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

Yasutomo Hoshika[1] | Elena Paoletti[1] | Mauro Centritto[2] |
Marcos Thiago Gaudio Gomes[2] | Jaime Puértolas[3] | Matthew Haworth[2]

[1]Institute of Research on Terrestrial Ecosystems (IRET), National Research Council of Italy (CNR), Sesto Fiorentino

[2]Institute of Sustainable Plant Protection (IPSP), National Research Council of Italy (CNR), Sesto Fiorentino, Italy

[3]Lancaster 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 Espírito Santo, Goiabeiras, CEP 29075-910, Vitória, Espírito Santo, Brazil

Jaime Puértolas, Department of Botany and Plant Ecology and Physiology, University of La Laguna, San Cristóbal de La Laguna, Spain

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Abstract

Mesophyll conductance (g_{mCO2}) is one of the most important components in plant photosynthesis. Tropospheric ozone (O_3) and drought impair physiological processes, causing damage to photosynthetic systems. However, the combined effects of O_3 and drought on g_{mCO2} are still largely unclear. We investigated leaf gas exchange during mid-summer in three Mediterranean oaks exposed to O_3 (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 CO_2 inside a leaf. The combination of O_3 and WD significantly decreased net photosynthetic rate (P_N) 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 O_3 and WD due to impaired stomatal physiological behaviour, indicating that the reduction of P_N 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 O_3 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 (O_3) 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 O_3 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 O_3 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 O_3 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 O_3 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 CO_2 (Larcher, 2003). Ozone frequently decreases net photosynthetic rate (P_N) in association with increased diffusion resistance to CO_2 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 CO_2 through the stomata and across the mesophyll layer represents the two greatest diffusive limitations to P_N (Centritto et al., 2003; Lauteri et al., 2014). Ozone causes stomatal closure due to the increased production of phytohormones such as abscisic acid (ABA) (Kangasjärvi et al., 2005; McAdam et al., 2017). In addition, several studies indicate that O_3 may also cause a reduction of mesophyll conductance (g_{mCO2}) 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., 2018) although Warren et al. (2007) did not find such an effect of O_3 on g_{mCO2} in *Fagus sylvatica*. Mesophyll conductance (g_{mCO2}) consists of both physical and biochemical components in the transport of CO_2 (Flexas et al., 2008; Loreto et al., 1992). Although the mechanisms of the effects of O_3 on g_{mCO2} 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 g_{mCO2} was accompanied by structural changes in mesophyll cells of O_3-exposed beech leaves. This may be supported by the fact that O_3 collapses mesophyll cells or alters mesophyll ultrastructure and other leaf morphological parameters, which may reduce the CO_2 diffusion inside leaves (Gao et al., 2016; Matyssek et al., 1991; Paoletti et al., 2009). In addition, O_3 also modifies the activation of aquaporins, thus increasing the mesophyll diffusion resistance to CO_2 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 g_{mCO2} to LMA was found according to a literature data review, which indicates that the maximum g_{mCO2} 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 O_3 exposure (Shang et al., 2019), suggesting that the effect of O_3 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 CO_2 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 g_{mCO2} observed as foliar [ABA] rises is caused by a reduction in stomatal conductance, lowering CO_2 availability in the internal leaf air-space, and a reduction in the biochemical transport of CO_2 across the mesophyll (likely associated with reduced aquaporin activity; Sorrentino et al., 2016). Abscisic acid plays a pivotal role in the reduction of g_{mCO2} 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 g_{mCO2} 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 O_3 flux and thus limit O_3 damage (Khan & Soja, 2003; Tingey & Hogsett, 1985). However, according to recent manipulative experiments, water deficits may instead exacerbate the negative effects of O_3 on plants (e.g. Cotrozzi et al., 2016). In fact, the combination of O_3 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; Cotrozzi 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 CO_2 diffusion in plants grown in combined O_3 fumigation and drought. However, to the best of our knowledge, no data are available for g_{mCO2} in plant leaves subjected to both high O_3 concentration and drought, and it is still unclear if there is an interaction of O_3 and drought on diffusion resistance to CO_2 transport in the mesophyll layer.

We examined the interactive effects of O_3 and drought on leaf gas exchange in a free-air O_3 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 O_3 and

drought on photosynthetic traits, especially on g_{mCO2} calculated from both the variable J (Harley et al., 1992; Loreto et al., 1992) and curve fitting methods (Ethier & Livingston, 2004)? (2) Which of the traits (ABA and LMA) is involved in the response of g_{mCO2} 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^{-3}] 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, and instead has to be gauged indirectly. This can lead to uncertainty and errors in the measurement of g_{mCO2}. It is preferable to utilise two complementary methodologies to reflect g_{mCO2}, especially under experimental conditions that could have pronounced effects on CO_2 transport across the mesophyll (Marino et al., 2020; Pons et al., 2009). In this study, we chose to utilise the variable J (Harley et al., 1992) and curve fitting (Ethier & Livingston, 2004) methods. Measurements were conducted under controlled condition of photosynthetic photon

