

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

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## Abstract

Mesophyll conductance ( $g_{mCO_2}$ ) 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  $g_{mCO_2}$  are still largely unclear. We investigated leaf gas exchange during mid-summer in three Mediterranean oaks exposed to  $O_3$  (ambient [ $35.2 \text{ nmol mol}^{-1}$  as daily mean];  $1.4 \times$  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  $g_{mCO_2}$  and stomatal conductance ( $g_{sCO_2}$ ) 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  $g_{mCO_2}$  was correlated with increased [ABA] in the three oaks, whereas there was a negative correlation between  $g_{mCO_2}$  with LMA in *Q. pubescens*. Interestingly, two deciduous oaks showed a weak or no significant correlation between  $g_{sCO_2}$  and ABA under high  $O_3$  and WD due to impaired stomatal physiological behaviour, indicating that the reduction of  $P_N$  was related to  $g_{mCO_2}$  rather than  $g_{sCO_2}$ . The results suggest that  $g_{mCO_2}$  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  $40 \text{ nmol mol}^{-1}$ ) induces a

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5% reduction of biomass growth for forest trees compared to that at the pre-industrial times (approximately 10–15 nmol mol<sup>-1</sup>; 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<sup>-1</sup> as daily mean O<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub> 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<sub>2</sub> (Larcher, 2003). Ozone frequently decreases net photosynthetic rate ( $P_N$ ) in association with increased diffusion resistance to CO<sub>2</sub> 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<sub>2</sub> 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<sub>3</sub> may also cause a reduction of mesophyll conductance ( $g_{mCO_2}$ ) 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<sub>3</sub> on  $g_{mCO_2}$  in *Fagus sylvatica*. Mesophyll conductance ( $g_{mCO_2}$ ) consists of both physical and biochemical components in the transport of CO<sub>2</sub> (Flexas et al., 2008; Loreto et al., 1992). Although the mechanisms of the effects of O<sub>3</sub> on  $g_{mCO_2}$  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  $g_{mCO_2}$  was accompanied by structural changes in mesophyll cells of O<sub>3</sub>-exposed beech leaves. This may be supported by the fact that O<sub>3</sub> collapses mesophyll cells or alters mesophyll ultrastructure and other leaf morphological parameters, which may reduce the CO<sub>2</sub> diffusion inside leaves (Gao et al., 2016; Matyssek et al., 1991; Paoletti et al., 2009). In addition, O<sub>3</sub> also modifies the activation of aquaporins, thus increasing the mesophyll diffusion resistance to CO<sub>2</sub> 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  $g_{mCO_2}$  to LMA was found according to a literature data review, which indicates that the maximum  $g_{mCO_2}$  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<sub>3</sub> exposure (Shang et al., 2019), suggesting that

the effect of O<sub>3</sub> 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<sub>2</sub> 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 & Brodrribb, 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  $g_{mCO_2}$  observed as foliar [ABA] rises is caused by a reduction in stomatal conductance, lowering CO<sub>2</sub> availability in the internal leaf air-space, and a reduction in the biochemical transport of CO<sub>2</sub> across the mesophyll (likely associated with reduced aquaporin activity; Sorrentino et al., 2016). Abscisic acid plays a pivotal role in the reduction of  $g_{mCO_2}$  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  $g_{mCO_2}$  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<sub>3</sub> flux and thus limit O<sub>3</sub> damage (Khan & Soja, 2003; Tingey & Hogsett, 1985). However, according to recent manipulative experiments, water deficits may instead exacerbate the negative effects of O<sub>3</sub> on plants (e.g. Cotrozzi et al., 2016). In fact, the combination of O<sub>3</sub> 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<sub>2</sub> diffusion in plants grown in combined O<sub>3</sub> fumigation and drought. However, to the best of our knowledge, no data are available for  $g_{mCO_2}$  in plant leaves subjected to both high O<sub>3</sub> concentration and drought, and it is still unclear if there is an interaction of O<sub>3</sub> and drought on diffusion resistance to CO<sub>2</sub> transport in the mesophyll layer.

