative to WW treatments).
335 No significant relationships were found between POD_0 and most of the parameters of oxidative
336 metabolism (*i.e.*, H_2O_2, Tot Car, Tot Phen, Tot Flav, AsA, GSH) (*data not shown*). However, we
337 found significant positive relationships between POD_0 and MDA for *Q. pubescens* and *Q. robur*,
338 although such a relationship was not found in *Q. ilex* (Fig. 6). The intercepts of those relationships
339 differed between *Q. pubescens* and *Q. robur* ($P \leq 0.001$ by ANCOVA), while slopes were
340 comparable ($\sim 8 \times 10^{-3}$ g DW m^2).

341 **4. Discussion**

342 Plants are frequently exposed to adverse environmental conditions such as drought and O_3. Under
343 these conditions, plants can survive thanks to the ability to adjust their metabolism to cope with
344 these stressors (Noctor et al., 2018). Much progress has been made in understanding the dual role of
345 ROS in plant biology. ROS are required for many important signalling reactions, but are also toxic
346 by-products of aerobic metabolism. This dual role is mainly dependent on (i) their concentration,
347 (ii) site and duration of their action, (iii) occurrence of previous stress events and (iv) concurrence
348 of other constrained conditions (Noctor et al., 2018). At high concentrations, ROS pose a significant
349 threat that may eventually lead to programmed cell death (PCD). At low doses, ROS are employed
350 as signals that mediate at least part of the responses towards stress. ROS production is a common
351 feature under abiotic stress conditions (Foyer and Noctor, 2011).

352 ROS accumulation can also be due to O_3 degradation into the leaf tissues (Czarnocka and
353 Karpińsky, 2018). Drought response mostly uses ROS as internally produced signalling molecules,
354 although severe drought may lead to photo-oxidative stress (Reddy et al., 2004). Recent evidence
355 shows that when subjected to a combination of multiple stresses, plants respond differently relative
356 to when they experience only a single type of stress (Cotrozzi et al., 2017b). Therefore, the first

357 question we wanted to address for the three species investigated in the present study was "How
358 much ROS are induced by realistic O_3 and water stress levels"? The impact of O_3 *per se* on ROS
359 production appeared to be species-specific in view of the accumulation of H_2O_2 observed only in *Q.*
360 *ilex*, where however the basal levels of this ROS were lower compared to the deciduous species,
361 likely due to a balancing relation with other ROS (e.g., anion superoxide, hydroxyl radical; Foyer
362 and Noctor, 2011). The induction of an oxidative burst by the two higher O_3 concentrations,
363 however, was not associated with membrane injury (as demonstrated by the unchanged MDA by-
364 product values). This result suggests that an activation of an efficient free radical scavenging system
365 minimized the adverse effects of a general peroxidation, thus contributing to (i) the maintenance of
366 membrane structure and integrity and (ii) the delay of leaf senescence (Miller et al., 1999).

367 Peroxidation control and cell membrane stability under increased ROS conditions are
368 usually characteristic of O_3-tolerant plants, which are able to cope with ROS by the activation of
369 enzymatic and non-enzymatic antioxidant compounds (Gill and Tuteja, 2010). In contrast, the
370 deciduous species increased MDA under O_3 as H_2O_2 likely reacted with some cell wall and plasma
371 membrane components, which resulted in lipid peroxidation (Czarnocka and Karpińsky, 2018).
372 Drought *per se* also induced a similar H_2O_2 accumulation in *Q. ilex*, but again, the maintenance of
373 membrane functionality (*i.e.*, unchanged MDA by-product values) suggests that a tight control of
374 ROS production occurred. In particular, H_2O_2 may be involved in the integration of cellular
375 processes and in the adaptation to environmental stimuli (Dizengremel et al., 2013). The reducing
376 water availability had a strong impact on the deciduous species. A marked over-production of H_2O_2
377 was observed in *Q. pubescens* SD and *Q. robur* MD plants and it was associated with membrane
378 denaturation. This result suggests that oxidative damage occurred, probably due to an inadequate
379 response of the antioxidative systems (Czarnocka and Karpińsky, 2018).

380 In light of the above, the second question was "Which antioxidant mechanisms are activated
381 in response to individual stresses and to the combination of the stressors at different intensities"? It
382 is known that leaf biochemical traits (*e.g.*, Car, AsA, GSH, Phen, Flav) are crucial for avoiding and

383 preventing oxidative damage during stress conditions (Sharma et al., 2012). Our results suggest that
384 the oxidative damage induced by the higher O_3 concentrations and the reduced water availability
385 (single or in combination) only slightly modified the pool (and the reduction level) of the
386 metabolites involved in the Halliwell-Asada cycle. Only O_3 *per se* increased the total abundance of
387 intracellular AsA in all the three species under AA conditions, confirming that AsA represents the
388 first line of defence against O_3-oxidative load (Conklin and Barth 2004). Particularly, a special role
389 could be attributed to the apoplastic ascorbate. This fraction could contribute to differences in O_3
390 tolerance for *Quercus* species as shown for other species (Burkey et al., 2000; Feng et al., 2010).
391 However, AsA did not seem to be sufficient to mitigate the negative effects of O_3 in terms of ROS
392 production (in *Q. ilex*) and membrane denaturation (in *Q. pubescens* and *Q. robur*), suggesting that
393 it may be more important in terms of regulation than in redox homeostasis (Foyer and Noctor,
394 2011). This is probably because AsA is a cofactor of several plant-specific enzymes that are
395 involved in important pathways leading to the biosynthesis of (i) cell wall hydroxyproline-rich
396 proteins, (ii) defence-related secondary metabolites and (iii) plant hormones (Gest et al., 2013). Our
397 results indicate that the phenylpropanoid pathway (including non-volatile isoprenoids such as
398 carotenoids) was very responsive in stressed plants.

399 It is known that Car are liposoluble antioxidants that play several functions in plant
400 metabolism including oxidative stress tolerance (Havaux et al., 2005). They serve an important
401 photoprotective role by dissipating excess excitation energy as heat or by scavenging ROS and
402 suppressing lipid peroxidation (Gill and Tuteja, 2010). Consequently, Car can transiently
403 complement the action of the primary antioxidants (*i.e.*, AsA and enzymatic antioxidant
404 compounds, Brunetti et al., 2015). Secondary metabolites (such as phenols and flavonoids) are well
405 suited to constitute a "secondary" antioxidant system with a central role in plant defence against
406 severe constraints by avoiding the generation of ROS and by quenching ROS once they are formed
407 (Brunetti et al., 2015). For all the three species tested in this work, it is possible to conclude that
408 distinct phenylpropanoid pathways were activated in response to O_3 and drought, when applied

409 singularly. Ozone and drought in combination generally had antagonistic effects on most
410 biochemical traits. This was most evident for Tot Car in *Q. ilex* (except when both stressors were of
411 severe intensity) and *Q. robur*, and for Tot Phen in *Q. ilex* and *Q. pubescens*, as well as for ROS
412 production, in which the accumulation of H_2O_2 in *Q. ilex* and *Q. pubescens* under 1.4 × AA-SD
413 conditions was not as severe as expected. However, Tot Flav (the most representative class of
414 phenylpropanoid compounds) was affected by both stressors in *Q. ilex* (at different intensities) and
415 in *Q. pubescens* and *Q. robur* under both 1.4 × AA-SD and 1.2 × AA-MD conditions, by exhibiting
416 relatively strong and synergistic effects relative to their combined impact. It is possible to
417 hypothesize that the strong decrease of Tot Phen could be related to the consumption of these
418 compounds by the cell to counteract the accumulation of H_2O_2, thus representing an important
419 defence mechanism against the increased oxidative metabolism induced by reduced water
420 availability. This result confirms that Tot Flav can be considered as a robust biochemical trait to
421 improve the adaptability of plants to harsh environments. However, this mechanism appeared to be
422 species-specific and depended on the so-called "metabolic plasticity" (Logemann et al., 2000).