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 (g_{mCO2}) was determined using the variable J method described by Harley et al. (1992):

$$g_{mCO2} = \frac{PN}{C_i - \frac{\Gamma^* \times [J_F + 8 \times (PN + R_d)]}{J_F - 4 \times (PN + R_d)}}, \quad (1)$$

where Γ^* is the CO_2 compensation point of photorespiration, which was calculated using the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) specificity factor estimated for woody hemideciduous (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_F) was calculated as:

$$J_F = PPFD \times \Phi PSII \times \alpha \times \beta, \quad (2)$$

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

$$\Phi PSII = \frac{F'_m - F_s}{F'_m}, \quad (3)$$

where F'_m is the maximal fluorescence and F_S is the steady-state fluorescence under light-adapted conditions (Genty et al., 1989). The concentration of [CO_2] within the chloroplast envelope (C_c) was calculated using the variable J g_{mCO2} as:

$$C_c = C_i - \frac{P_N}{g_{mCO2}}, \quad (4)$$

where C_i is the concentration of [CO_2] within the internal substomatal air-space. Photorespiration (R_{PR}) was calculated following Sharkey (1988):

$$R_{PR} = \frac{P_N + R_d}{\frac{C_c}{\Gamma^*} - 1}. \quad (5)$$

Total conductance to CO_2 (g_{totCO2}) was calculated as Haworth, Marino, et al. (2018b):

$$g_{totCO2} = [g_{sCO2} \cdot g_{mCO2}] / [g_{sCO2} + g_{mCO2}], \tag{6}$$

where g_{sCO2} is the stomatal conductance for CO_2 ($g_{sH2O}/1.6$, where g_{sH2O} 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 μmol mol^{-1} [CO_2], the [CO_2] within the cuvette was lowered to 50 μmol mol^{-1} [CO_2] concentration for 30–60 min to fully open stomata and eliminate any stomatal diffusion constraints to P_N. The 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 μmol mol^{-1}) and 8 steps for WD plants (Ca: 50, 100, 200, 400, 600, 900, 1300, 1700 μmol 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 RubisCO (V_{cmax}), the maximum rate of electron transport driving regeneration of ribulose-1,5-bisphosphate (RuBP) (J_{max}) and mesophyll conductance (g_{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-Seisakujo) 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, O_3 and 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 O_3 and 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 CO_2 transport and to describe the possible relative association of ABA or LMA with leaf diffusive conductance to CO_2. In addition, principal component analysis (PCA) was applied to characterise the photosynthetic response, diffusive limitations to CO_2 transport (g_{sCO2}, g_{mCO2}, and g_{totCO2}), and leaf traits (ABA, LMA) of three oak species (Q. ilex, Q. pubescens, and Q. robur) grown under different 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 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 O_3 or water deficit treatments.

Abscisic acid (ABA) content was affected by both O_3 and water deficit treatments. Elevated 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 ($O_3 \times W \times$ Sp.) was significant, confirming that the combined treatment of 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 (O_3, 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 O_3 and WD on R_n among the treatments in three oaks (Figure 1B). A lower R_{PR} 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 O_3 and water-deficit treatments in the other two oak species. Ozone and WD treatments significantly affected g_{sH2O}, g_{mCO2} and g_{totCO2}, although the effects were dependent on species (Figure 1D–F). In evergreen Q. ilex, g_{sH2O} was significantly reduced by WD treatments (−37%). The values of g_{mCO2} were not affected by elevated O_3 and WD singly in this species. However, the combined treatment of O_3 and WD significantly decreased this parameter (g_{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 O_3 (AA, ambient O_3 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	1132 ± 180	a	1356 ± 84	ab
WD	177.9 ± 6.5	a	185.8 ± 6.2	a	2050 ± 253	bc	2665 ± 84	c
Quercus pubescens								
WW	89.7 ± 3.1	A	101.3 ± 6.0	AB	283 ± 85	A	383 ± 12	A
WD	107.2 ± 1.0	B	113.1 ± 3.5	B	979 ± 279	AB	1230 ± 223	B
Quercus robur								
WW	71.8 ± 4.3	a′	75.2 ± 3.7	a′	994 ± 152	a′	1649 ± 30	b′
WD	76.7 ± 3.0	a′	82.6 ± 5.1	a′	2030 ± 93	b′	1790 ± 157	b′
ANOVA results								
O_3	ns				**			
W	**				***			
Sp.	***				***			
O_3 × W	ns				ns			
O_3 × Sp.	ns				ns			
W × Sp.	ns				ns			
O_3 × 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).

decreased by both O_3 (*Q. pubescens*, g_{sH2O}: −31%, g_{mCO2}: −50%, g_{totCO2}: −43%; *Q. robur*, g_{sH2O}: −57%, g_{mCO2}: −43%, g_{totCO2}: −60%) and WD (*Q. pubescens*, g_{sH2O}: −40%, g_{mCO2}: −78%, g_{totCO2}: −54%; *Q. robur*, g_{sH2O}: −62%, g_{mCO2}: −86%, g_{totCO2}: −86%), although the negative effect was more pronounced in WD compared to elevated O_3 treatments. The reduction of P_N was closely correlated with low diffusive conductance of CO_2 (g_{sCO2}, g_{mCO2}, and g_{totCO2}) in AA, with the largest correlation coefficient being observed in relationships between P_N and g_{totCO2} in all three oak species (Figure 2). However, no significant correlation between g_{sCO2} and P_N was found in 1.4 × AA for deciduous oaks, although P_N was tightly related to g_{mCO2} in this condition. Exposure to 1.4 × AA O_3 induced a reduction in g_{mCO2}, but g_{sCO2} remained broadly similar in *Q. pubescens*, resulting in a breakdown of the positive correlation between g_{sCO2} and g_{mCO2} observed in *Q. ilex* and *Q. robur* (Figure 3). The combined treatments of O_3 and WD significantly reduced CO_2 concentration within the chloroplast envelope (C_c) in the three oak species due to a significant limitation of CO_2 diffusive conductance, although the response of the internal sub-stomatal air-space (C_i) to O_3 and WD treatments was dependent on species (Table S2). The fluorescence measurements indicated that the combined treatment of O_3 and WD reduced ΦPSII, especially in two deciduous oaks (Figure 1G). The enhanced O_3 and WD treatment did not affect V_{cmax} and J_{max} in *Q. ilex*, as confirmed in the P_N/C_c curves (Figures 1H–I and 4). On the other hand, significant reductions in the values of V_{cmax} and J_{max} were found in O_3- and WD-treated leaves of *Q. pubescens* and *Q. robur*. Values of g_{mCO2} estimated

