ECOPHYSIOLoGY, STRESS AND ADAPTATION

Species-specific variation of photosynthesis and mesophyll conductance to ozone and drought in three Mediterranean oaks

Yasutomo Hoshika1 | Elena Paoletti1 | Mauro Centritto2 | Marcos Thiago Gaudio Gomes2 | Jaime Puértolas3 | Matthew Haworth2

1Institute of Research on Terrestrial Ecosystems (IRET), National Research Council of Italy (CNR), Sesto Fiorentino
2Institute of Sustainable Plant Protection (IPSP), National Research Council of Italy (CNR), Sesto Fiorentino, Italy
3Lancaster Environment Centre, Lancaster University, Lancaster, UK

Correspondence
Yasutomo Hoshika, Institute of Research on Terrestrial Ecosystems (IRET), National Research Council of Italy (CNR), Via Madonna del Piano, I-50019 Sesto Fiorentino, Italy. Email: yasutomo.hoshika@cnr.it

Present address
Marcos Thiago Gaudio Gomes, Department of Biological Sciences, Center for Human and Natural Sciences, Federal University of Espirito Santo, Vitoria, Espirito Santo, Brazil
Jaime Puértolas, Department of Botany and Plant Ecology and Physiology, University of La Laguna, San Cristóbal de La Laguna, Spain

Funding information
European Commission, Grant/Award Number: MOTTLES (LIFE15 ENV/IT/000183); Fondazione Cassa di Risparmio di Firenze, Grant/Award Number: 2013/7956

Edited by A. Krieger-Liszkay

Abstract
Mesophyll conductance (g_{mCO2}) is one of the most important components in plant photosynthesis. Tropospheric ozone (O3) and drought impair physiological processes, causing damage to photosynthetic systems. However, the combined effects of O3 and drought on g_{mCO2} are still largely unclear. We investigated leaf gas exchange during mid-summer in three Mediterranean oaks exposed to O3 (ambient [35.2 nmol mol$^{-1}$ as daily mean]; 1.4× ambient) and water treatments (WW [well-watered] and WD [water-deficit]). We also examined if leaf traits (leaf mass per area [LMA], foliar abscisic acid concentration [ABA]) could influence the diffusion of CO2 inside a leaf. The combination of O3 and WD significantly decreased net photosynthetic rate (PN) regardless of the species. The reduction of photosynthesis was associated with a decrease in g_{mCO2} and stomatal conductance (g_{SCO2}) in evergreen Quercus ilex, while the two deciduous oaks (Q. pubescens, Q. robur) also showed a reduction of the maximum rate of carboxylation (V_{cmax}) and maximum electron transport rate (J_{max}) with decreased diffusive conductance parameters. The reduction of g_{mCO2} was correlated with increased [ABA] in the three oaks, whereas there was a negative correlation between g_{mCO2} with LMA in Q. pubescens. Interestingly, two deciduous oaks showed a weak or no significant correlation between g_{SCO2} and ABA under high O3 and WD due to impaired stomatal physiological behaviour, indicating that the reduction of PN was related to g_{mCO2} rather than g_{SCO2}. The results suggest that g_{mCO2} plays an important role in plant carbon gain under concurrent increases in the severity of drought and O3 pollution.

1 | INTRODUCTION

The Mediterranean basin is an area where photochemical smog episodes are often observed due to high solar radiation and temperature, low precipitation, and recirculation of the polluted air mass during summer seasons (Ochoa-Hueso et al., 2017). Tropospheric ozone (O3) is a widespread phytotoxic air pollutant impairing plant physiological function and growth (Grulke & Heath, 2020). A recent meta-analysis indicates that the current background level of O3 in many areas in the world (approximately 40 nmol mol$^{-1}$) induces a
5% reduction of biomass growth for forest trees compared to that at the pre-industrial times (approximately 10–15 nmol mol⁻¹; Wittig et al., 2009; Li et al., 2017). Ozone is often elevated during hot and dry summers, especially in the Mediterranean area (30–55 nmol mol⁻¹ as daily mean O₃ concentration during summer: Ochoa-Hueso et al., 2017; Paoletti, 2006; Paoletti et al., 2019), where water availability limits plant growth and reduces productivity (Haworth et al., 2017; Killi et al., 2016). Recent modelling studies predict that O₃ concentrations will remain high alongside an increased frequency of drought events in the future (Giorgi & Gutowski, 2016; Mills et al., 2018). Research on the interacting impacts of O₃ and drought is needed to study if these two factors have antagonistic or synergistic effects on plant physiology (Paoletti et al., 2010).

Plant growth is closely related to photosynthetic assimilation of CO₂ (Larcher, 2003). Ozone frequently decreases net photosynthetic rate (Pₚₑₐ) in association with increased diffusion resistance to CO₂ and reduced ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity in leaves (Bagard et al., 2015; Feng et al., 2011; Hoshika, Haworth, et al., 2020b; Watanabe et al., 2013). The movement of CO₂ through the stomata and across the mesophyll layer represents the two greatest diffusive limitations to photosynthesis (Howarth et al., 2017; Killi et al., 2016). Recent modelling studies where water availability limits plant growth and reduces productivity (Flexas et al., 2008). Mesophyll conductance (gₘCO₂) as reported in snap bean (Flowers et al., 2007), poplars (Xu et al., 2019) and beech trees (Hoshika, Fares, et al., 2020a; Watanabe et al., 2013) although Warren et al. (2007) did not find such an effect of O₃ on gₘCO₂ in Fagus sylvatica. Mesophyll conductance (gₘCO₂) consists of both physical and biochemical components in the transport of CO₂ (Flexas et al., 2008; Loreto et al., 1992). Although the mechanisms of the effects of O₃ on gₘCO₂ are still unclear, several potential causes have been recognised (Nadal et al., 2021). In fact, Hoshika, Haworth, et al. (2020b) suggested that the decrease of gₘCO₂ was accompanied by structural changes in mesophyll cells of O₃-exposed beech leaves. This may be supported by the fact that O₃ collapses mesophyll cells or alters mesophyll ultrastructure and other leaf morphological parameters, which may reduce the CO₂ diffusion inside leaves (Gao et al., 2016; Matyssek et al., 1991; Paoletti et al., 2009). In addition, O₃ also modifies the activation of aquaporins, thus increasing the mesophyll diffusion resistance to CO₂ transport (Eichelmann et al., 2004). Leaf mass per area (LMA) is a parameter reflecting the leaf thickness and tissue density, which indicate an allocation of resources to structural strength against biotic and/or abiotic stress factors (Haworth & Raschi, 2014; Milla-Moreno et al., 2016). An asymptotic response of gₘCO₂ to LMA was found according to a literature data review, which indicates that the maximum gₘCO₂ may be limited by LMA values (Flexas et al., 2008). Ozone increased LMA in European silver birch (Günthardt-Goerg et al., 1993), whereas a reduction of this parameter was found in a hybrid poplar after O₃ exposure (Shang et al., 2019), suggesting that the effect of O₃ on LMA is not likely one-directional and is species-specific (Poorter et al., 2009).

Under water deficit conditions, decreases in both stomatal and mesophyll conductance to CO₂ uptake have been considered to be a major constraint of photosynthesis (Centritto et al., 2003, 2009; Killi & Haworth, 2017; Marino et al., 2020). As soil dries, an increase in ABA synthesis in above-ground tissues occurs (Brunetti et al., 2019), leading to an increase in [ABA] in mesophyll cells that triggers stomatal closure (McAdam & Brodribb, 2018). Many studies have shown that stomatal conductance is negatively correlated with the ABA content of leaves (Brunetti et al., 2019; Haworth, Cosentino, et al., 2018a; Mizokami et al., 2015) and xylem sap (Brunetti et al., 2019; Tardieu & Davies, 1993). The reduction in gₘCO₂ observed as foliar [ABA] rises is caused by a reduction in stomatal conductance, lowering CO₂ availability in the internal leaf air-space, and a reduction in the biochemical transport of CO₂ across the mesophyll (likely associated with reduced aquaporin activity; Sorrentino et al., 2016). Abscisic acid plays a pivotal role in the reduction of gₘCO₂ under drought conditions as reported in rose, cherry, olive and poplar (Brunetti et al., 2019; Sorrentino et al., 2016) and confirmed using wild type and ABA-deficient mutants of Arabidopsis (Mizokami et al., 2015). Over longer term, the decrease in gₘCO₂ induced by reduced water availability may also be related to morphological acclimation to drought stress, such as an increase of LMA (Fleck et al., 2010). Meta-analysis indicates a tendency of increase in LMA with decreasing soil water availability, although this depends on species and drought tolerance (Poorter et al., 2009).