423 In light of the above, the third question was "Are metabolic responses markedly species-
424 specific"? In *Q. ilex*, O_3 *per se* induced an increase of Tot Car that inhibited and/or prevented the
425 peroxidation action of free radicals, as confirmed by unchanged MDA by-product values. The
426 concomitant decrease of Tot Flav could be related to the consumption of these compounds by the
427 cell to counteract the accumulation of H_2O_2, thus representing an important defense mechanism
428 against the increased oxidative metabolism induced by O_3 (Pellegrini et al., 2018). The unchanged
429 Tot Phen values confirmed that *Q. ilex* can be considered O_3-tolerant according to the evidence that
430 the biosynthesis of phenylpropanoids increases more in stress-sensitive than in tolerant species (Fini
431 et al., 2012; Cotrozzi et al., 2018b). Drought *per se* did not give rise to the same effects induced by
432 O_3 confirming that the biochemical features found in *Q. ilex* are enough to explain its superior
433 ability to counteract unfavorable environmental conditions, also in terms of the reducing water
434 availability. In *Q. pubescens*, high O_3 concentrations *per se* induced a concomitant reduction of Tot

435 Car and Tot Flav suggesting that these antioxidants could be consumed by the cell to counteract the
436 possible ROS generation due to increased oxidative metabolism and cellular damages. It is known
437 that Car and Flav are involved in non-photochemical quenching mechanisms, thus reducing the risk
438 of photo-oxidative stress (Niinemets et al., 2003). Based on relative physical-chemical features and
439 intra-cellular distribution, they may serve distinct and complementary functions (Close and Beadle,
440 2003). Consequently, the utilization of these compounds could improve the tolerance of *Q.*
441 *pubescens* to O_3, as confirmed by the unchanged Tot Phen values. For this species, moderate
442 drought *per se* did not give rise to the same effects induced by O_3. This result confirms that the
443 biochemical features found in *Q. pubescens* increased the ability of cells to scavenge stress-derived
444 ROS, but they counteracted the negative impact of both stressors (at different intensities) less
445 efficiently than in the sympatric *Q. ilex*. In *Q. robur*, high O_3 concentrations *per se* induced a partial
446 rearrangement of the phenylpropanoid pathways with different functions in order to alleviate the
447 excess of excitation pressure and to provide antioxidative protection to chloroplasts.

448 Phenylpropanoid pathways contribute to all aspects of plant responses towards biotic and
449 abiotic stimuli (Vogt, 2010). Generally, the increase in phenylpropanoid concentration can be
450 considered a repair process that can equip stressed plants with an additional antioxidant system
451 capable of avoiding and scavenging ROS (Cotrozzi et al., 2018b). It is possible to conclude that the
452 reduction of Tot Car and the concomitant increase of Tot Flav were not enough to counteract and/or
453 reduce the photo-oxidative stress induced by high O_3 concentrations. The concomitant induction of
454 Tot Phen would indicate a better capacity to regulate the level of ROS, and hence the cellular redox
455 state. However, these additional antioxidative mechanisms were not able to protect and/or repair the
456 cell structure and to prevent the occurrence of the oxidative load (as confirmed by the increase of
457 MDA by-products values). Severe drought *per se* did not give rise to the same effects induced by
458 high O_3 concentrations confirming that *Q. robur* is least adapted to unfavorable environmental
459 conditions because of a lower degree of tolerance compared with the other two species. Even
460 though no clear relationship between the activation of phenylpropanoids and stress tolerance has

461 been established, it is known that the biosynthesis of these secondary metabolites increases more in
462 stress-sensitive than in tolerant species (Fini et al., 2012). The variation in O_3-sensitivity among the
463 three species tested in this work can be ascribed not only to the ability of cells to scavenge O_3-
464 derived ROS and to raise detoxifying barriers, but also to the stomatal O_3 uptake. According to
465 Reich (1987), cell fate in an O_3-polluted environment depends on exposure, uptake and biological
466 responses, so a powerful tool to estimate plant susceptibility to O_3 should take into account both
467 cellular biochemical defenses and O_3 flux through stomata.

468 In light of the above, the fourth question was "Are oxidative metabolism and stomatal
469 uptake of O_3 correlated"? Treutter (2005) reported that the antioxidant compounds can be divided
470 into two groups: "preformed" and "induced" compounds, the latter being synthetized by plants in
471 response to physical injury, infection or stress. Concerning our results, it is possible to conclude that
472 no significant relationships were found between most of the parameters of antioxidant metabolism
473 and POD_0 suggesting that an unbalance between repair (*i.e.*, the capacity to activate detoxifying
474 systems) and avoidance strategies (*i.e.*, the ability of leaves to partially close stomata to exclude O_3
475 from leaf intercellular space) occurred. Although recent studies documented that the POD approach
476 can be considered the best metric to assess O_3 effects on (i) plant productivity (*i.e.*, biomass and
477 yield losses, leaf mass per area etc.), (ii) photosynthetic performance and (iii) visible foliar injury
478 (Gao et al., 2017; Hoshika et al., 2018), the internal mechanism of O_3 sensitivity is controversial.
479 Some studies found that O_3 sensitivity was associated with high stomatal conductance (g_s, Wittig et
480 al., 2007; Cotrozzi et al., 2016; Yang et al., 2016), but others argued that it depended on antioxidant
481 levels (Nali et al., 2004; Dai et al., 2017). In addition, a wide range of different biochemical,
482 structural and physiological leaf traits can play a pivotal role in determining O_3 response. The
483 relative contributions of these different traits in controlling the interspecific variation in O_3
484 sensitivity among a wide range of species remain elusive (Li et al., 2017). A significant and positive
485 relationship between MDA and POD_0 was found in *Q. pubescens* and *Q. robur*, confirming that the

486 greater stomatal O_3 flux in these deciduous oak species (according to biomass results from Hoshika
487 et al., 2018) resulted in pronounced membrane denaturation.

488 It is possible to conclude that the POD approach can be used for assessing accelerated leaf
489 senescence in deciduous oak species. In fact, no similar relationship was found in *Q. ilex* that under
490 AA-WW conditions displayed not only lower amounts of AsA, GSH, Tot Car and Tot Phen than
491 the other species, but also the lowest values of POD_0 suggesting that intrinsic physiological and
492 biochemical mechanisms can contribute significantly to the stress tolerance. To conclude, O_3 and
493 drought had antagonistic effects on most biochemical traits which depended on plant species. In
494 particular, we untangled the species-specific biochemical adjustments that may reduce the impact of
495 O_3 when combined with the effect of drought.

496 **Acknowledgments**
497 This work was financially supported by the Fondazione Cassa di Risparmio di Firenze (2013/7956)
498 and the LIFE15ENV/IT/000183 project MOTTLES. We would like to thank Alessandro Materassi,
499 Gianni Fasano and Moreno Lazzara for their support during the field work.

500 **References**

501 Alonso, R., Elvira, S., Gonzalez-Fernandez, I., Calvete, H., Garcia-Gomez, H., Bermejo, V., 2014.
502 Drought stress does not protect *Quercus ilex* L. from ozone effects: results from a comparative
503 study of two subspecies differing in ozone sensitivity. Plant Biol. 16, 375-384.
504 Assumpção, C.F., Herms, V.S., Pagno, C., Castagna, A., Mannucci, A., Sgherri, C., et al.
505 (2018).Phenolic enrichment in apple skin following post-harvest fruit UV-B treatment. Postharvest
506 Biolg. Technol. 138, 37-45.
507 Avery, R.J., 2006. Reactivity-based VOC control for solvent products: more efficient ozone
508 reduction strategies. Environ. Sci. Technol. 40, 4845-4850.
509 Bansal, S., Hallsby, G., Löfvenius, M.O., Nilsson, M.-C., 2013. Synergistic, additive and
510 antagonistic impacts of drought and herbivory on *Pinus sylvestris*: leaf, tissue and whole-plant
511 responses and recovery. Tree Physiol. 33, 451-463.