by the curve fitting method were consistent with those calculated by the variable J method in all three species (Figure S1).

Mesophyll conductance (g_{mCO2}) was negatively correlated with foliar ABA in three oak species (Figure 5). Although there was a negative correlation in the relationships between g_{sCO2} 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 g_{mCO2} only in *Q. pubescens* leaves, while there was no significant correlation between LMA and g_{sCO2} (Figure S2).

3.3 | Principal component analysis

Principle component analysis (PCA) allowed the characterisation of photosynthetic parameters and leaf traits in each oak species grown under different O_3 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 O_3 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
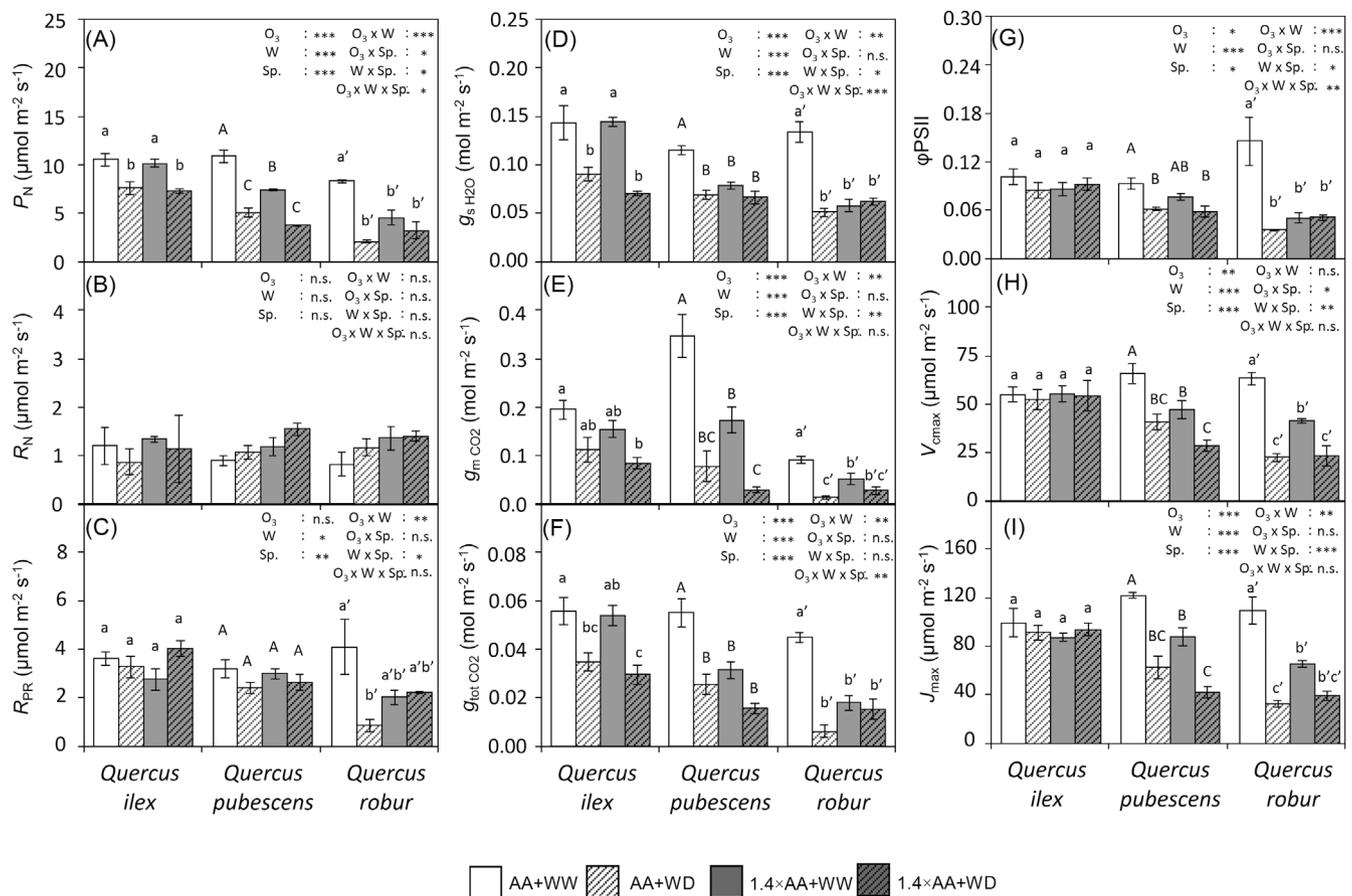
FIGURE 1 Photosynthetic parameters of three Mediterranean oaks (*Quercus ilex*, *Q. pubescens*, and *Q. robur*) grown under two levels of O_3 treatments (AA, ambient O_3 concentrations; $1.4 \times$ AA) and two levels of water treatments (WW, well-watered; WD, water-deficit). (A) Light-saturated net photosynthetic rate [P_{Nsat}], (B) dark respiration rate [R_N], (C) photorespiration [R_{PR}], (D) stomatal conductance to water vapour [g_{sH2O}], (E) mesophyll conductance to CO_2 [g_{mCO2}], (F) total conductance to CO_2 [g_{totCO2}], (G) quantum yield of photosystem II photochemistry [φPSII], (H) maximum rate of carboxylation [V_{cmax}], and (I) maximum rate of electron transport for RuBP regeneration [J_{max}]. Each value is the mean ± standard error ($n = 3$ plots). Three-way ANOVA: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns denotes not significant. Different letters show significant differences among treatments within each species ($P < 0.05$, Tukey test)