It has been considered that stomatal closure during drought may reduce stomatal O₃ flux and thus limit O₃ damage (Khan & Soja, 2003; Tingeey & Hogsett, 1985). However, according to recent manipulative experiments, water deficits may instead exacerbate the negative effects of O₃ on plants (e.g. Cotrozi et al., 2016). In fact, the combination of O₃ and drought may cause the generation of excessive reactive oxygen species (ROS), and may thus overwhelm the detoxification capacity of plants (Alonso et al., 2001; Cotrozi et al., 2016; Hoshika, Fares, et al., 2020a). Reactive oxygen species have been proposed to function as secondary messengers in ABA signalling in plant cells (Vainonen & Kangasjärvi, 2015). Elevated ABA production may further limit stomatal and mesophyll CO₂ diffusion in plants grown in combined O₃ fumigation and drought. However, to the best of our knowledge, no data are available for gₘCO₂ in plant leaves subjected to both high O₃ concentration and drought, and it is still unclear if there is an interaction of O₃ and drought on diffusion resistance to CO₂ transport in the mesophyll layer.

We examined the interactive effects of O₃ and drought on leaf gas exchange in a free-air O₃ exposure experiment on three common oak species in Mediterranean Europe (Quercus ilex L., Q. pubescens Willd., Q. robur L.). Q. ilex is an evergreen species with sclerophyllous leaves known to be drought-tolerant. Deciduous Quercus pubescens has medium-sized pubescent leaves with the capacity to withstand summer drought. Quercus robur is a long-lived, widely distributed deciduous species that requires high water availability. We sought answers to two questions: (1) is there any interaction of O₃ and
drought on photosynthetic traits, especially on $\delta_{\text{in}CO_2}$ calculated from both the variable $J$ (Harley et al., 1992; Loreto et al., 1992) and curve fitting methods (Etherie & Livingston, 2004)? (2) Which of the traits (ABA and LMA) is involved in the response of $\delta_{\text{in}CO_2}$ in combinations of $O_3$ and drought?

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental site and plant material

Experiments were carried out in an O$_3$ Free-Air Controlled Exposure (FACE) facility at Sesto Fiorentino, Florence, in central Italy (43°48′59″N, 11°12′01″E, 55 m a.s.l). Detailed information on the FACE system can be found in our previous papers (Hoshika, Fares, et al., 2020a; Paoletti et al., 2017). Two-year-old seedlings of three oak species (Q. ilex, Q. pubescens, and Q. robur) were obtained from nearby nurseries in autumn 2014, and transplanted into 10 L circular plastic pots filled with a mixture of sand:peat:soil = 1:1:1 (vol:vol:vol). Plants were treated with the combination of two levels of O$_3$ concentration (ambient air [AA] and 1.4 times ambient O$_3$ concentration [1.4 × AA]) and two levels of water irrigation (WW [well-watered, 100% field capacity ≈ 0.295 m$^3$ m$^{-2}$] and WD [water-deficit, 40% field capacity]) during one growing season (1st June to 15th October 2015). Mean hourly O$_3$ concentrations were 35.2 nmol mol$^{-1}$ in AA and 48.9 nmol mol$^{-1}$ in 1.4 × AA. The values of AOT40 (Accumulated exposure Over a Threshold of 40 nmol mol$^{-1}$) were 17.8 μmol mol$^{-1}$ h in AA and 40.3 μmol mol$^{-1}$ h in 1.4 × AA. These levels of O$_3$ have been frequently observed in highly polluted areas of the Northern Hemisphere (Mills et al., 2018). We set three replicated plots (5 × 5 × 2 m) to each O$_3$ treatment with three plants per species per combination of O$_3$ and water (O$_3$ × W). In total, 36 plants per species were utilised in this experiment.

### 2.2 | Measurement of leaf gas exchange

Leaf gas exchange measurements were performed on fully expanded sun leaves with a healthy appearance (one leaf per plant [5th leaf from the tip of the shoot]), on 1–2 plants in each replicated plot of the combination of O$_3$ and W treatments [statistical units: N = 3 plots]) using a Li6400XT portable infra-red gas analyser (Li-Cor instruments) in August 2015 on days with clear sky between 8:00 h and 12:00 h. All target leaves were developed during the experimental treatments (June 2015). Mesophyll conductance cannot be measured directly, target leaves were developed during the experimental treatments August 2015 on days with clear sky between 8:00 h and 12:00 h. All plants were treated with the combination of two levels of O$_3$ concentration (ambient air [AA] and 1.4 times ambient O$_3$ concentration [1.4 × AA]). The values of AOT40 (Accumulated exposure Over a Threshold of 40 nmol mol$^{-1}$) were 17.8 μmol mol$^{-1}$ h in AA and 40.3 μmol mol$^{-1}$ h in 1.4 × AA. These levels of O$_3$ have been frequently observed in highly polluted areas of the Northern Hemisphere (Mills et al., 2018). We set three replicated plots (5 × 5 × 2 m) to each O$_3$ treatment with three plants per species per combination of O$_3$ and water (O$_3$ × W). In total, 36 plants per species were utilised in this experiment.

Flux density (PPFD, 1500 μmol m$^{-2}$ s$^{-1}$), leaf temperature (25°C) and leaf-to-air vapour pressure deficit of 1.6 ± 0.2 KPa. Punctual point measurements of leaf gas exchange and chlorophyll fluorescence were performed at 400 μmol mol$^{-1}$ [CO$_2$]. The leaves were placed into the leaf cuvette and allowed to acclimatise to the cuvette conditions for 15–20 min until gas exchange parameters had remained stable for approximately 5 min. Chlorophyll fluorescence estimation was performed using a single multi-phase flash with an initial saturating pulse of 8000 μmol m$^{-2}$ s$^{-1}$ (Loriaux et al., 2013). Mesophyll conductance ($\delta_{\text{in}CO_2}$) was determined using the variable $J$ method described by Harley et al. (1992):

$$\delta_{\text{in}CO_2} = \frac{PN}{C_i - \frac{I^*}{\Phi_{PSII}} \times (PN + K_i)}$$  

where $I^*$ is the CO$_2$ compensation point of photorespiration, which was calculated using the rubulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) specificity factor estimated for woody hemi-deciduous (for Q. pubescens and Q. robur) and woody evergreen (for Q. ilex) (Galmes et al., 2005). The Kok (1948) method was used to determine respiration in the light ($R_d$) (PPFD levels of 400, 300, 200, 100, 80, 60, 30 μmol m$^{-2}$ s$^{-1}$). At the end of the $R_d$ measurements, the LED light source was turned off for 30 min and respiration in the dark ($R_N$) was measured. The PSII electron transport rate ($J_e$) was calculated as:

$$J_e = PPFD \times \Phi_{PSII} \times \alpha \times \beta,$$

where the partitioning factor between photosystems I and II is considered to be 0.5 ($\beta$); leaf absorbance ($\alpha$) is assumed to be 0.85 (Laisk & Loreto, 1996), and the actual quantum efficiency of PSII ($\Phi_{PSII}$) can be determined as:

$$\Phi_{PSII} = \frac{F_m - F_o}{F_m},$$

where $F_m$ is the maximal fluorescence and $F_o$ is the steady-state fluorescence under light-adapted conditions (Genty et al., 1989). The concentration of [CO$_2$] within the chloroplast envelope ($C_i$) was calculated using the variable $J$ $\delta_{\text{in}CO_2}$ as:

$$C_i = C_0 - \frac{P_N}{\delta_{\text{in}CO_2}},$$

where $C_0$ is the concentration of [CO$_2$] within the internal substomatal air-space. Photorespiration ($R_{ph}$) was calculated following Sharkey (1988):