We examined the interactive effects of O<sub>3</sub> and drought on leaf gas exchange in a free-air O<sub>3</sub> 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<sub>3</sub> and

drought on photosynthetic traits, especially on  $g_{mCO_2}$  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  $g_{mCO_2}$  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 [ $1.4 \times AA$ ]) and two levels of water irrigation (WW [well-watered, 100% field capacity  $\approx 0.295 \text{ m}^3 \text{ m}^{-3}$ ] and WD [water-deficit, 40% field capacity]) during one growing season (1st June to 15th October 2015). Mean hourly  $O_3$  concentrations were  $35.2 \text{ nmol mol}^{-1}$  in AA and  $48.9 \text{ nmol mol}^{-1}$  in  $1.4 \times AA$ . The values of AOT40 (Accumulated exposure Over a Threshold of  $40 \text{ nmol mol}^{-1}$ ) were  $17.8 \text{ } \mu\text{mol mol}^{-1} \text{ h}$  in AA and  $40.3 \text{ } \mu\text{mol mol}^{-1} \text{ h}$  in  $1.4 \times AA$ . 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 ( $5 \times 5 \times 2 \text{ m}$ ) to each  $O_3$  treatment with three plants per species per combination of  $O_3$  and water ( $O_3 \times W$ ). 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  $g_{mCO_2}$ . It is preferable to utilise two complementary methodologies to reflect  $g_{mCO_2}$ , 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,  $1500 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ ), leaf temperature ( $25^\circ\text{C}$ ) and leaf-to-air vapour pressure deficit of  $1.6 \pm 0.2 \text{ KPa}$ . Punctual point measurements of leaf gas exchange and chlorophyll fluorescence were performed at  $400 \text{ } \mu\text{mol mol}^{-1} [\text{CO}_2]$ . 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  $8000 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$  (Loriaux et al., 2013). Mesophyll conductance ( $g_{mCO_2}$ ) was determined using the variable  $J$  method described by Harley et al. (1992):

$$g_{mCO_2} = \frac{PN}{C_i - \frac{\Gamma \times [J_F + 8 \times (PN + R_d)]}{J_F - 4 \times (PN + R_d)}}, \quad (1)$$

where  $\Gamma^*$  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,  $30 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ ). 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:

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

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 ( $\Phi\text{PSII}$ ) can be determined as:

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

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  $J$   $g_{mCO_2}$  as:

$$C_c = C_i - \frac{PN}{g_{mCO_2}}, \quad (4)$$

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

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

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

$$g_{\text{totCO}_2} = [g_{\text{sCO}_2} \cdot g_{\text{mCO}_2}] / [g_{\text{sCO}_2} + g_{\text{mCO}_2}], \quad (6)$$

where  $g_{\text{sCO}_2}$  is the stomatal conductance for  $\text{CO}_2$  ( $g_{\text{sH}_2\text{O}}/1.6$ , where  $g_{\text{sH}_2\text{O}}$  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 RubisCO ( $V_{\text{cmax}}$ ), the maximum rate of electron transport driving regeneration of ribulose-1,5-bisphosphate (RuBP) ( $J_{\text{max}}$ ) and mesophyll conductance ( $g_{\text{mCO}_2}$ ).

### 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  $70^\circ\text{C}$  for 1 week to reach a constant weight. The LMA was calculated as the ratio of the dry mass to the projected leaf area ( $\text{g m}^{-2}$ ).