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_{cmax}, J_{max} 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_{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 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
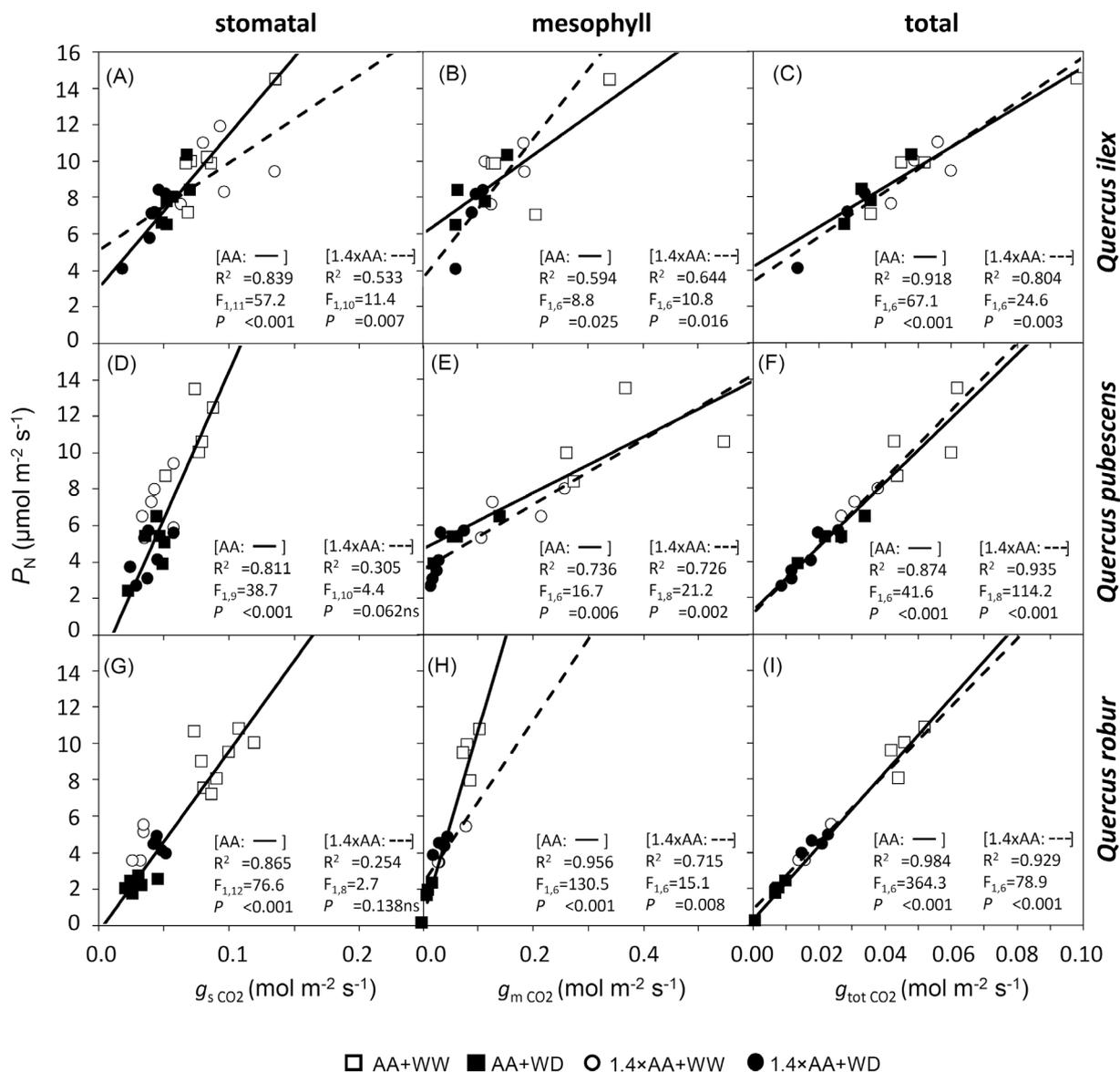
FIGURE 2 Relationships between net photosynthetic rate (P_N) and stomatal conductance for CO_2 (g_{sCO2}), mesophyll conductance to CO_2 (g_{mCO2}), and total conductance to CO_2 (g_{totCO2}) in three Mediterranean oaks (*Quercus ilex*, *Q. pubescens* and *Q. robur*) grown under two levels of O_3 treatments (AA, ambient O_3 concentrations; $1.4 \times$ AA) and two levels of water treatments (WW, well-watered; WD, water-deficit). Linear regression analyses were applied: black line, AA; dotted line, $1.4 \times$ AA