$$R_{ph} = \frac{P_N + R_d}{\frac{PN}{PN} - 1}.$$
where \( g_{s\text{CO2}} \) is the stomatal conductance for \( \text{CO}_2 \) (\( g_{s\text{H2O}}/1.6 \), where \( g_{s\text{H2O}} \) is the stomatal conductance for water vapour). The responses of net photosynthetic rate (\( P_n \)) to \( C_i \) (\( P_n/C_i \)) curves were obtained according to Centritto et al. (2003). After measurement of the gas exchange and chlorophyll fluorescence parameters at 400 \( \mu \text{mol mol}^{-1} \) [\( \text{CO}_2 \)], the [\( \text{CO}_2 \)] within the cuvette was lowered to 50 \( \mu \text{mol mol}^{-1} \) [\( \text{CO}_2 \)] concentration for 30–60 min to fully open stomata and eliminate any stomatal diffusion constraints to \( P_n \). The \( \text{CO}_2 \) concentration was then increased every 3 min at 10 steps for WW plants (Ca: 50, 140, 220, 300, 400, 600, 800, 1100, 1400, 1700 \( \mu \text{mol mol}^{-1} \)) and 8 steps for WD plants (Ca: 50, 100, 200, 400, 600, 900, 1300, 1700 \( \mu \text{mol mol}^{-1} \)). According to the protocol of Ethier and Livingston (2004), the response of \( P_n \) to \( C_i \) was then utilised to estimate the maximum rate of carboxylation of Ribulose-1,5-bisphosphate (RuBP) (\( V_{\text{max}C} \)) and mesophyll conductance (\( g_{\text{mCO2}} \)).

### 2.3 Measurements of leaf mass per area

After the measurements of photosynthetic parameters, three leaf-disks (1 cm diameter) were taken using a leaf punch (Fujiwara-Seisakujyo) for the determination of the leaf mass per unit area (LMA). The samples were dried in an oven at 70°C for 1 week to reach a constant weight. The LMA was calculated as the ratio of the dry mass to the projected leaf area (g m\(^{-2}\)).

### 2.4 Analysis of foliar abscisic acid concentration

To determine foliar abscisic acid (ABA) concentration, additional leaf disks were collected. They were frozen in liquid N immediately after collection, stored in a deep freezer at −80°C. Leaf samples were freeze-dried and finely ground. Deionised water was added (1:50 weight ratio), the sample incubated on a shaker at 4°C overnight, then centrifuged to collect the aqueous extract. Abscisic acid (ABA) concentration in this extract was determined using a radioimmunoassay using the monoclonal antibody MAC252 (Quarrie et al., 1988).

### 2.5 Data analysis

Data were checked for normal distribution (Kolmogorov–Smirnov test) and homogeneity of variance (Levene’s test). A three-way analysis of variance (ANOVA) was used to examine the effects of species, \( \text{O}_3 \) and \( \text{W} \) treatments on photosynthetic parameters, LMA and ABA contents. In addition, the best ANOVA model was also tested and confirmed by AIC (Akaike Information Criterion). If specific factors or factorial combinations were statistically significant in ANOVA, Tukey’s HSD tests were applied to test significant differences among the \( \text{O}_3 \) and \( \text{W} \) treatments combinations in each species because most parameters were affected by species. A linear regression analysis was applied to investigate possible relationships between \( P_n \) and diffusive limitations to \( \text{CO}_2 \) transport and to describe the possible relative association of ABA or LMA with leaf diffusive conductance to \( \text{CO}_2 \). In addition, principal component analysis (PCA) was applied to characterise the photosynthetic response, diffusive limitations to \( \text{CO}_2 \) transport (\( g_{s\text{CO2}}, g_{\text{mCO2}}, \) and \( g_{\text{mH2O}} \)), and leaf traits (ABA, LMA) of three oak species (\( Q. \) ilex, \( Q. \) pubescens, and \( Q. \) robur) grown under different \( \text{O}_3 \) and water levels. Results were considered significant at \( P < 0.05 \). All statistical analyses were made using R software (R 4.1.2; R Core Team, 2021).

### 3 RESULTS

#### 3.1 Leaf mass per area and abscisic acid content

Averaged LMA values were higher in evergreen than deciduous oak species (Table 1). Leaf mass per area (LMA) of \( Q. \) pubescens was significantly increased by water deficit treatment (+20%), although no \( \text{O}_3 \) effect on LMA was observed in this species. In the other two species (\( Q. \) ilex and \( Q. \) robur), LMA was not affected by either \( \text{O}_3 \) or water deficit treatments.

Abscisic acid (ABA) content was affected by both \( \text{O}_3 \) and water deficit treatments. Elevated \( \text{O}_3 \) singly increased ABA in \( Q. \) robur leaves (+66%). On the other hand, water deficit significantly increased ABA contents in \( Q. \) ilex (+81%) and \( Q. \) robur leaves (+104%). The interaction of three factors (\( \text{O}_3 \times \text{W} \times \text{Sp} \)) was significant, confirming that the combined treatment of \( \text{O}_3 \) and water deficit significantly increased foliar ABA in all three species, and this increase was especially pronounced in \( Q. \) pubescens (+335%).

#### 3.2 Photosynthetic traits

For all parameters except \( R_n \), a full-factorial ANOVA model (\( \text{O}_3, \text{W}, \) Species and their full factorial interactions) was selected as the best one according to AIC (Table S1). Ozone exposure significantly decreased \( P_n \) in two deciduous oaks (\( Q. \) pubescens, \( Q. \) robur), while water deficit stress reduced \( P_n \) in all three species (Figure 1A). There was no effect of \( \text{O}_3 \) and WD on \( R_n \) among the treatments in three oaks (Figure 1B). A lower \( R_n \) was found in WD-treated \( Q. \) robur leaves (−79%, Figure 1C) concomitant with reduced \( P_n \) (−74%, Figure 1A), although no difference in this parameter was found among the \( \text{O}_3 \) and water-deficit treatments in the other two oak species. Ozone and WD treatments significantly affected \( g_{s\text{H2O}}, g_{\text{mCO2}}, \) and \( g_{\text{mH2O}} \), although the effects were dependent on species (Figure 1D–F). In evergreen \( Q. \) ilex, \( g_{s\text{H2O}} \) was significantly reduced by WD treatments (−37%). The values of \( g_{\text{mCO2}} \) were not affected by elevated \( \text{O}_3 \) and WD singly in this species. However, the combined treatment of \( \text{O}_3 \) and WD significantly decreased this parameter (\( g_{\text{mCO2}}: −57\% \)). On the other hand, in deciduous \( Q. \) pubescens and \( Q. \) robur, diffusive conductance parameters were significantly
TABLE 1  Leaf mass per area (LMA) and abscisic acid (ABA) contents in leaves of three Mediterranean oaks (Quercus ilex, Q. pubescens and Q. robur) under different O3 (AA, ambient O3 concentration; 1.4 × AA) and water treatments (WW, well-watered; WD, water-deficit)

|          | LMA (g m⁻²) | ABA (ng g⁻¹) |
|----------|-------------|--------------|
|          | AA          | 1.4 × AA     | AA                        | 1.4 × AA     |
| Quercus ilex |             |              |                          |              |
| WW       | 185.7 ± 3.0 | a            | 172.0 ± 4.3              | a            |
| WD       | 177.9 ± 6.5 | a            | 185.8 ± 6.2              | a            |
|          |             |              | 1132 ± 180               | 1356 ± 84    |
|          |             |              | 2050 ± 253               | 2665 ± 84    |
| Quercus pubescens |             |              |                          |              |
| WW       | 89.7 ± 3.1  | A            | 101.3 ± 6.0              | AB           |
| WD       | 107.2 ± 1.0 | B            | 113.1 ± 3.5              | B            |
|          |             |              | 283 ± 85                 | 383 ± 12     |
|          |             |              | 979 ± 279                | 1230 ± 223   |
| Quercus robur |             |              |                          |              |
| WW       | 71.8 ± 4.3  | a'           | 75.2 ± 3.7               | a'           |
| WD       | 76.7 ± 3.0  | a'           | 82.6 ± 5.1               | a'           |
|          |             |              | 994 ± 152                | 1649 ± 30    |
|          |             |              | 2030 ± 93                | 1790 ± 157   |

ANOVA results

|          |            |            |
|----------|------------|------------|
| O₃       | ns         | **         |
| W        | **         | ***        |
| Sp.      | ***        |            |
| O₃ × W   | ns         |            |
| O₃ × Sp. | ns         |            |
| W × Sp.  | ns         |            |
| O₃ × W × Sp. | ns |            |

Note: Each value is the mean ± SE (n = 3 plots). Asterisks show the significance of ANOVA: ***P < 0.001, **P < 0.01, *P < 0.05, ns: not significant. Different letters show significant differences among treatments within each species (P < 0.05, Tukey test).