512 Bohler, S., Cuypers, A., Vangronsveld, J., 2015. Interactive effects between ozone and drought:
513 sorrow or joy?, in: Mahalingam, R., (Eds.), Combined stresses in plants - physiological, molecular,
514 and biochemical aspects. Springer International Publishing, Switzerland, pp. 147-157.
515 Brunetti, C., Guidi, L., Sebastiani, F., Tattini, M., 2015. Isoprenoids and phenylpropanoids are key
516 components of the antioxidant defense system of plants facing severe excess light stress. Environ.
517 Exp. Bot. 119, 54-62.
518 Burkey, K.O., Wei, C., Eason, G., Ghosh, P., Fenner, G.P., 2000. Antioxidant metabolite levels in
519 ozone-sensitive and tolerant genotypes of snap bean. Physiol. Plant. 110, 195-200.
520 Bussotti, F., Bettini, D., Grossoni, P., Mansuino, S., Nibbi, R., Soda, C., Tani, C., 2002. Structural
521 and functional traits of *Quercus ilex* in response to water availability. Environ. Exp. Bot. 47, 11-23
522 Butkovic, V., Cvitas, T., Klasing L., 1990. Photochemical ozone in the Mediterranean. Sci. Total
523 Environ. 99, 145-151.
524 Chaves, M.M., Maroco, J.P., Pereira, J.S., 2003. Understanding plant responses to drought: from
525 genes to the whole plant. Funct. Plant Biol. 30, 239-264.
526 Close, D.C., Beadle, C.L., 2003. Alternate energy dissipation? Phenolic metabolites and the
527 xanthophyll cycle. J. Plant Physiol. 160, 431-434.
528 CLRTAP 2017. Mapping Critical Levels for Vegetation. Chapter III of Manual on methodologies
529 and criteria for modelling and mapping critical loads and levels and air pollution effects, risks and
530 trends. UNECE Convention on Long-range Transboundary Air Pollution; accessed on 28 Feb 2018
531 www.icpmapping.org.
532 Conklin, P.L., Barth, C., 2004. Ascorbic acid, a familiar small molecule intertwined in the response
533 of plants to ozone, pathogens, and the onset of senescence. Plant Cell Environ. 27, 959-970.
534 Cotrozzi, L., Remorini, D., Pellegrini, E., Landi, M., Massai, R., Nali, C., Guidi, L., Lorenzini, G.
535 2016. Variations in physiological and biochemical traits of oak seedlings grown under drought and
536 ozone stress. Physiol. Plant 157, 69-84.
537 Cotrozzi, L., Remorini, D., Pellegrini, E., Guidi, L., Lorenzini, G., Massai, R., Nali, C., Landi, M.,
538 2017a. Cross-talk between physiological and metabolic adjustments adopted by *Quercus cerris* to

539 mitigate the effects of severe drought and realistic future ozone concentrations. Forests 8, 148,
540 doi:10.3390/f8050148.
541 Cotrozzi, L., Pellegrini, E., Guidi, L., Landi, M., Lorenzini, G., Massai, R., Remorini, D., Tonelli,
542 M., Trivellini, A., Vernieri, P., Nali, C., 2017b. Losing the warning signal: drought compromises
543 the cross-talk of signaling molecules in *Quercus ilex* exposed to ozone. Front. Plant Sci. 8, 1020,
544 doi: 10.3389/fpls.2017.01020.
545 Cotrozzi, L., Remorini, D., Pellegrini, E., Guidi, L., Nali, C., Lorenzini, G., Massai, R., Landi, M.,
546 2018a. Living in a Mediterranean city in 2050: broadleaf or evergreen "citizens"? Environ. Sci.
547 Pollut. Res. 25, 8161-8173.
548 Cotrozzi, L., Campanella, A., Pellegrini, E., Lorenzini, G., Nali, C., Paoletti, E., 2018b.
549 Phenylpropanoids are key players in the antioxidant defense to ozone of European ash, *Fraxinus*
550 *excelsior*. Environ. Sci. Pollut. Res. 25, 8137-8147.
551 Curtu, A.L., Şofletea, N., Toader, A.V., Enescu, C.M., 2011. Leaf morphological and genetic
552 differentiation between *Quercus robur* L. and its closest relative, the drought tolerant *Q.*
553 *pedunculiflora* K Kock. Ann. For. Sci. 68, 1163-1172.
554 Czarnocka, W., Karpińsky, S., 2018. Friend or foe? Reactive oxygen species production,
555 scavenging and signaling in plant response to environmental stresses. Free Radic. Biol. Med.,
556 doi.org/10.1016/j.freeradbiomed.2018.01.011.
557 Dai, L., Li, P., Shang, B., Liu, S., Yang, A., Wang, Y., Feng, Z., 2017. Differential responses of
558 peach (*Prunus persica*) seedlings to elevated ozone are related with leaf mass per area, antioxidant
559 enzymes activity rather than stomatal conductance. Environ. Pollut. 227, 380-388.
560 Davey, M.W., Dekempenner, E., Keulemans, J., 2003. Rocket-powered high-performance liquid
561 chromatographic analysis of plant ascorbate and glutathione. Anal. Biochem. 316, 74-81.
562 Dizengremel, P., Jolivet, Y., Tuzet, A., Ranieri, A., Le Thiec, D., 2013. Integrative leaf-level
563 phytotoxic ozone dose assesment for forest risk modelling, in: Matyssek. R., Clarke, N., Cudlin, P.,
564 de Carvalho, M.H.C., 2008. Drought stress and reactive oxygen species. Plant Signal. Behav. 3,
565 156-165.

566 EEA, 2017 Air quality in Europe – 2017 report. EEA Report No17/20217, European Environment
567 Agency (https://www.eea.europa.eu/publications/air quality-in-europe-2017).
568 Feng, Z., Pang, J., Nouchi, I., Kobayashi, K., Yamakawa, T., Zhu, J., 2010. Apoplastic ascorbate
569 contributes to the differential ozone sensitivity in two varieties of winter wheat under fully open-air
570 field conditions. Environ. Pollut. 158, 3539-3545.
571 Fini, A., Guidi, L., Ferrini, F., Brunetti, C., Di Fernando, M., Biricolti, S., Pollastri, S., Calamai, L.,
572 Tattini, M., 2012. Drought stress has contrasting effects on antioxidant enzymes activity and
573 phenylpropanoid biosynthesis in *Fraxinus ornus* leaves: an excess light stress affair? J. Plant
574 Physiol. 169, 929-939.
575 Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol.
576 155, 2-18.
577 Gao, F., Catalayud, V., Paoletti, E., Hoshika Y., Feng Z., 2017. Water stress mitigates the negative
578 effects of ozone on photosynthesis and biomass in poplar plants. Environ. Pollut. 230, 268-279.
579 Gest, N., Gautier, H., Stevens, R., 2013. Ascorbate as seen through plant evolution: the rise of a
580 successful molecule? J. Exp. Bot. 64, 33-53.
581 Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress
582 tolerance in crop plants. Plant Physiol. Biochem. 48, 909-930.
583 Guidi, L., Remorini, D., Cotrozzi, L., Giordani, T., Lorenzini, G., Massai, R., Nali, C., Natali, L.,
584 Pellegrini, E., Trivellini, A., Vangelisti, A., Vernieri, P., Landi, M., 2017. The harsh life of an urban
585 tree: the effect of a single pulse of ozone in salt-stressed *Quercus ilex* saplings. Tree Physiol. 37,
586 246-260.
587 Havaux, M., Eymery, F., Porfirova, S., Rey, P., Dörmann, P., 2005. Vitamin E protects against
588 photoinhibition and photooxidative stress in *Arabidopsis thaliana*. Plant Cell 17, 3451-3469.
589 Hoshika, Y., Moura, B., Paoletti, E., 2018. Ozone risk assessment in three oak species as affected
590 by soil water availability. Environ. Sci. Pollut. Res. 25, 8125-8136.