than deciduous species (Feng et al., 2018) because they have thicker leaves, which restricts O_3 diffusion inside a leaf due to their lower intercellular air space (Wieser et al., 2002). In addition, O_3-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 O_3 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 CO_2 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_{cmax}) and RuBP regeneration (expressed as J_{max}) (Figure 1A, D, H, I), suggesting that a photosynthetic downregulation was mainly caused by stomatal CO_2 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
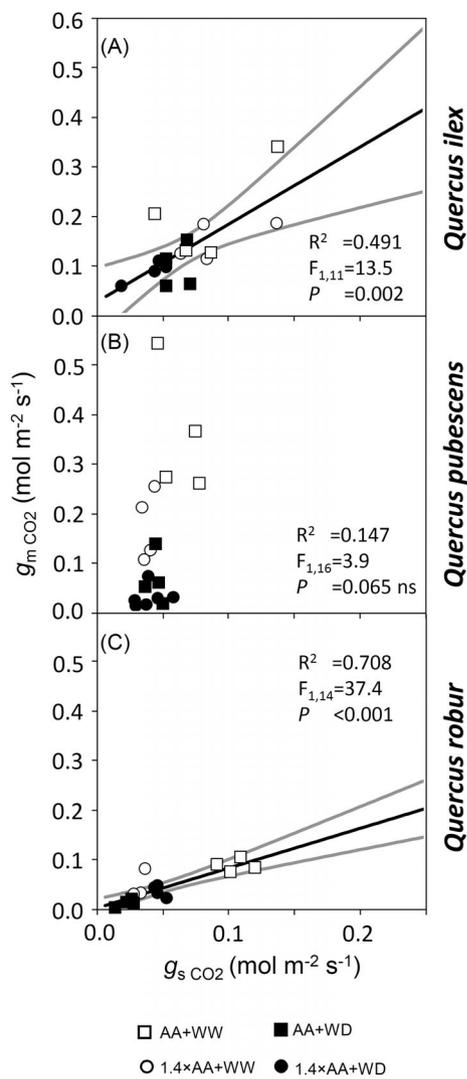
FIGURE 3   Stomatal conductance to CO_2 (g_{sCO2}) versus mesophyll conductance to CO_2 (g_{mCO2}) in *Quercus ilex*, *Quercus pubescens* and *Quercus robur* under combinations of ambient ozone levels (square symbols) and ozone fumigation (circles symbols) with full irrigation (white fill) and water deficit (black fill). Linear regression analyses were applied. Grey lines show the 95% confidential intervals of mean

stomatal conductance, significant reductions of g_{mCO2}, V_{cmax} and J_{max}, were found in WD treatments (Figure 1A, D, E, H, I). The lack of positive relationship between g_{sCO2} and g_{mCO2} 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_{mCO2} and g_{totCO2} 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_c) 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_{mCO2}) may be affected by both physical and biochemical traits (Flexas et al., 2008). We observed negative relationships between g_{mCO2} and ABA in three oak species under O_3 and drought (Figure 5), while g_{mCO2} 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_{mCO2} (Flexas et al., 2021).