Deviated by both O₃ (Q. pubescens, g₅H₂O: −31%, g₅CO₂: −50%, g₅mCO₂: −43%; Q. robur, g₅H₂O: −57%, g₅mCO₂: −43%, g₅mCO₂: −60%) and WD (Q. pubescens, g₅H₂O: −40%, g₅mCO₂: −78%, g₅mCO₂: −54%; Q. robur, g₅H₂O: −62%, g₅mCO₂: −86%, g₅mCO₂: −86%), although the negative effect was more pronounced in WD compared to elevated O₃ treatments. The reduction of Pn was closely correlated with low diffusive conductance of CO₂ (g₅CO₂, g₅mCO₂, and g₅mCO₂) in AA, with the largest correlation coefficient being observed in relationships between Pn and g₅mCO₂ in all three oak species (Figure 2). However, no significant correlation between g₅CO₂ and Pn was found in 1.4 × AA for deciduous oaks, although Pn was tightly related to g₅mCO₂ in this condition. Exposure to 1.4 × AA O₃ induced a reduction in g₅mCO₂, but g₅CO₂ remained broadly similar in Q. pubescens, resulting in a breakdown of the positive correlation between g₅CO₂ and g₅mCO₂ observed in Q. ilex and Q. robur (Figure 3). The combined treatments of O₃ and WD significantly reduced CO₂ concentration within the chloroplast envelope (Cₖ) in the three oak species due to a significant limitation of CO₂ diffusive conductance, although the response of the internal sub-stomatal air-space (Cₖ) to O₃ and WD treatments was dependent on species (Table S2). The fluorescence measurements indicated that the combined treatment of O₃ and WD reduced ΦPSII, especially in two deciduous oaks (Figure 1G). The enhanced O₃ and WD treatment did not affect Vₚmax and Jₚmax in Q. ilex, as confirmed in the Pn/Cₖ curves (Figures 1H–I and 4). On the other hand, significant reductions in the values of Vₚmax and Jₚmax were found in O₃- and WD-treated leaves of Q. pubescens and Q. robur. Values of g₅mCO₂ estimated by the curve fitting method were consistent with those calculated by the variable J method in all three species (Figure S1).

Mesophyll conductance (g₅mCO₂) was negatively correlated with foliar ABA in three oak species (Figure 5). Although there was a negative correlation in the relationships between g₅CO₂ and ABA in Q. ilex and Q. robur, such a negative relationship was not found in Q. pubescens (Figure 5). On the other hand, LMA was negatively correlated with g₅mCO₂ only in Q. pubescens leaves, while there was no significant correlation between LMA and g₅CO₂ (Figure S2).

### 3.3 Principal component analysis

Principal component analysis (PCA) allowed the characterisation of photosynthetic parameters and leaf traits in each oak species grown under different O₃ and water regimes (Figure 6). This analysis indicated two principal components that together explained 76.0% of the variance within the measured data. The principal component 1 explains 62.1% of the variance, showing the effects of O₃ and WD on biochemical and diffusive components of photosynthesis. On the other hand, the second principal component 2 explains 13.9% of variance in relation to leaf morphological traits (i.e. LMA), ABA content and leaf respiratory mechanisms. The multivariate space of two deciduous oaks overlapped and mainly occupied the third and fourth quadrants of the main two PCA coordinates, whereas evergreen Q. ilex occurred in distinct multivariate space compared with the two
deciduous species (Tukey test on PCA scores) Q. ilex versus Q. pubescens, component 1: \( P = 0.058 \), component 2: \( P < 0.001 \); Q. ilex vs. Q. robur, component 1: \( P < 0.001 \), component 2: \( P < 0.001 \).

### 4 DISCUSSION

#### 4.1 Effects of ozone or drought on leaf gas exchange

The PCA analysis revealed that the photosynthetic response to O\(_3\) and WD was different between evergreen Q. ilex and the two deciduous oaks (Figure 6). In the elevated O\(_3\) treatment (1.4 \( \times \) AA O\(_3\) + WW), the two deciduous oaks showed significant decreases in photosynthesis after O\(_3\) exposure with concomitant reductions in \( V_{\text{cmax}} \) and stomatal and mesophyll diffusive conductance (Figure 1A, D, E, H, I), as observed in other deciduous tree species such as Siebold’s beech (Hoshika, Haworth, et al., 2020b). In contrast, Warren et al. (2007) did not find an effect of increased O\(_3\) concentration on \( g_{\text{mCO2}} \) in a deciduous F. sylvatica. Kitao et al. (2009) reported a strong decrease of stomatal conductance in this species during O\(_3\) exposure, which may be a primary cause of the reduction of photosynthetic activity. At the same time, a decrease of stomatal conductance may reduce stomatal O\(_3\) flux to leaves, thus limiting the O\(_3\) damages to photosynthetic systems in mesophyll cells (Hoshika, Fares, et al., 2020a). In fact, Warren et al. (2007) pointed that the difference in stomatal O\(_3\) flux was relatively small between control (ambient O\(_3\)) and elevated O\(_3\) (twice ambient O\(_3\)) treatments in a deciduous F. sylvatica due to an O\(_3\)-induced stomatal closure. Therefore, it should also be noted that the photosynthetic response to O\(_3\) may be species-specific depending on the capacity of avoidance of O\(_3\) entry through stomata. On the other hand, in evergreen Q. ilex, 1.4 \( \times \) AA O\(_3\) exposure in isolation did not affect \( P_n \) (Figure 1A), demonstrating a high resistance capacity to O\(_3\) in Q. ilex as indicated before by a response of biomass to O\(_3\) in this species (Alonso et al., 2014; Hoshika et al., 2018). In general, evergreen species are more resistant to O\(_3\).
than deciduous species (Feng et al., 2018) because they have thicker leaves, which restricts O₃ diffusion inside a leaf due to their lower intercellular air space (Wieser et al., 2002). In addition, O₃-treated *Q. ilex* leaves may activate the phenylpropanoid pathway, which plays a significant role in plant tolerance against oxidative stress through quenching ROS and reducing lipid peroxidation (Cotrozzi et al., 2016; Pellegrini et al., 2019). This is unlikely to be related to the protective antioxidant role of isoprene during O₃ fumigation (Loreto & Velikova, 2001) as *Q. ilex* mainly emits monoterpenes while the deciduous *Quercus* emits isoprenoids (Lehning et al., 1999; Rapparini et al., 2004).

Water deficit significantly reduced *P*ₙ in all three oaks, although different mechanisms of photosynthetic damage were observed between deciduous and evergreen oak species (Figure 1A–I). Previous studies have established that stomatal closure is a major factor in reducing photosynthetic rates due to limited CO₂ supply to the carboxylation sites during water-deficit stress for Mediterranean tree species (Keenan et al., 2009). The reduction of *P*ₙ in *Q. ilex* was associated with a significant decrease of stomatal conductance without any biochemical reduction of RuBP carboxylation capacity (*V*ₖₜₘₐₓ) and RuBP regeneration (expressed as *J*ₘₜₐₓ) (Figure 1A, D, H, I), suggesting that a photosynthetic downregulation was mainly caused by stomatal CO₂ diffusive limitation to photosynthesis in a WD condition. Similar results were also observed in olive (Centritto et al., 2003) and giant reed (*Arundo donax*) (Haworth et al., 2019) subject to drought. On the other hand, in two deciduous oaks, in addition to the reduction of
stomatal conductance, significant reductions of $g_{m\,CO_2}$, $V_c$, and $J_{\text{max}}$ were found in WD treatments (Figure 1A, D, E, H, I). The lack of positive relationship between $g_{s\,CO_2}$ and $g_{m\,CO_2}$ in Q. pubescens may indicate that, while stomata remained open under O$_3$ fumigation, a decline in CO$_2$ uptake resulted in lower transport of CO$_2$ across the mesophyll (Figure 3). In a drought-sensitive oak (Q. robur), an increase in $C_i$ was observed (Table S2), consistent with non-stomatal limitations of photosynthesis such as reduced photosynthesis photochemistry or biochemistry (Killi et al., 2016). This discrepancy in the degree of photosynthetic damage between species was dependent on plant resistance to water deficit. In fact, non-stomatal limitations to photosynthesis due to water deficit are often observed when the stress becomes severe (Flexas et al., 2004; Michelozzi et al., 2011).