### 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  $-80^\circ\text{C}$ . Leaf samples were freeze-dried and finely ground. Deionised water was added (1:50 weight ratio), the sample incubated on a shaker at  $4^\circ\text{C}$  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 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 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 ( $g_{\text{sCO}_2}$ ,  $g_{\text{mCO}_2}$ , and  $g_{\text{totCO}_2}$ ), 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 ( $\text{O}_3 \times W \times \text{Sp}$ ) 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 ( $\text{O}_3$ , 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  $R_{\text{PR}}$  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  $\text{O}_3$  and water-deficit treatments in the other two oak species. Ozone and WD treatments significantly affected  $g_{\text{sH}_2\text{O}}$ ,  $g_{\text{mCO}_2}$  and  $g_{\text{totCO}_2}$ , although the effects were dependent on species (Figure 1D–F). In evergreen *Q. ilex*,  $g_{\text{sH}_2\text{O}}$  was significantly reduced by WD treatments (−37%). The values of  $g_{\text{mCO}_2}$  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 ( $g_{\text{mCO}_2}$ : −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<sub>3</sub> (AA, ambient O<sub>3</sub> concentration; 1.4 × AA) and water treatments (WW, well-watered; WD, water-deficit)

	LMA (g m <sup>-2</sup> )				ABA (ng g <sup>-1</sup> )			
	AA		1.4 × AA		AA		1.4 × AA	
<i>Quercus ilex</i>								
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
<i>Quercus pubescens</i>								
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
<i>Quercus robur</i>								
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'
<b>ANOVA results</b>								
O <sub>3</sub>	ns				**			
W	**				***			
Sp.	***				***			
O <sub>3</sub> × W	ns				ns			
O <sub>3</sub> × Sp.	ns				ns			
W × Sp.	ns				ns			
O <sub>3</sub> × 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<sub>3</sub> (*Q. pubescens*,  $g_{sH_2O}$ : -31%,  $g_{mCO_2}$ : -50%,  $g_{totCO_2}$ : -43%; *Q. robur*,  $g_{sH_2O}$ : -57%,  $g_{mCO_2}$ : -43%,  $g_{totCO_2}$ : -60%) and WD (*Q. pubescens*,  $g_{sH_2O}$ : -40%,  $g_{mCO_2}$ : -78%,  $g_{totCO_2}$ : -54%; *Q. robur*,  $g_{sH_2O}$ : -62%,  $g_{mCO_2}$ : -86%,  $g_{totCO_2}$ : -86%), although the negative effect was more pronounced in WD compared to elevated O<sub>3</sub> treatments. The reduction of  $P_N$  was closely correlated with low diffusive conductance of CO<sub>2</sub> ( $g_{sCO_2}$ ,  $g_{mCO_2}$ , and  $g_{totCO_2}$ ) in AA, with the largest correlation coefficient being observed in relationships between  $P_N$  and  $g_{totCO_2}$  in all three oak species (Figure 2). However, no significant correlation between  $g_{sCO_2}$  and  $P_N$  was found in 1.4 × AA for deciduous oaks, although  $P_N$  was tightly related to  $g_{mCO_2}$  in this condition. Exposure to 1.4 × AA O<sub>3</sub> induced a reduction in  $g_{mCO_2}$ , but  $g_{sCO_2}$  remained broadly similar in *Q. pubescens*, resulting in a breakdown of the positive correlation between  $g_{sCO_2}$  and  $g_{mCO_2}$  observed in *Q. ilex* and *Q. robur* (Figure 3). The combined treatments of O<sub>3</sub> and WD significantly reduced CO<sub>2</sub> concentration within the chloroplast envelope ( $C_c$ ) in the three oak species due to a significant limitation of CO<sub>2</sub> diffusive conductance, although the response of the internal sub-stomatal air-space ( $C_i$ ) to O<sub>3</sub> and WD treatments was dependent on species (Table S2). The fluorescence measurements indicated that the combined treatment of O<sub>3</sub> and WD reduced ΦPSII, especially in two deciduous oaks (Figure 1G). The enhanced O<sub>3</sub> 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<sub>3</sub>- and WD-treated leaves of *Q. pubescens* and *Q. robur*. Values of  $g_{mCO_2}$  estimated