591 Jolivet, Y., Bagard, M., Cabané, M., Vaultier M.N., Gandin, A., Afif, D., Dizengremel, P., Le
592 Thiec, D., 2016. Deciphering the ozone-induced changes in cellular processes: a prerequisite for
593 ozone risk assessment at the tree and forest levels. Annals Forest Sci. 73, 923-943.
594 Li P., Feng Z., Catalayud V., Yuan X., Xu Y., Paoletti E., 2017. A meta-analysis on growth,
595 physiological, and biochemical responses of woody species to ground-level ozone highlights the
596 role of plant functional types. Plant Cell Environ. 40, 2369-2380.
597 Logemann, E., Tavernaro, A., Schulz, W., Somssich, I.E., Hahlbrock, K., 2000. UV light selectively
598 coinduces supply pathways from primary metabolism and flavonoid secondary product formation in
599 parsley. Proc. Natl. Acad. Sci. 97, 1903-1907.
600 Miller, J.D., Arteca, R.N., Pell, E.J., 1999. Senescence-associated gene expression during ozone-
601 induced leaf senescence in *Arabidopsis*. Plant Physiol. 120, 1015-1023.
602 Nali, C., Paoletti, E., Marabottini, R., Della Rocca, G., Lorenzini, G., Paolacci, A.R., Ciaffi, M.,
603 Badiani, M., 2004. Ecophysiological and biochemical strategies of response to ozone in
604 Mediterranean evergreen broadleaf species. Atmos. Environ. 38, 2247-2257.
605 Niinements, Ü., Kollist, H., García-Plazaola, J.I., Hernández, A., Becerril, J.M., 2003. Do the
606 capacity and kinetics for modification of xanthophyll cycle pool size depend on growth irradiance
607 in temperate trees? Plant Cell Environ. 26, 1787-1802.
608 1648.
609 Noctor, G., Reichheld, J.P., Foyer, C.H., 2018. ROS-related redox regulation and signaling in
610 plants. Semin. Cell Dev. Biol. in press.
611 Paoletti, E., Materassi, A., Fasano, G., Hoshika, Y., Carriero G., Silaghi D., Badea, O., 2017. A
612 new-generation 3D ozone Face (Free Air Controlled Exposure). Sci. Tot. Environ. 575, 1407-1414.
613 Pellegrini, E., Campanella, A., Cotrozzi, L., Tonelli, M., Nali, C., Lorenzini, G., 2018. What about
614 the detoxification mechanisms underlying ozone sensitivity in *Liriodendron tulipifera*? Environ.
615 Sci. Pollut. Res. 25, 8148-8160.

616 Pollastrini, M., Desotgiu, R., Camin, F., Ziller, L., Marzuoli, R., Gerosa, G., Bussotti, F., 2013.
617 Intra-annual pattern of photosynthesis, growth and stable isotope partitioning in a poplar clone
618 subjected to ozone and water stress. Water Air Soil Pollut., 224, 1761-1772.
619 Reddy, A.R., Chaitanya, K.V., Vivekanandan, M., 2004. Drought-induced responses of
620 photosynthesis and antioxidant metabolism in higher plants. J. Plant Physiol. 161, 1189-1202.
621 Reich, P.B., 1987. Quantifying plant response to ozone: a unifying theory. Tree Physiol. 3, 63-91.
622 Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative
623 damage, and antioxidative defense mechanisms in plants under stressful conditions. J. Bot. 217037,
624 https://doi:10.1155/2012/217037.
625 Treutter, D., 2005. Significance of flavonoids in plant resistance and enhancement of their
626 biosynthesis. Plant Biol. 7, 581-591.
627 Vranckx, G., Jacquemyn, H., Mergeay, J., Cox, K., Janssens, P., Gielem B.A.S., Muys, B., Honnay,
628 O., 2014. The effect of drought stress on heterozygosity-fitness correlations in pedunculated oak
629 (*Quercus robur*). Ann. Bot. 113, 1057-1069.
630 Vogt, T., 2010. Phenylpropanoid biosynthesis. Mol. Plant 3, 2-20.
631 Witting, V.E., Ainsworth E.A., Long S.P., 2007. To what extent do current and projected increases
632 in surface ozone affect photosynthesis and stomatal conductance of trees? A meta-analytic review
633 of the last 3 decades of experiments. Plant Cell Environ. 30, 1150-1162.
634 Yang, N., Wang, X., Cotrozzi, L., Chen, Y., Zheng, F., 2016. Ozone effects on photosynthesis of
635 ornamental species suitable for urban green spaces of China. Urban For. Urban Green. 20, 437-447.
636 Yuan, X., Calatayud, V., Gao, F., Fares, S., Paoletti, E., Tian, Y., Feng, Z., 2016. Interaction of
637 drought and ozone exposure on isoprene emission from extensively cultivated poplar. Plant Cell
638 Environ. 39, 2276-2287.

Table 1. *P* values of three-way ANOVA of the effects of ozone (O_3), drought and plant species on malondialdehyde (MDA), hydrogen peroxide (H_2O_2), total carotenoids (Tot Car), total ascorbate (Tot AsA), total glutathione (Tot Glu), total phenolic acids (Tot Phen) and total flavonoids (Tot Flav). Asterisks show the significance of factors/interaction: *** $P\leq0.001$, ** $P\leq0.01$; ns $P>0.05$. d.f. represents the degrees of freedom.

Effects	d.f.	MDA	H_2O_2	Tot Car	Tot AsA	Tot GSH	Tot Phen	Tot Flav
O_3	2	***	***	***	***	*	***	***
Drought	2	***	***	**	**	ns	ns	ns
Plant species	2	***	***	***	***	***	***	***
O_3 × drought	4	**	***	***	ns	ns	***	***
O_3 × plant species	4	**	***	***	ns	ns	***	***
Drought × plant species	4	***	***	***	*	ns	***	***
O_3 × drought × plant species	8	ns	***	***	ns	ns	***	***