Previous studies discussed whether an ABA production could reduce g_{mCO2}, which plays an important role in carbon uptake in abiotic-stressed plants (Brunetti et al., 2019; Mizokami et al., 2015; Sorrentino et al., 2016). However, no study has been reported about the relationship between g_{mCO2} and ABA under elevated O_3 or the combination of O_3 and drought, although several studies have
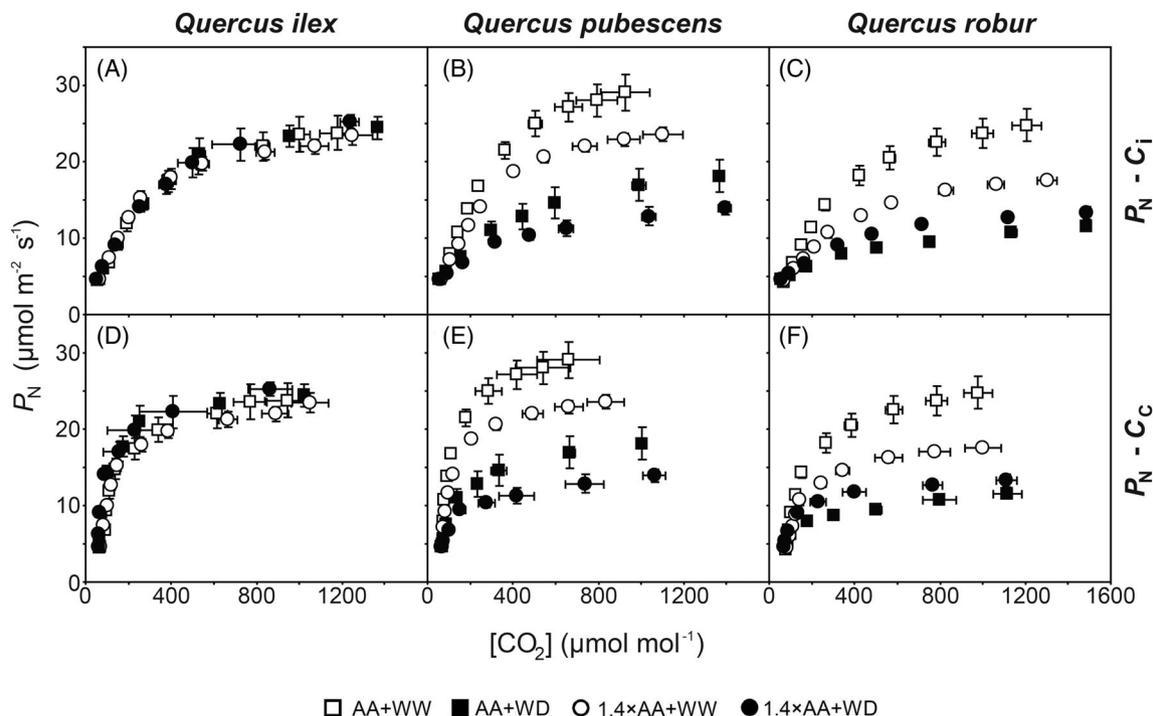
FIGURE 4 Responses of net photosynthetic rate (P_N) to the sub-stomatal concentration of CO_2 (C_i) (upper figures) or the concentration of CO_2 inside the chloroplast envelope (C_c) (bottom figures) for *Quercus ilex* (A, D) *Q. pubescens* (B, E) and *Q. robur* (C, F) grown under two levels of O_3 treatments (AA, ambient O_3 concentrations; $1.4 \times$ 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; $1.4 \times$ 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
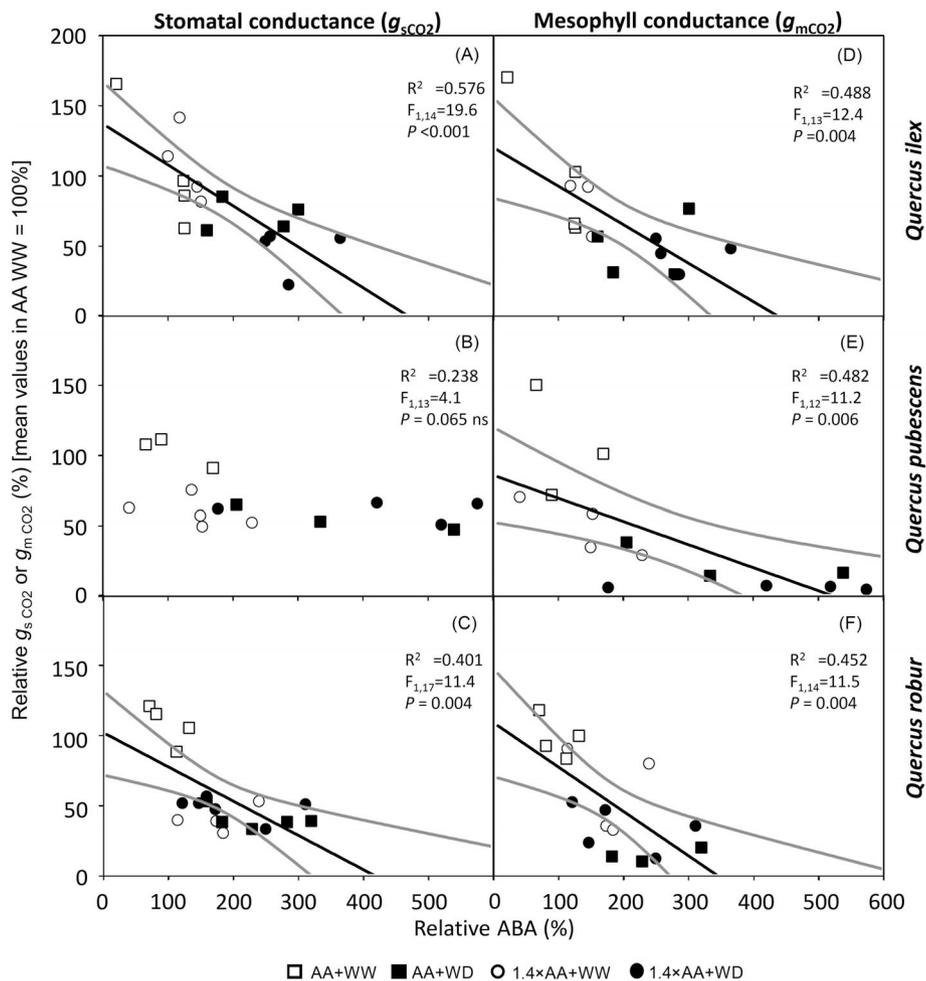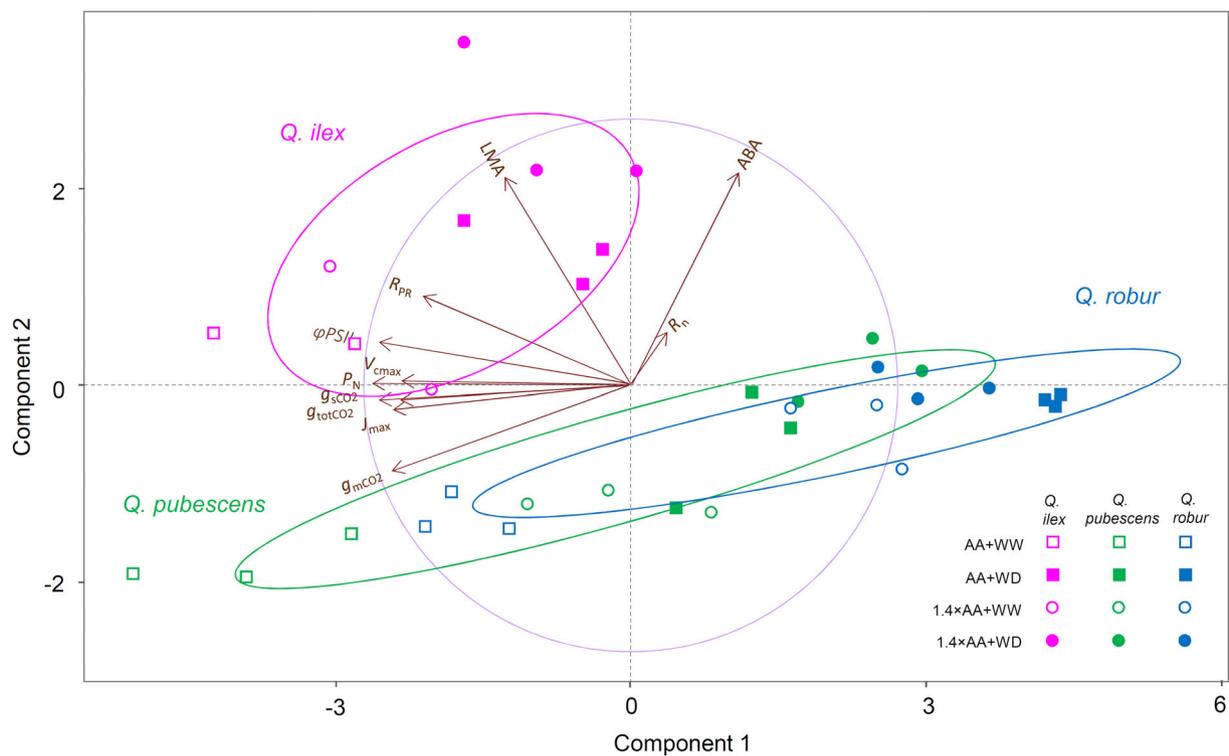
FIGURE 6 Principle component analysis of parameters derived from analyses of the photosynthetic parameters, diffusive limitations to CO_2 transport (g_{sCO2}, g_{mCO2}, and g_{totCO2}), and leaf traits (ABA, LMA) of three oak species (*Quercus ilex*, *Q. pubescens*, and *Q. robur*) grown under two levels of O_3 treatments (AA, ambient O_3 concentrations; $1.4 \times$ AA) and two levels of water treatments (WW, well-watered; WD, water-deficit). Ellipses represent 95% confidence intervals for measurements for three oak species. The correlation circle is also shown. Component 1 accounts for 62.1% of the variance within the dataset and component 2 accounts for 13.9% of variance