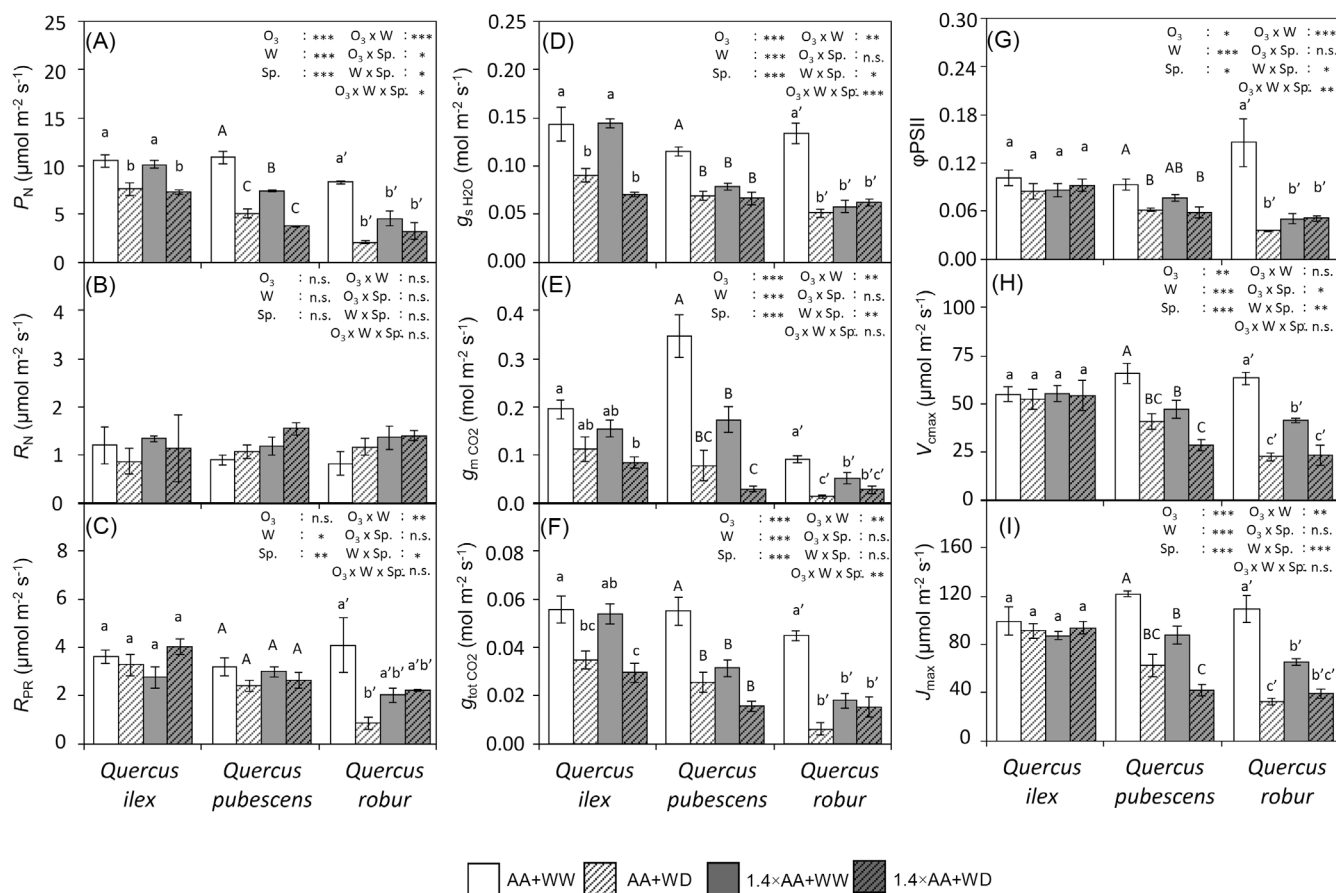
by the curve fitting method were consistent with those calculated by the variable J method in all three species (Figure S1).