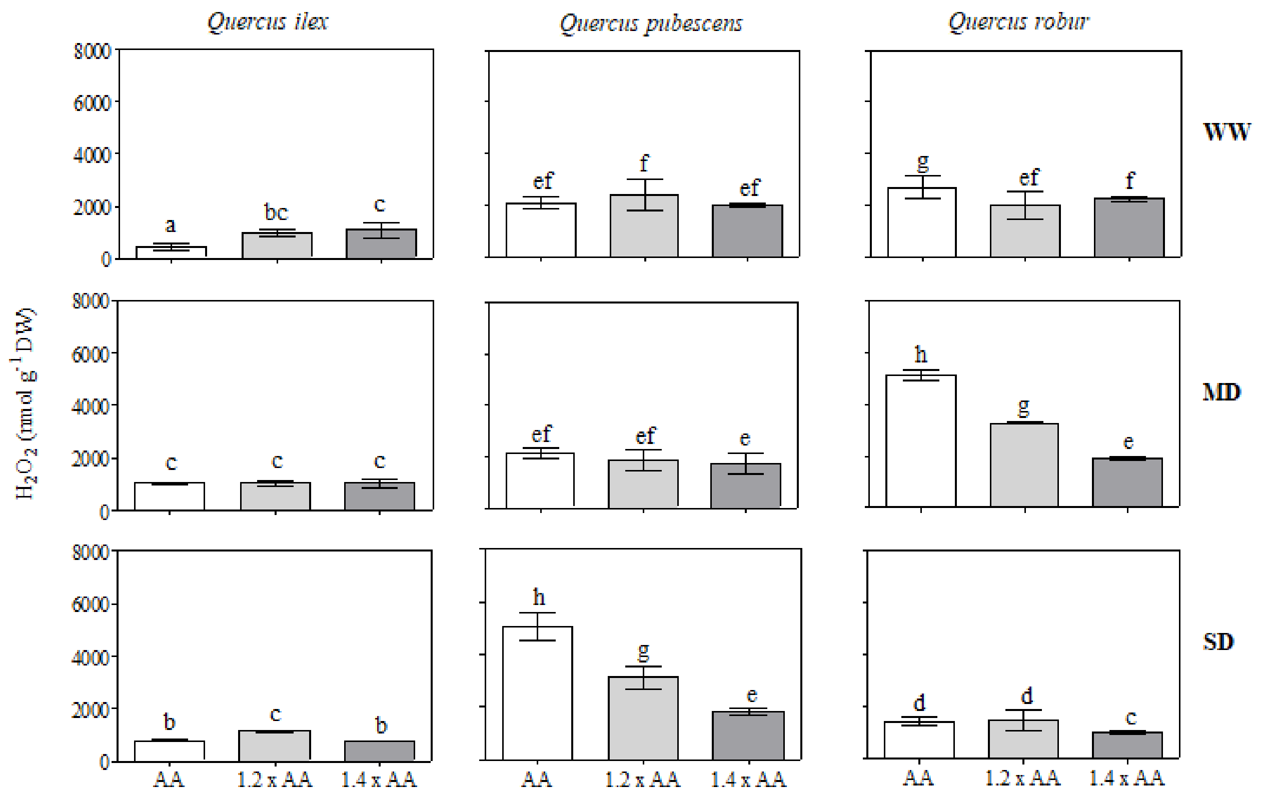

Fig. 1. Hydrogen peroxide [H_2O_2, nmol g^{-1} dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O_3 exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O_3 (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean (n = 3) ± standard deviation. According to a three-way ANOVA with O_3, drought and plant species as factors, different letters show significant differences among bars in the nine graphs (Tukey test, $P \leq 0.05$).

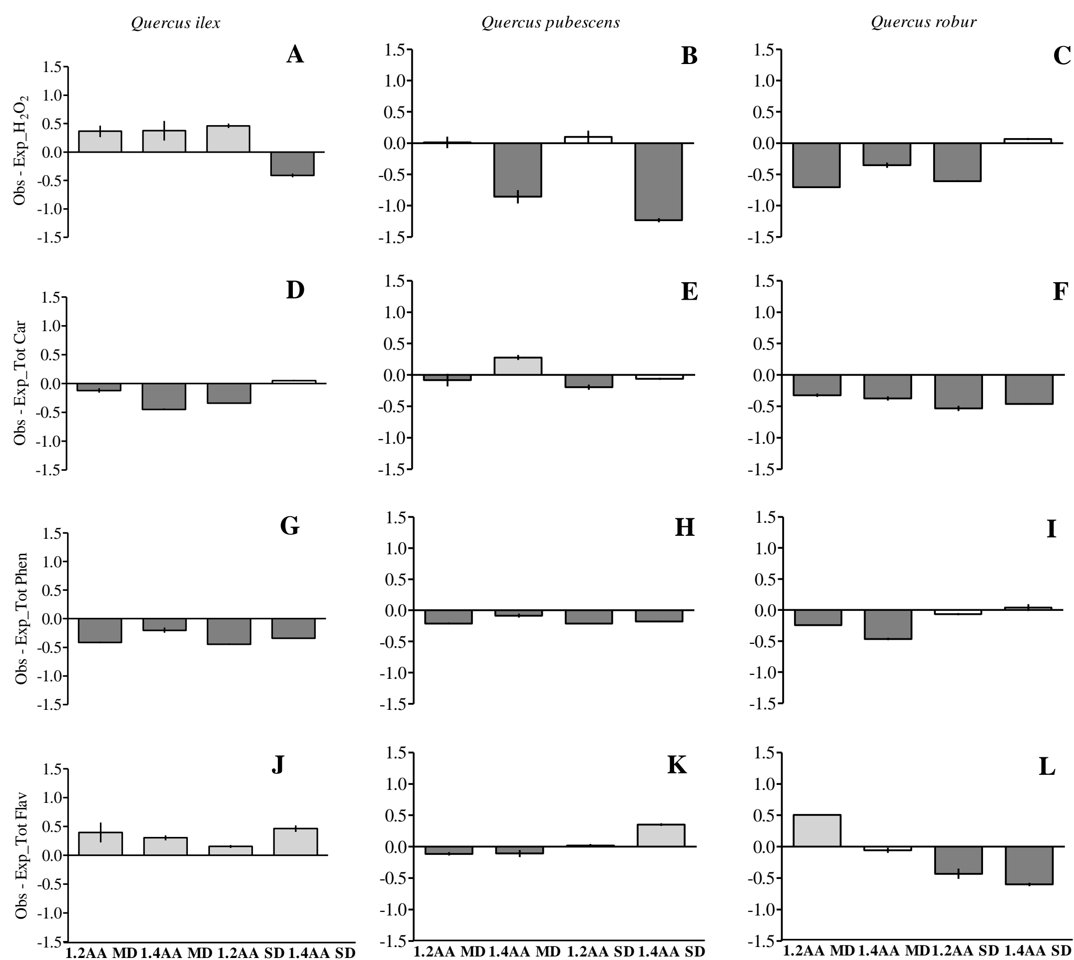

Fig. 2. The combined impact of increasing ozone levels [applied for 4.5 months: 1.2 × and 1.4 × ambient air (1.2AA and 1.4AA)] and reducing water availability [moderate drought (MD) and severe drought (SD)] on hydrogen peroxide (H_2O_2, A-C), total carotenoids (Tot Car, D-F), total phenols (Tot Phen, G-I) and total flavonoids (Tot Flav, J-L) in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves. The bars represent the intensity-specific effect size difference (mean ± 95% confidence interval) between the observed (Obs) and expected (Exp) additive effects from the combination of the two stressors. The zero line represents the expected additive effects from combined stressors. When the means were greater than or less than the zero line, they were considered synergistic (gray bars) or antagonistic (dark gray bars; Bansal et al., 2013).