debated the role of ABA in the response of g_{mCO2} to drought (e.g. Brunetti et al., 2019). In our experiment, we found that WD increased leaf ABA content in three oaks, while O_3 increased [ABA] in *Q. robur* leaves (Table 1). As a result, g_{mCO2} declined with an increasing concentration of ABA in leaves of the three oak species grown under elevated O_3 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 O_3 and drought in the same experiment (Cocozza et al., 2020). Shrinkage of mesophyll cells due to low leaf water potential may negatively affect g_{mCO2} by physically constraining CO_2 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_{sCO2} and ABA was found under the combination of O_3 and drought (Figure 5B, C). It is known that O_3 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 O_3 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 O_3-induced ethylene emissions (Hoshika et al., 2019; Wilkinson & Davies, 2010). Changes

in stomatal function will influence the availability of CO_2 in the internal sub-stomatal leaf air-space (Killi et al., 2016). In fact, a significant increase in C_i was observed in *Q. pubescens* subjected to the combined treatment of O_3 and WD (Table S2). This may feedback into the rate of CO_2 movement across the mesophyll to the chloroplast. As g_{mCO2} is a flux-weighted function affected by the interaction of CO_2 availability, biochemical/physical transport constraints and CO_2 uptake in the chloroplast (Tholen et al., 2012), shifts in stomatal physiological function, mesophyll properties or the biochemistry of photosynthesis induced by O_3 fumigation are likely to be evident in rates of CO_2 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 CO_2 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 CO_2 diffusion to sub-stomatal cavities under elevated O_3 (Watanabe et al., 2014). However, this was not sufficient to enhance total diffusive conductance to CO_2 (g_{totCO2}) under elevated O_3 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 O_3 exposure (Omasa et al., 2002; Terashima, 1992). Stomatal patchiness may result in an overestimation of C_i, thus leading to an

underestimation of g_{mCO2}. 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_{mCO2} in the three oak species and it is reinforced by the close correlation between the variable J and the curve-fitting estimates of g_{mCO2} 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_{sCO2} and g_{mCO2} in *Q. ilex*, while the two deciduous oaks also showed a reduction of V_{cmax} and J_{max} with increased diffusive resistance to CO_2 transport. The reduction of g_{mCO2} was correlated with foliar [ABA] in the three oaks, while a negative correlation of g_{mCO2} with LMA was found in *Q. pubescens*. However, in two deciduous oaks, a weak or no significant correlation between g_{sCO2} 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_{mCO2} rather than g_{sCO2} in the two deciduous oaks when treated with O_3 and WD factors together. The results suggest that g_{mCO2} 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.