Mesophyll conductance ( $g_{mCO_2}$ ) was negatively correlated with foliar ABA in three oak species (Figure 5). Although there was a negative correlation in the relationships between  $g_{sCO_2}$  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  $g_{mCO_2}$  only in *Q. pubescens* leaves, while there was no significant correlation between LMA and  $g_{sCO_2}$  (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<sub>3</sub> 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<sub>3</sub> 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 [ $P_{N_{Sat}}$ ], (B) dark respiration rate [ $R_N$ ], (C) photorespiration [ $R_{PR}$ ], (D) stomatal conductance to water vapour [ $g_{sH_2O}$ ], (E) mesophyll conductance to  $CO_2$  [ $g_{mCO_2}$ ], (F) total conductance to  $CO_2$  [ $g_{totCO_2}$ ], (G) quantum yield of photosystem II photochemistry [ $\phi_{PSII}$ ], (H) maximum rate of carboxylation [ $V_{cmax}$ ], and (I) maximum rate of electron transport for RuBP regeneration [ $J_{max}$ ]. Each value is the mean  $\pm$  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)

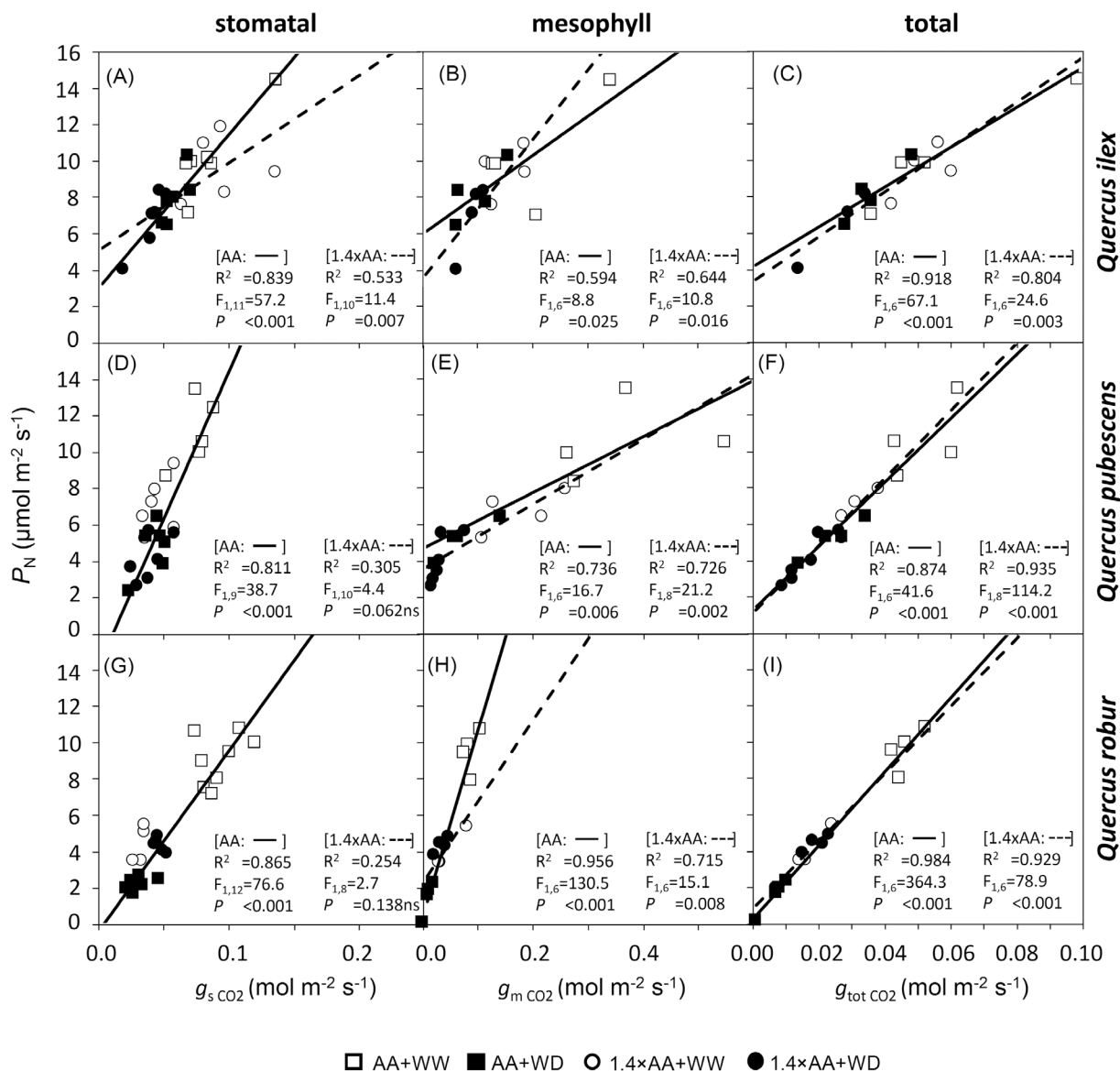
deciduous species ([Tukey test on PCA scores] *Q. ilex* versus *Q. pubescens*, component 1:  $P = 0.058$ , component 2:  $P < 0.001$ ; *Q. ilex* vs. *Q. robur*, component 1:  $P < 0.001$ , component 2:  $P < 0.001$ ).