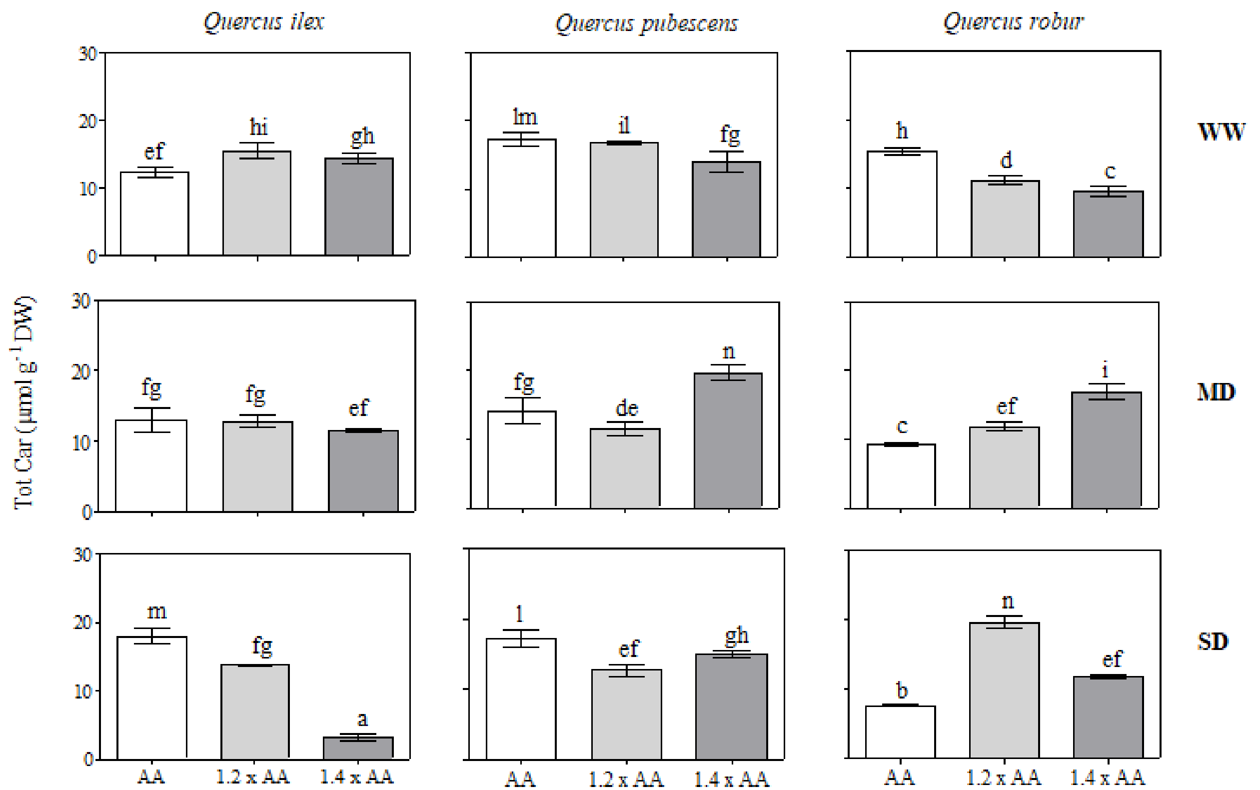

Fig. 3. Total carotenoid (Tot Car) content [μmol g^{-1} dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O_3 exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O_3 (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean (n = 3) ± standard deviation. According to a three-way ANOVA with O_3, drought and plant species as factors, different letters show significant differences among bars in the nine graphs (Tukey test, $P \leq 0.05$). Tot Car = neoxanthin + violaxanthin + anteraxanthin + lutein + zeaxanthin + β-carotene.

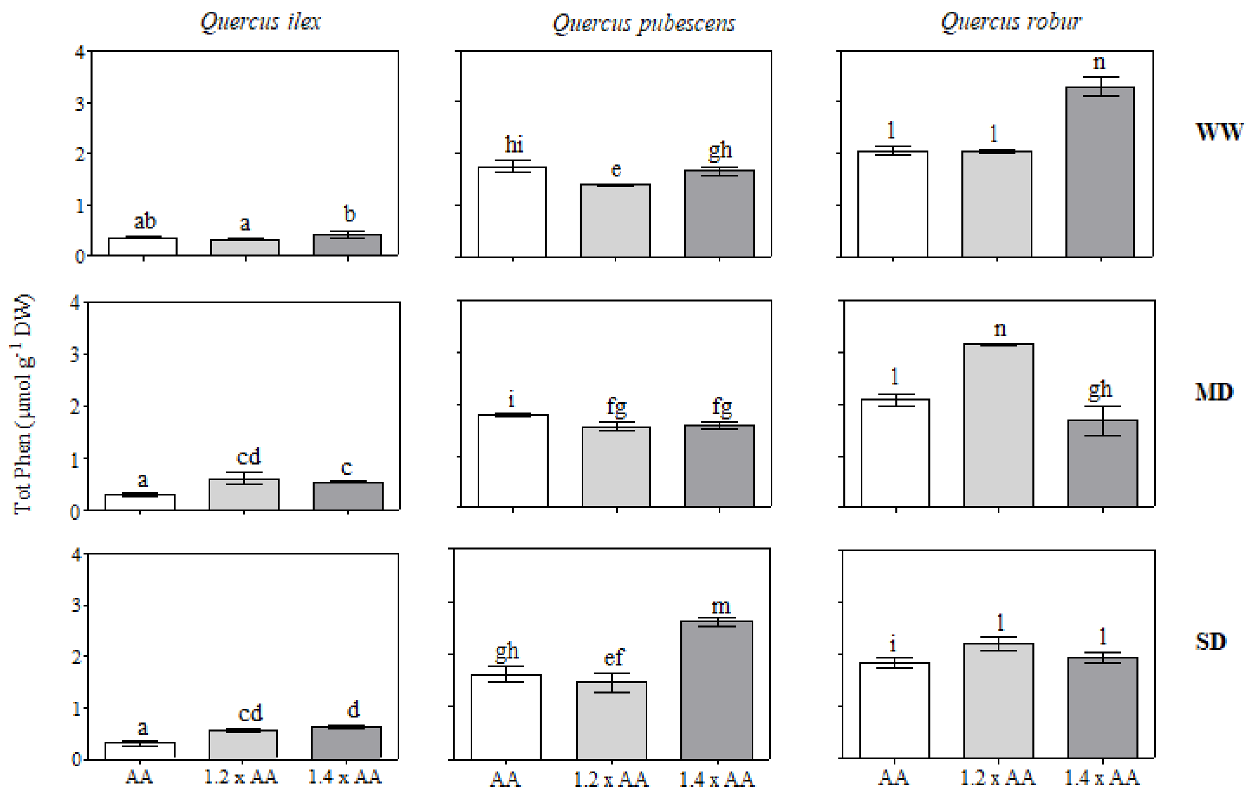

Fig. 4. Total free phenolic (Tot Phen) acid content [µmol g^{-1} dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O_3 exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O_3 (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean (n = 3) ± standard deviation. According to a three-way ANOVA with O_3, drought and plant species as factors, different letters show significant differences among bars in the nine graphs (Tukey test, $P \leq 0.05$). Tot Phen = caffeoyl-glucoside + chlorogenic acid + coumaric acid (only in deciduous species) + coumaroylquinic (only in deciduous species) + coumaroyl-glucoside + coumaroyl-glucoside 2 + cryptochlorogenic acid (only in *Q. pubescens*) + gallic acid + galloyl-glucoside + gentisic acid + neochlorogenic acid (only in deciduous species).