### 4.2 Interactive effects of ozone and drought on leaf gas exchange

Net photosynthetic rate was significantly decreased by the combined treatment of $1.4\times$ AA O$_3$ + WD in all three oak species (Figure 1A). However, the interaction of O$_3$ and drought on photosynthetic gas exchange parameters appeared to vary with species. Previous studies suggest that drought stress may induce stomatal closure and thus might protect plants from O$_3$ damage (Khan & Soja, 2003; Tingey & Hogsett, 1985). However, WD did not prevent O$_3$ damage to the photosynthetic physiology, especially in Q. ilex, but rather amplified the negative effects. In fact, the negative effects on $g_{m\,CO_2}$ and $g_{\text{tot}\,CO_2}$ were exacerbated by the combined treatment of O$_3$ and WD in Q. ilex (Figure 1E, F), which results in a significant decrease of CO$_2$ concentration within the chloroplast envelope ($C_i$) in this species, leading to a reduction of photosynthetic rate (Table S2). Hoshika, Fares, et al. (2020a) found that Q. ilex may have activated biochemical defence against O$_3$ stress singly, which may contribute to limiting the accumulation of ROS, such as hydrogen peroxide. However, the combination of O$_3$ and drought alters the antioxidant system in Q. ilex, which may not be sufficient to counteract the severe oxidative damage to photosynthetic mechanisms mediated by the excess generation of ROS in this condition (Alonso et al., 2001; Hoshika, Fares, et al., 2020a; Pellegrini et al., 2019).

### 4.3 Are stomatal and mesophyll conductance under high ozone and drought related to leaf abscisic acid content or leaf mass per area?

Mesophyll conductance ($g_{m\,CO_2}$) may be affected by both physical and biochemical traits (Flexas et al., 2008). We observed negative relationships between $g_{m\,CO_2}$ and ABA in three oak species under O$_3$ and drought (Figure 5), while $g_{m\,CO_2}$ negatively correlated with LMA in Q. pubescens (Figure S2). We found that Q. pubescens trees showed a leaf morphological acclimation to WD treatments by high investment in leaf structural components that resulted in increased LMA values (Table 1). These leaf morphological changes may help to increase the tolerance against hydraulic dysfunction in plants subjected to water deficit conditions (Sancho-Knapik et al., 2021). However, the increase of LMA may be associated with thicker leaves leading to a limitation of CO$_2$ diffusion in the gas phase due to a low sub-stomatal air space and/or liquid phase due to increased cell density and thick cell walls (Fini et al., 2016; Niinemets et al., 2005; Peguero-Pina et al., 2017). In addition, a recent study suggests that the increase in LMA may be related to a change in the cell wall composition upon abiotic stresses, which may affect the variation of $g_{m\,CO_2}$ (Flexas et al., 2021).
FIGURE 4  Responses of net photosynthetic rate ($P_N$) to the sub-stomatal concentration of CO$_2$ ($C_i$) (upper figures) or the concentration of CO$_2$ inside the chloroplast envelope ($C_c$) (bottom figures) for *Quercus ilex* (A, D) *Q. pubescens* (B, E) and *Q. robur* (C, F) grown under two levels of O$_3$ treatments (AA, ambient O$_3$ concentrations; 1.4 × AA) and two levels of water treatments (WW, well-watered; WD, water-deficit). Each value is the mean ± standard error ($n$ = 3 plots).

FIGURE 5  Relationships between relative stomatal conductance ($g_{sCO2}$) or mesophyll conductance ($g_{mCO2}$) and relative leaf abscisic acid (ABA) contents in three Mediterranean oaks (*Quercus ilex*, *Q. pubescens*, and *Q. robur*) grown under two levels of O$_3$ treatments (AA, ambient O$_3$ concentrations; 1.4 × AA) and two levels of water treatments (WW, well-watered; WD, water-deficit). The mean $g_{sCO2}$, $g_{mCO2}$, and ABA values in AA were considered as 100%. Linear regression analyses were applied. Grey lines show the 95% confidential intervals of mean values.
debated the role of ABA in the response of \( g_{\text{mCO2}} \) to drought (e.g. Brunetti et al., 2019). In our experiment, we found that WD increased leaf ABA content in three oaks, while O3 increased [ABA] in \( Q. \) robur leaves (Table 1). As a result, \( g_{\text{mCO2}} \) declined with an increasing concentration of ABA in leaves of the three oak species grown under elevated O3 and drought (Figure 5). Shatil-Cohen et al. (2011) reported that ABA may modify the activation of aquaporins in bundle-sheath cells leading to a decrease of leaf water potential. In fact, three oak species showed a reduction of leaf water potential under O3 and drought in the same experiment (Cocozza et al., 2020). Shrinkage of mesophyll cells due to low leaf water potential may negatively affect \( g_{\text{mCO2}} \) by physically constraining CO2 diffusion within the leaf.

Abscisic acid (ABA) is a crucial phytohormone that induces stomatal closure under WD conditions (Bharath et al., 2021; Davies & Zhang, 1991). However, in two deciduous oaks, a weak or no significant correlation between \( g_{\text{scCO2}} \) and ABA was found under the combination of O3 and drought (Figure 5B, C). It is known that O3 frequently affects stomatal physiological function resulting in the impaired physiological control of stomatal aperture, i.e. a slower or less sensitive stomatal control (Hoshika et al., 2019). Mills et al. (2009) reported that O3 may reduce the sensitivity of stomata to ABA in Leontodon hispidus. Although the mechanisms are still unclear, the less sensitive stomatal response may be related to O3-induced ethylene emissions (Hoshika et al., 2019; Wilkinson & Davies, 2010). Changes in stomatal function will influence the availability of CO2 in the internal sub-stomatal leaf air-space (Klll et al., 2016). In fact, a significant increase in \( C_i \) was observed in \( Q. \) pubescens subjected to the combined treatment of O3 and WD (Table S2). This may feedback into the rate of CO2 movement across the mesophyll to the chloroplast. As \( g_{\text{mCO2}} \) is a flux-weighted function affected by the interaction of CO2 availability, biochemical/physical transport constraints and CO2 uptake in the chloroplast (Tholen et al., 2012), shifts in stomatal physiological function, mesophyll properties or the biochemistry of photosynthesis induced by O3 fumigation are likely to be evident in rates of CO2 transport across the mesophyll layer. It has been reported that the mesophyll signal may control stomatal response to several environmental stimuli, suggesting that there is a coordination between mesophyll CO2 demand and stomatal behaviour (Fujita et al., 2013, 2019; Mott et al., 2008). Therefore, the impaired physiological response of stomata might be a compensatory one to promote CO2 diffusion to sub-stomatal cavities under elevated O3 (Watanabe et al., 2014). However, this was not sufficient to enhance total diffusive conductance to CO2 (\( g_{\text{totCO2}} \)) under elevated O3 in the three oak species examined in this study (Figure 1F).

Another important factor influencing \( C_i \) is non-uniform stomatal apertures, i.e. stomatal patchiness, which may be caused by drought or O3 exposure (Omasa et al., 2002; Terashima, 1992). Stomatal patchiness may result in an overestimation of \( C_i \) thus leading to an
underestimation of $g_{\text{mCO}_2}$. In the present study, however, the minimum value of measured stomatal conductance was 35–40 mmol m$^{-2}$ in the three oak species, where large errors in $C_i$ calculation due to stomatal patchiness are not expected (Buckley et al., 1997). This suggests that the overestimation of $C_i$ was negligible on the calculation of $g_{\text{mCO}_2}$ in the three oak species and it is reinforced by the close correlation between the variable $J$ and the curve-fitting estimates of $g_{\text{mCO}_2}$ in all three species (Figure S1).