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; *Funding acquisition*: Elena Paoletti; *Investigation*: 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.

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 halepensis*. *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., Zavadlav, 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.

Cotrozzi, 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.

Ethier, G.J. & Livingston, N.J. (2004) On the need to incorporate sensitivity to CO2 transfer conductance into the Farquhar-von Caemmerer-berry leaf photosynthesis model. *Plant, Cell & Environment*, 27, 137–153.

Feng, Z., Büker, P., Pleijel, H., Emberson, L., Karlsson, P.E. & Uddling, J. (2018) A unifying explanation for variation in ozone sensitivity among woody plants. *Global Change Biology*, 24, 78–84.

Feng, Z., Pang, J., Kobayashi, K., Zhu, J. & Ort, D.R. (2011) Differential responses in two varieties of winter wheat to elevated ozone concentration under fully open-air field conditions. *Global Change Biology*, 17, 580–591.

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 CO_2 and leaf morphological characteristics under drought stress during *Quercus ilex* L. resprouting. *Annals of Forest Science*, 67, 308.

Flexas, J., Bota, J., Loreto, F., Cornic, 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-Carbó, M., Diaz-Espejo, A., Galmés, J. & Medrano, H. (2008) Mesophyll conductance to CO_2: 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 CO_2 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.

Galmes, 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-Armiñana, 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(S1), 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 CO_2 flux by analysis of the response of photosynthesis to CO_2. *Plant Physiology*, 98, 1429–1436.

Haworth, M., Cosentino, 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., Consentino, 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. *GCB 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 O_3-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 CO_2 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.

Kitao, M., Löw, M., Heerdt, C., Grams, T.E.E., Häberle, K.-H. & Matyssek, 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.

Laisk, A. & Loreto, F. (1996) Determining photosynthetic parameters from leaf CO_2 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. & Paoletti, 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 CO_2 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.

Loriaux, S.D., Avenson, T.J., Welles, J.M., Mcdermitt, D.K., Eckles, R.D., Riensche, 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., Scartazza, 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. & Brodribb, T.J. (2018) Mesophyll cells are the main site of abscisic acid biosynthesis in water-stressed leaves. *Plant Physiology*, 177, 911–917.

McAdam, E., Brodribb, 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 day 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., Sibbernsen, E.D. & Shope, J.C. (2008) The role of the mesophyll in stomatal responses to light and CO_2. *Plant, Cell & Environment*, 31, 1299–1306.

Nadal, M., Carriquí, M. & Flexas, J. (2021) Chapter 3: Mesophyll conductance to CO_2 diffusion in a climate change scenario: effects of elevated CO_2, 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.

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

Paoletti, 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.

Paoletti, 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.

Paoletti, E., Materassi, A., Fasano, G., Hoshika, Y., Carriero, G., Silaghi, D. et al. (2017) A new-generation 3D ozone FACE (free air controlled exposure). *Science of the Total Environment*, 575, 1407–1414.

Paoletti, E., Schaub, M., Matyssek, R., Wieser, G., Augustaitis, A., Bastrup-Birk, A.M. et al. (2010) Advances of air pollution science: from forest decline to multiple-stress effects on forest ecosystem services. *Environmental Pollution*, 158, 1986–1989.

Peguero-Pina, J.J., Sisó, S., Flexas, J., Galmés, J., García-Nogales, A., Niinemets, Ü. et al. (2017) Cell-level anatomical characteristics explain high mesophyll conductance and photosynthetic capacity in sclerophyllous Mediterranean oaks. *The New Phytologist*, 214, 585–596.

Pellegrini, E., Hoshika, Y., Dusart, N., Cotrozzi, L., Gérard, J., Nali, C. et al. (2019) Antioxidative responses of three oak species under ozone and water stress conditions. *Science of the Total Environment*, 647, 390–399.

Pons, T.L., Flexas, J., von Caemmerer, S., Evans, J.R., Genty, B., Ribas-Carbo, M. et al. (2009) Estimating mesophyll conductance to CO_2: methodology, potential errors, and recommendations. *Journal of Experimental Botany*, 60, 2217–2234.

Poorter, H., Niinemets, Ü., Poorter, I., Wright, I.J. & Villar, R. (2009) Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *The New Phytologist*, 182, 565–588.

Quarrie, S.A., Whitford, P.N., Appleford, N.E.J., Wang, T.L., Cook, S.K., Henson, I.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 cerealand lupin leaves. *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 CO_2 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*, 230, 521–534.

Shang, B., Xu, Y., Dai, L., Yuan, X. & Feng, Z. (2019) Elevated ozone reduced leaf nitrogen allocation to photosynthesis in poplar. *Science of the Total Environment*, 657, 169–178.

Sharkey, T.D. (1988) Estimating the rate of photorespiration in leaves. *Physiologia Plantarum*, 73, 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 CO_2 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 CO_2 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.

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