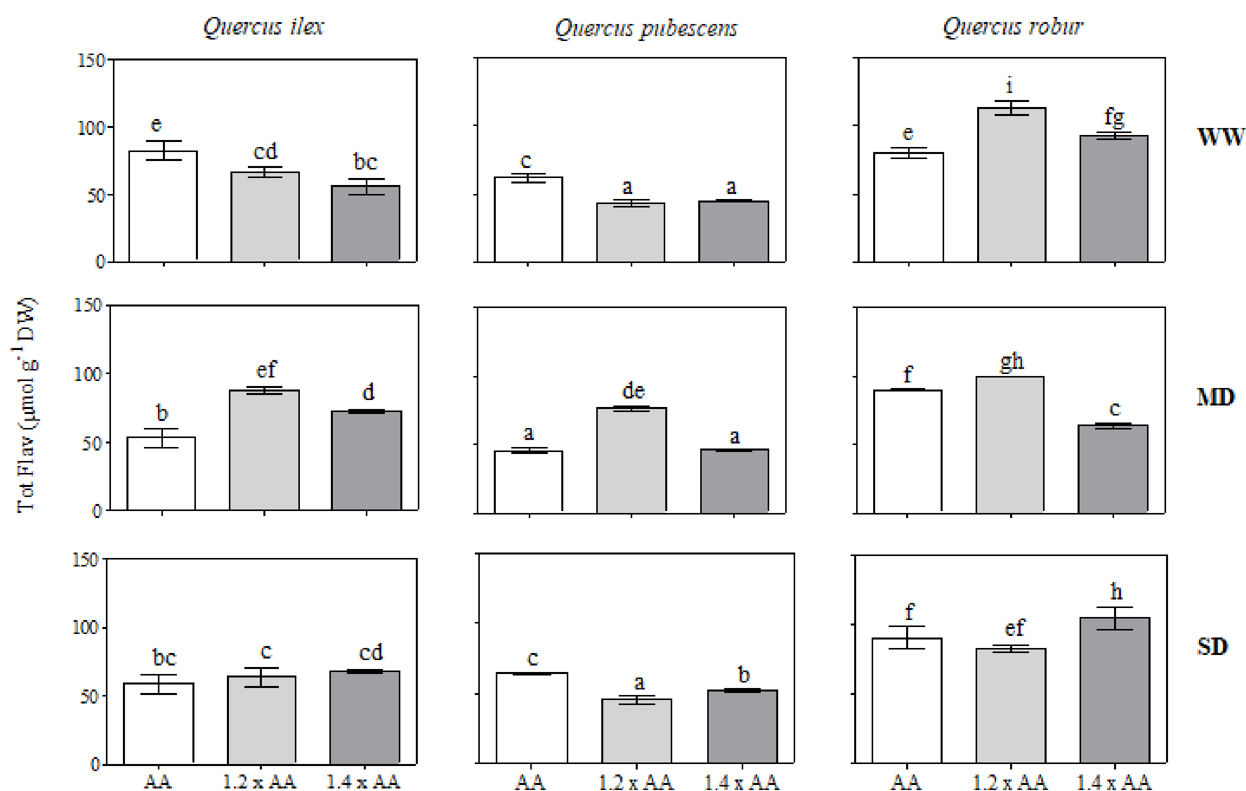

Fig. 5. Total flavonoid (Tot Flav) content [µmol g^{-1} dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O_3 exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O_3 (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean (n = 3) ± standard deviation. According to a three-way ANOVA with O_3, drought and plant species as factors, different letters show significant differences among bars in the nine graphs (Tukey test, $P \leq 0.05$). Tot Flav = catechin + epicatechin + epigallocatechin + hesperidin + isorhamnetin-glucoside + isorhamnetin-rutinoside + kaempferol (only in *Q. robur*) + kaempferol-glucoside + kaempferol-rutinoside + phloridzin + procyanidin B1 + pelargonidin-rutinoside + quercetin + quercetin arabinoside + quercetin-galactoside + quercetin-galloyl-glucoside + quercetin-glucoside + quercetin-rhamnoside + quercetin-xyloside + rhamnetin-glucoside + rutin.

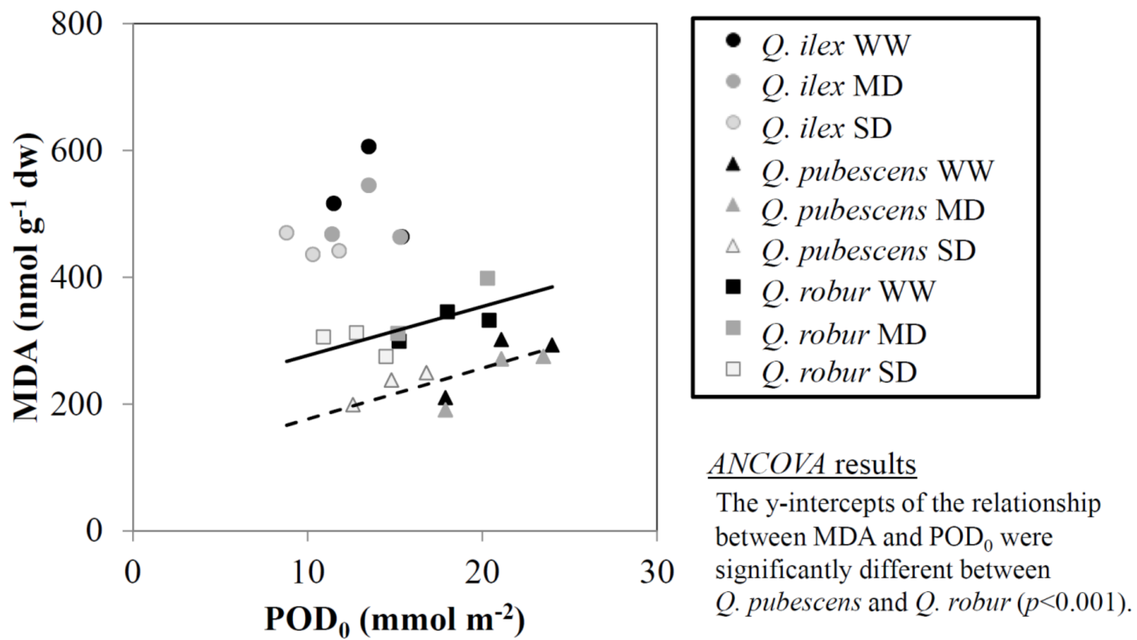

Fig. 6. Relationships between phytotoxic O_3 dose (POD_0, mmol m^{-2}) and malondialdehyde (MDA, nmol g^{-1} dry weight (DW)) in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O_3 exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O_3 (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Significant relationships were found in *Q. pubescens* (dotted line, $y = 8.05x + 95.74$, $R^2 = 0.58$, $P < 0.05$) and *Q. robur* (black line, $y = 7.72x + 199.49$, $R^2 = 0.50$, $P < 0.05$), while no significant regression was found in *Q. ilex* ($y = 6.21x + 413.15$, $R^2 = 0.06$, $P = 0.52$). The y-intercepts were significantly different between *Q. pubescens* and *Q. robur* by an ANCOVA test ($P < 0.001$). ns denotes not significant.

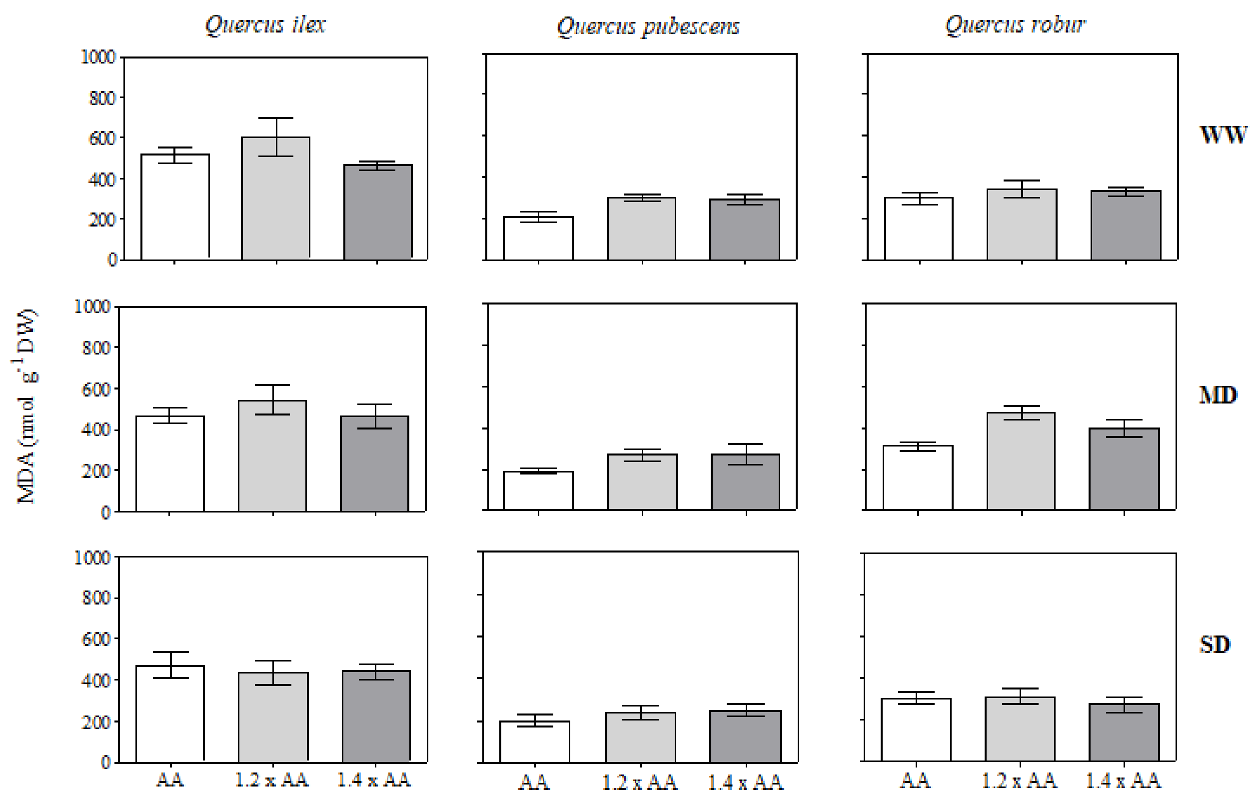

Fig. 1S. Quantification of malondialdehyde (MDA) by-product [nmol g^{-1} dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O_3 exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O_3 (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean (n = 3) ± standard deviation. According to a three-way ANOVA with O_3, drought and plant species as factors, the absence of letters indicates not significant interaction between variability factors (see Table 1).