5 | CONCLUSION

The results of this study indicate that the photosynthetic response to $O_3$ and drought was different between a Mediterranean evergreen $Q$. ilex, and deciduous $Q$. pubescens and $Q$. robur. Drought or elevated $O_3$ caused oxidative damage to the photosynthetic systems in the two deciduous oaks. In contrast, drought stress negatively affected photosynthesis in evergreen $Q$. ilex, although single $O_3$ treatment did not. However, the combination of $O_3$ and drought reduced the net photosynthetic rate regardless of the species. The reduction of photosynthesis was associated with a reduction of $g_{\text{CO}_2}$ and $g_{\text{mCO}_2}$ in $Q$. ilex, while the two deciduous oaks also showed a reduction of $V_{\text{cmax}}$ and $J_{\text{max}}$ with increased diffusive resistance to CO$_2$ transport. The reduction of $g_{\text{mCO}_2}$ was correlated with foliar [ABA] in the three oaks, while a negative correlation of $g_{\text{mCO}_2}$ with LMA was found in $Q$. pubescens. However, in two deciduous oaks, a weak or no significant correlation between $g_{\text{CO}_2}$ and [ABA] was found under the combination of $O_3$ and drought due to the impaired physiological response of stomata. In fact, net photosynthesis was closely correlated with $g_{\text{mCO}_2}$ rather than $g_{\text{CO}_2}$ in the two deciduous oaks when treated with $O_3$ and WD factors together. The results suggest that $g_{\text{mCO}_2}$ plays a significant role in carbon gain in plants under concurrent increases in the severity of droughts and $O_3$ pollution. Since several studies found a recovery from photosynthetic damage after cessation of $O_3$ fumigation (Watanabe et al., 2014) or drought treatment (Michelozzi et al., 2011), further studies focusing on the recovery phase will also be needed to better understand photosynthetic acclimations in complex environments such as the Mediterranean that are frequently subject to $O_3$ and drought.

ACKNOWLEDGEMENTS

We are grateful to Moreno Lazzara and Alessandro Materassi for technical support. We are also grateful for financial support to the Fondazione Cassa di Risparmio di Firenze (2013/7956) and the LIFE+ project MOT-TLES (LIFE15 ENV/IT/000183) of the European Commission.

Open access funding enabled and organized by Projekt DEAL.

AUTHOR CONTRIBUTIONS

Conceptualization: Yasutomo Hoshika, Matthew Haworth, Mauro Centritto and Elena Paoletti; Data curation: Yasutomo Hoshika, Matthew Haworth, Marcos Thiago Gaudio Gomes and Jaime Puértolas; Formal analysis: Yasutomo Hoshika and Matthew Haworth; Methodology: Yasutomo Hoshika, Matthew Haworth and Mauro Centritto; Writing - original draft: Yasutomo Hoshika; Writing-review & editing: Matthew Haworth, Marcos Thiago Gaudio Gomes, Jaime Puértolas, Mauro Centritto and Elena Paoletti.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as all new created data is already contained within this article.

ORCID

Yasutomo Hoshika https://orcid.org/0000-0002-5263-2945

REFERENCES

Alonso, R., Elvira, S., Castillo, F.J. & Gimeno, B.S. (2001) Interactive effects of ozone and drought stress on pigments and activities of antioxidative enzymes in Pinus halophila. Plant, Cell & Environment, 24, 905–916.

Alonso, R., Elvira, S., González-Fernández, I., Calvete, H., García-Gómez, H. & Bermejo, V. (2014) Drought stress does not protect Quercus ilex L. from ozone effects: results from a comparative study of two subspecies differing in ozone sensitivity. Plant Biology, 16, 375–384.

Bagard, M., Jolivet, Y., Hasenfratz-Sauder, M.-P., Gérard, J., Dizengremel, P. & Le Thiec, D. (2015) Ozone exposure and flux-based response functions for photosynthetic traits in wheat, maize and poplar. Environmental Pollution, 206, 411–420.

Bharath, P., Gahir, S. & Raghavendra, A.S. (2021) Abscisic acid-induced stomatal closure: an important component of plant defense against abiotic and biotic stress. Frontiers in Plant Science, 12, 615114.

Brunetti, C., Gori, A., Marino, G., Latini, P., Sobolev, A.P., Nardini, A. et al. (2019) Dynamic changes in ABA content in water-stressed Populus nigra: effects on carbon fixation and soluble carbohydrates. Annals of Botany, 124, 627–644.

Buckley, T.N., Farquhar, G.D. & Mott, K.A. (1997) Qualitative effects of patchy stomatal conductance distribution features on gas-exchange calculations. Plant, Cell & Environment, 20, 867–880.

Centritto, M., Lauteri, M., Monteverdi, M.C. & Serraj, R. (2009) Leaf gas exchange, carbon isotope discrimination, and grain yield in contrasting rice genotypes subjected to water deficits during the reproductive stage. Journal of Experimental Botany, 60, 2325–2339.

Centritto, M., Loreto, F. & Chartzoulakis, K. (2003) The use of low [CO$_2$] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. Plant, Cell & Environment, 26, 585–594.

Cocozza, C., Paoletti, E., Mrak, T., Zavaadlav, S., Levanič, T., Kraigher, H. et al. (2020) Isotopic and water relation responses to ozone and water stress in three oak species with different adaptation strategies. Forests, 11, 864.

Cotrozzì, L., Remorini, D., Pellegrini, E., Landi, M., Massai, R., Nali, C. et al. (2016) Variations in physiological and biochemical traits of oak seedlings grown under drought and ozone stress. Physiologia Plantarum, 157, 69–84.

Davies, W.J. & Zhang, J.H. (1991) Root signals and the regulation of growth and development of plants in drying soil. Annual Review of Plant Physiology and Plant Molecular Biology, 42, 55–76.

Eichelmann, H., Oja, V., Rasulov, B., Padu, E., Bichele, I., Pettai, H. et al. (2004) Photosynthetic parameters of birch (Betula pendula Roth) leaves growing in normal and in CO$_2$- and O$_3$-enriched atmospheres. Plant, Cell & Environment, 27, 479–495.

Ether, G.J. & Livingston, N.J. (2004) On the need to incorporate sensitivity to CO$_2$ transfer conductance into the Farquhar-von Caemmerer-berry leaf photosynthesis model. Plant, Cell & Environment, 27, 137–153.
Fleck, I., Peña-Rojas, K. & Aranda, X. (2010) Mesophyll conductance to

Harley, P.C., Loreto, F., Dimarco, G. & Sharkey, T.D. (1992) Theoretical

Fini, A., Loreto, F., Tattini, M., Giordano, C., Ferrini, F., Brunetti, C. et al. (2016) Mesophyll conductance plays a central role in leaf functioning of Oleaceae species exposed to contrasting sunlight irradiance. Physiologia Plantarum, 157, 54–68.

Fleck, I., Peña-Rojas, K. & Aranda, X. (2010) Mesophyll conductance to CO2 and leaf morphological characteristics under drought stress during Quercus ilex L. respouting. Annals of Forest Science, 67, 308.

Flexas, J., Bota, J., Loreto, F., Comín, G. & Sharkey, T.D. (2004) Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biology, 6, 269–279.

Flexas, J., Clemente-Moreno, M.J., Bota, J., Brodribb, T.J., Gago, J., Mizokami, Y. et al. (2021) Cell wall thickness and composition are involved in photosynthetic limitation. Journal of Experimental Botany, 72, 3971–3986.

Flexas, J., Ribas-Carbo, M., Díaz-Espejo, A., Galmés, J. & Medrano, H. (2008) Mesophyll conductance to CO2: current knowledge and future prospects. Plant, Cell & Environment, 31, 602–621.

Flowers, M.D., Fiscus, E.L., Burkey, K.O., Booker, F.L. & Dubois, J.B. (2007) Photosynthesis, chlorophyll fluorescence, and yield of snap bean (Phaseolus vulgaris L.) genotypes differing in sensitivity to ozone. Environmental and Experimental Botany, 61, 190–198.

Fujita, T., Noguchi, K. & Terashima, I. (2013) Apoplastic mesophyll signals induce rapid stomatal responses to CO2 in Commelina communis. The New Phytologist, 199, 395–406.

Fujita, T., Noguchi, K. & Terashima, I. (2019) Confirmation of mesophyll signals controlling stomatal responses by a newly devised transplanting method. Functional Plant Biology, 46, 467–481.

Galmés, J., Flexas, J., Keys, A.J., Cifre, J., Mitchell, R.A.C., Madgwick, P.J. et al. (2005) Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. Plant, Cell & Environment, 28, 571–579.

Gao, F., Calatayud, V., García-Breijo, F., Reig-Armiriana, J. & Feng, Z. (2016) Effects of elevated ozone on physiological, anatomical and ultrastructural characteristics of four common urban tree species in China. Ecological Indicators, 67, 367–379. https://doi.org/10.1016/j.ecolind.2016.03.012

Genty, B., Briantais, J.-M. & Baker, N.R. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta (BBA) - General Subjects, 990, 87–92.

Giorgi, F. & Gutowski, W.J. (2016) Coordinated experiments for projections of regional climate change. Current Climate Change Reports, 2, 202–210.

Grulke, N.E. & Heath, R.L. (2020) Ozone effects on plants in natural ecosystems. Plant Biology, 22(1), 12–37.

Günthardt-Goerg, M.S., Matyssek, R., Scheidegger, C. & Keller, T. (1993) Differentiation and structural decline in the leaves and bark of birch (Betula pendula) under low ozone concentrations. Trees, 7, 104–114.

Harley, P.C., Loreto, F., Dimarco, G. & Sharkey, T.D. (1992) Theoretical considerations when estimating the mesophyll conductance to CO2 flux by analysis of the response of photosynthesis to CO2. Plant Physiology, 98, 1429–1436.

Haworth, M., Consantino, S.L., Marino, G., Brunetti, C., Riggi, E., Avola, G. et al. (2018a) Increased free abscisic acid during drought enhances stomatal sensitivity and modifies stomatal behaviour in fast growing giant reed (Arundo donax L.). Environmental and Experimental Botany, 147, 116–124.

Haworth, M., Consantino, S.L., Marino, G., Brunetti, C., Scordia, D., Testa, G. et al. (2017) Physiological responses of Arundo donax ecotypes to drought: a common garden study. CCB Bioenergy, 9, 132–143.

Haworth, M., Marino, G. & Centritto, M. (2018b) An introductory guide to gas exchange analysis of photosynthesis and its application to plant phenotyping and precision irrigation to enhance water use efficiency. Journal of Water and Climate Change, 9, 786–808.

Haworth, M., Marino, G., Riggi, E., Avola, G., Brunetti, C., Scordia, D. et al. (2019) The effect of summer drought on the yield of Arundo donax is reduced by the retention of photosynthetic capacity and leaf growth later in the growing season. Annals of Botany, 124, 567–579.

Haworth, M. & Raschi, A. (2014) An assessment of the use of epidermal micro-morphological features to estimate leaf economics of Late Triassic–Early Jurassic fossil Ginkgoales. Review of Palaeobotany and Palynology, 205, 1–8.

Hoshika, Y., De Carlo, A., Baraldi, R., Neri, L., Carrari, E., Agathokleous, E. et al. (2019) Ozone-induced impairment of night-time stomatal closure in O3-sensitive poplar clone is affected by nitrogen but not by phosphorus enrichment. Science of the Total Environment, 692, 713–722.

Hoshika, Y., Fares, S., Pellegrini, E., Conte, A. & Paoletti, E. (2020a) Water use strategy affects avoidance of ozone stress by stomatal closure in Mediterranean trees—a modelling analysis. Plant, Cell & Environment, 43, 611–623.

Hoshika, Y., Haworth, M., Watanabe, M. & Koike, T. (2020b) Interactive effect of leaf age and ozone on mesophyll conductance in Siebold’s beech. Physiologia Plantarum, 170, 172–186.

Hoshika, Y., Moura, B. & Paoletti, E. (2018) Ozone risk assessment in three oak species as affected by soil water availability. Environmental Science and Pollution Research, 25, 8125–8136.

Khan, S. & Soja, G. (2003) Yield responses of wheat to ozone exposure as modified by drought-induced differences in ozone uptake. Water, Air and Soil Pollution, 147, 299–315.

Kangasjärvi, J., Jaspers, P. & Kollist, H. (2005) Signalling and cell death in ozone-exposed plants. Plant, Cell & Environment, 28, 1021–1036.

Keenan, T., García, R., Friend, A.D., Zaehle, S., Gracia, C. & Sabate, S. (2009) Improved understanding of drought controls on seasonal variation in Mediterranean forest canopy CO2 and water fluxes through combined in situ measurements and ecosystem modelling. Biogeosciences, 6, 1423–1444.

Killi, D., Bussotti, F., Raschi, A. & Haworth, M. (2016) Adaptation to high temperature mitigates the impact of water deficit during combined heat and drought stress in C3 sunflower and C4 maize varieties with contrasting drought tolerance. Physiologia Plantarum, 159, 130–147.

Killi, D. & Haworth, M. (2017) Diffusive and metabolic constraints to photosynthesis in quinoa during drought and salt stress. Plants, 6, 49.

Kitaö, M., Löw, M., Heerdt, C., Grams, T.E.E., Häberle, K.-H. & Matsuyssek, R. (2009) Effects of chronic elevated ozone exposure on gas exchange responses of adult beech trees (Fagus sylvatica) as related to the within-canopy light gradient. Environmental Pollution, 157, 537–544.

Kok, B. (1948) A critical consideration of the quantum yield of chlorella photosynthesis. Enzymologia, 13, 1–56.

Laik, A. & Loreto, F. (1996) Determining photosynthetic parameters from leaf CO2 exchange and chlorophyll fluorescence - ribulose-1,5-bisphosphate carboxylase oxygenase specificity factor, dark respiration in the light, excitation distribution between photosystems, alternative electron transport rate, and mesophyll diffusion resistance. Plant Physiology, 110, 903–912.

Larcher, W. (2003) Physiological plant ecology 4th edition. New York: Springer-Verlag.

Lauteri, M., Haworth, M., Serraj, R., Monteverdi, M.C. & Centritto, M. (2014) Photosynthetic diffusional constraints affect yield in drought stressed rice cultivars during flowering. PLoS One, 9, e109054.
Lehning, A., Zimmer, I., Steinbrecher, R., Brüggemann, N. & Schnitzler, J.P. (1999) Isoprene synthase activity and its relation to isoprene emission in Quercus robur L. leaves. Plant, Cell & Environment, 22, 495–504. https://doi.org/10.1046/j.1365-3040.1999.00425.x

Li, P., Feng, Z., Catalayud, V., Yuan, X., Xu, Y. & Paolletti, E. (2017) A meta-analysis on growth, physiological, and biochemical responses of woody species to ground-level ozone highlights the role of plant functional types. Plant, Cell & Environment, 40, 2369–2380.

Loreto, F., Harley, P.C., Di Marco, G. & Sharkey, T.D. (1992) Estimation of mesophyll conductance to CO₂ flux by three different methods. Plant Physiology, 98, 1437–1443.

Loreto, F. & Velikova, V. (2001) Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiology, 127, 1781–1787.

Loroux, S.D., Avenson, T.J., Welles, J.M., Mcdermitt, D.K., Eckles, R.D., Riense, B. et al. (2013) Closing in on maximum yield of chlorophyll fluorescence using a single multiphase flash of sub-saturating intensity. Plant, Cell & Environment, 36, 1755–1770.

Marino, G., Haworth, M., Scartazzia, A., Tognetti, R. & Centritto, M. (2020) A comparison of the variable J and carbon-isotopic composition of sugars methods to assess mesophyll conductance from the leaf to the canopy scale in drought-stressed cherry. International Journal of Molecular Sciences, 21(4), 1222.

Matyssek, R., Günthardt-Goerg, M.S., Keller, T. & Scheidegger, C. (1991) Impairment of gas exchange and structure in birch leaves (Betula pendula) caused by low ozone concentrations. Trees, 5, 5–13.

McAdam, S.A.M. & Brodrrib, T.J. (2018) Mesophyll cells are the main site of abscisic acid biosynthesis in water-stressed leaves. Plant Physiology, 177, 911–917.

McAdam, E., Brodrrib, T.J. & McAdam, S.A.M. (2017) Does ozone increase ABA levels by non-enzymatic synthesis causing stomata to close? Plant, Cell & Environment, 40, 741–747.

Michelozzi, M., Loreto, F., Colom, R., Rossi, F. & Calamassi, R. (2011) Drought responses in Aleppo pine seedlings from two wild provenances with different climatic features. Photosynthetica, 49, 564–572.