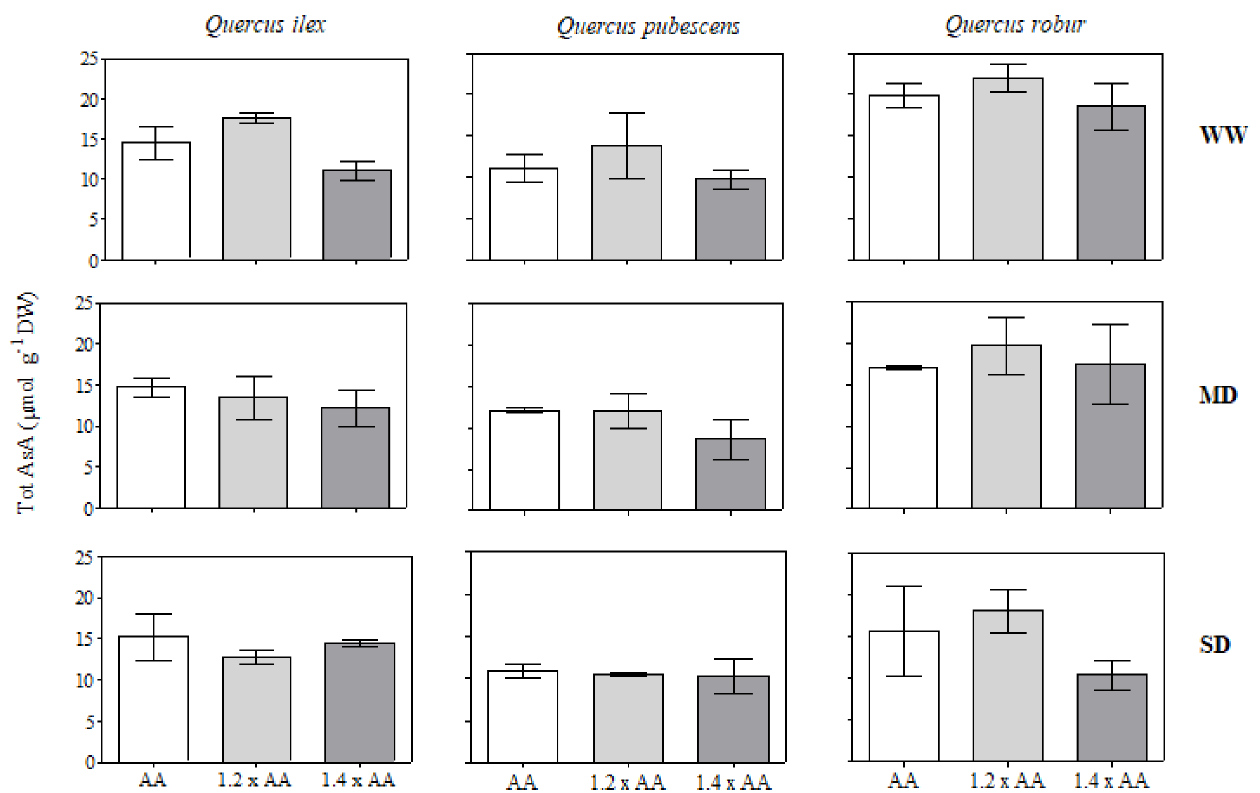

Fig. 2S. Quantification of total ascorbate (AsA) content [µmol g^{-1} dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O_3 exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O_3 (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean (n = 3) ± standard deviation. According to a three-way ANOVA with O_3, drought and plant species as factors, the absence of letters indicates not significant interaction between variability factors (see Table 1).

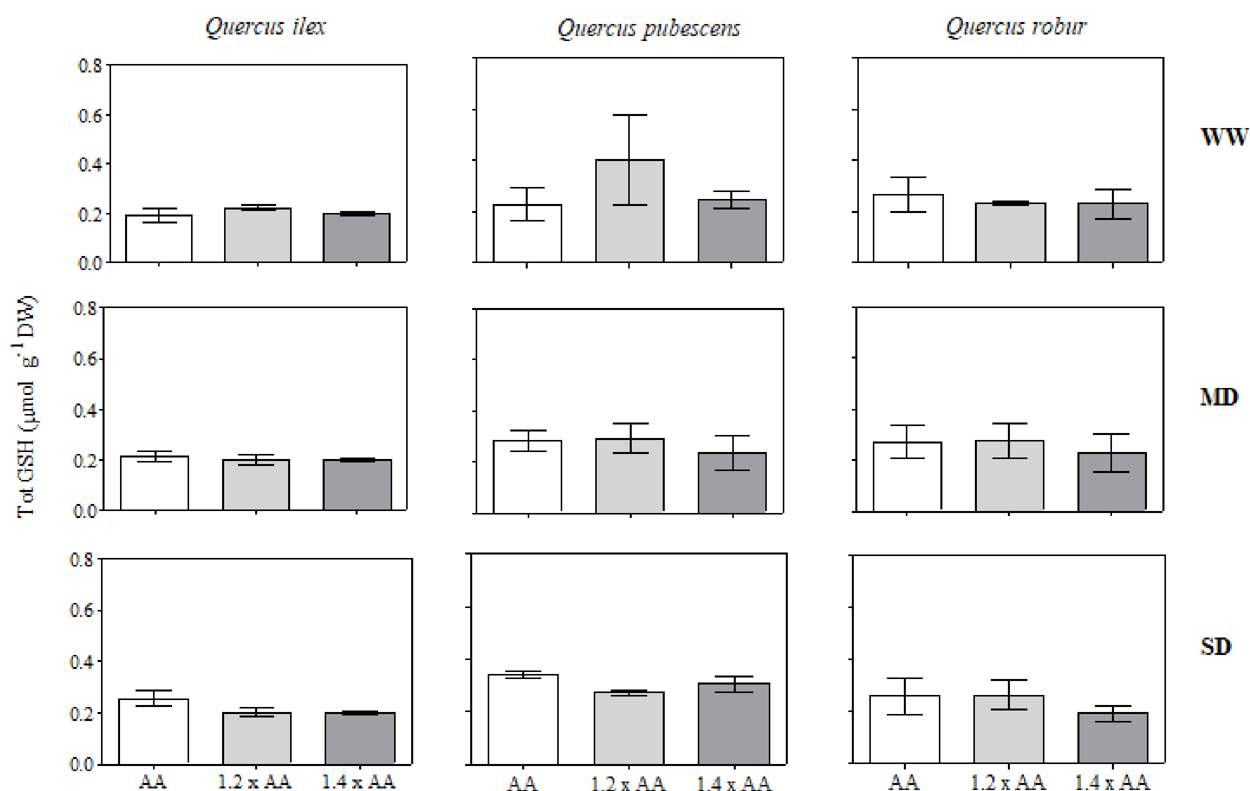

Fig. 3S. Quantification of total glutathione (GSH) content [µmol g-1 dry weight (DW)] in Quercus ilex, Q. pubescens and Q. robur leaves under free air O_3 exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O_3 (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean (n = 3) ± standard deviation. According to a three-way ANOVA with O_3, drought and plant species as factors, the absence of letters indicates not significant interaction between variability factors (see Table 1).