Milla-Moreno, E.A., McKown, A.D., Guy, R.D. & Soolanayakanahally, R.Y. (2016) Leaf mass per area predicts palisade structural properties linked to mesophyll conductance in balsam poplar (Populus balsamifera L.). Botany, 94, 1–45.

Mills, G., Hayes, F., Wilkinson, S. & Davies, W.J. (2009) Chronic exposure to increasing background ozone impairs stomatal functioning in grassland species. Global Change Biology, 15, 1522–1533.

Mills, G., Pleijel, H., Malley, C.S., Sinha, B., Cooper, O., Schultz, M. et al. (2018) Tropospheric ozone assessment report: present-dy tropospheric ozone distribution and trends relevant to vegetation. Elementa Science of the Anthropocene, 6(1), 47. https://doi.org/10.1525/elementa.302

Mizokami, Y., Noguchi, K., Kojima, M., Sakakibara, H. & Terashima, I. (2015) Mesophyll conductance decreases in the wild type but not in an ABA-deficient mutant (aba1) of Nicotiana plumbaginifolia under drought conditions. Plant, Cell & Environment, 38, 388–398.

Mott, K.A., Sibbern, E.D. & Shope, J.C. (2008) The role of the mesophyll in stomatal responses to light and CO₂. Plant, Cell & Environment, 31, 1299–1306.

Nadal, M., Carriqui, M. & Flexas, J. (2021) Chapter 3: Mesophyll conductance to CO₂ diffusion in a climate change scenario: effects of elevated CO₂, temperature and water stress. In: Becklin, K.M., Ward, J. K. & Way, D.A. (Eds.) Photosynthesis, respiration, and climate change. Advances in photosynthesis and respiration, including bioenergy and related processes, Vol. 46. Dordrecht: Springer, pp. 49–78.

Niinemets, Ü., Cescatti, A., Rodeghiero, M. & Tosens, T. (2005) Leaf internal diffusion conductance limits photosynthesis more strongly in older leaves of Mediterranean evergreen broad-leaved species. Plant, Cell & Environment, 28, 1552–1566.

Ochoa-Hueso, R., Munzi, S., Alonso, R., Arróniz-Crespo, M., Avila, A., Bermejo, V. et al. (2017) Ecological impacts of atmospheric pollution and interactions with climate change in terrestrial ecosystems of the Mediterranean Basin: current research and future directions. Environmental Pollution, 227, 194–206.

Omasa, K., Saji, H., Youssefian, S. & Kondo, K. (2002) Air pollution and plant biotechnology. Tokyo: Springer-Verlag.

Paolletti, E. (2006) Impact of ozone on Mediterranean forests: a review. Environmental Pollution, 144, 463–474.

Paolletti, E., Alivernini, A., Anav, A., Badea, O., Carrari, E., Chivulescu, S. et al. (2019) Toward stomatal–flux based forest protection against ozone: the MOTTLES approach. Science of the Total Environment, 691, 516–527.

Paolletti, E., ConTRAN, N., Bernasconi, P., Günthardt-Goerg, M.S. & Vollenweider, P. (2009) Structural and physiological responses to ozone in manna ash (Fraxinus ornus L.) leaves of seedlings and mature trees under controlled and ambient conditions. Science of the Total Environment, 407, 1631–1643.

Pons, T.L., Flexas, J., von Caemmerer, S., Evans, J.R., Genty, B., Ribas-Carbo, M. et al. (2009) Estimating mesophyll conductance to CO₂: methodology, potential errors, and recommendations. Journal of Experimental Botany, 60, 2217–2234.

Quarrie, S.A., Whitford, P.N., Appleford, N.E.J., Wang, T.L., Cook, S.K., Henson, L.E. et al. (1988) A monoclonal antibody to (S)-abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereals leaf lupins. Planta, 173, 330–339.

R Core Team. (2021) R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.R-project.org/

Rapparini, F., Baraldi, R., Miglietta, F. & Loreto, F. (2004) Isoprenoid emission in trees of Quercus pubescens and Quercus ilex with lifetime exposure to naturally high CO₂ environment. Plant, Cell & Environment, 27, 381–391.

Sancho-Knapik, D., Escudero, A., Mediavilla, S., Scoffoni, C., Zailaa, J., Cavender-Bares, J. et al. (2021) Deciduous and evergreen oaks show contrasting adaptive responses in leaf mass per area across environments. The New Phytologist, 238, 1182–1197.

Sharkey, T.D. (1988) Estimating the rate of photorespiration in leaves. Physiology Plantarum, 74, 147–152.

Shatil-Cohen, A., Attia, Z. & Moshelion, M. (2011) Bundle-sheath cell regulation of xylem-mesophyll water transport via aquaporins under drought stress: a target of xylem-borne ABA? The Plant Journal, 67, 72–80.
Sorrentino, G., Haworth, M., Wahbi, S., Mahmood, T., Zuomin, S. & Centritto, M. (2016) Abscisic acid induces rapid reductions in mesophyll conductance to carbon dioxide. PLoS One, 11, e0148554.

Tardieu, F. & Davies, W.J. (1993) Integration of hydraulic and chemical signalling in the control of stomatal conductance and water status of droughted plants. Plant, Cell & Environment, 16, 341–349.

Terashima, I. (1992) Anatomy of non-uniform leaf photosynthesis. Photosynthesis Research, 31, 195–212.

Tholen, D., Ethier, G., Genty, B., Pepin, S. & Zhu, X.-G. (2012) Variable mesophyll conductance revisited: theoretical background and experimental implications. Plant, Cell & Environment, 35, 2087–2103.

Tingey, D.T. & Hogsett, W. (1985) Water stress reduces ozone injury via a stomatal mechanism. Plant Physiology, 77, 944–947.

Vainonen, J.P. & Kangasjärvi, J. (2015) Plant signaling in acute ozone exposure. Plant, Cell & Environment, 38, 240–252.

Warren, C.R., Löw, M., Matyssek, R. & Tausz, M. (2007) Internal conductance to CO2 transfer of adult Fagus sylvatica: variation between sun and shade leaves and due to free-air ozone fumigation. Environmental and Experimental Botany, 59, 130–138.

Watanabe, M., Hoshika, Y., Inada, N., Wang, X., Mao, Q. & Koike, T. (2013) Photosynthetic traits of Siebold’s beech and oak saplings grown under free air ozone exposure. Environmental Pollution, 174, 50–56.

Watanabe, M., Hoshika, Y. & Koike, T. (2014) Photosynthetic responses of monarch birch seedlings to differing timings of free air ozone fumigation. Journal of Plant Research, 127, 339–345.

Watanabe, M., Komimaki, Y., Mori, M., Okabe, S., Arakawa, I., Kinose, Y. et al. (2018) Mesophyll conductance to CO2 in leaves of Siebold’s beech (Fagus crenata) seedlings under elevated ozone. Journal of Plant Research, 131, 907–914.

Wieser, G., Tegischer, K., Tausz, M., Häberle, K.-H., Grams, T.E.E. & Matyssek, R. (2002) Age effects on Norway spruce (Picea abies) susceptibility to ozone uptake: a novel approach relating stress avoidance to defense. Tree Physiology, 22, 583–590.

Wilkinson, S. & Davies, W.J. (2010) Drought, ozone, ABA and ethylene: new insights from cell to plant to community. Plant, Cell & Environment, 33, 510–525.

Wittig, V.E., Ainsworth, E.A., Naidu, S.L., Karnosky, D.F. & Long, S.P. (2009) Quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: a quantitative meta-analysis. Global Change Biology, 15, 396–424.

Xu, Y., Feng, Z., Shang, B., Dai, L., Uddling, J. & Tarvainen, L. (2019) Mesophyll conductance limitation of photosynthesis in poplar under elevated ozone. Science of the Total Environment, 657, 136–145.

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

**How to cite this article:** Hoshika, Y., Paoletti, E., Centritto, M., Gomes, M.T.G., Puértolas, J. & Haworth, M. (2022) Species-specific variation of photosynthesis and mesophyll conductance to ozone and drought in three Mediterranean oaks. Physiologia Plantarum, 174(1), e13639. Available from: https://doi.org/10.1111/ppl.13639