## 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  $g_{mCO_2}$  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  $O_3$

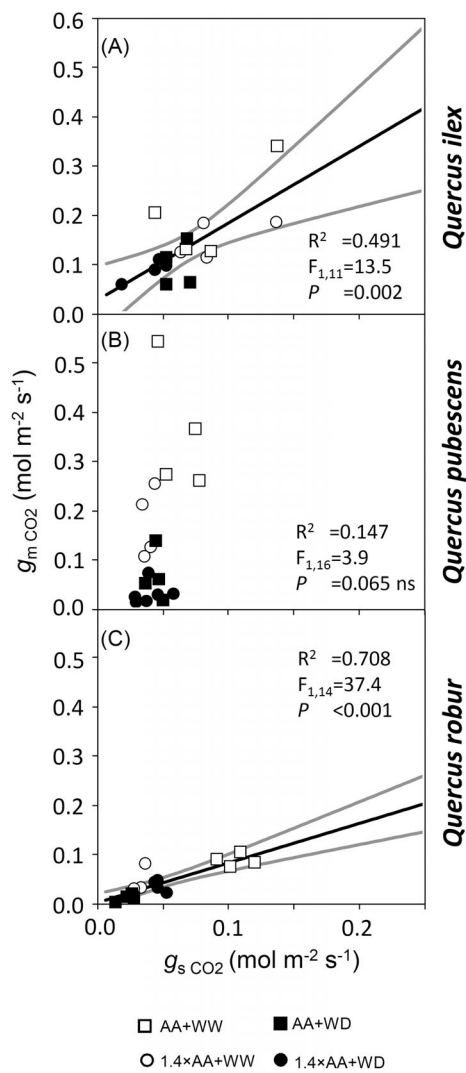


**FIGURE 2** Relationships between net photosynthetic rate ( $P_N$ ) and stomatal conductance for  $\text{CO}_2$  ( $g_{s\text{CO}_2}$ ), mesophyll conductance to  $\text{CO}_2$  ( $g_{m\text{CO}_2}$ ), and total conductance to  $\text{CO}_2$  ( $g_{\text{totCO}_2}$ ) in three Mediterranean oaks (*Quercus ilex*, *Q. pubescens* and *Q. robur*) grown under two levels of  $\text{O}_3$  treatments (AA, ambient  $\text{O}_3$  concentrations;  $1.4 \times \text{AA}$ ) and two levels of water treatments (WW, well-watered; WD, water-deficit). Linear regression analyses were applied: black line, AA; dotted line,  $1.4 \times \text{AA}$

than deciduous species (Feng et al., 2018) because they have thicker leaves, which restricts  $\text{O}_3$  diffusion inside a leaf due to their lower intercellular air space (Wieser et al., 2002). In addition,  $\text{O}_3$ -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  $\text{O}_3$  fumigation (Loreto & Velikova, 2001) as *Q. ilex* mainly emits monoterpenes while the deciduous *Quercus* emits isoprenoids (Lehning et al., 1999; Rapparin 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  $\text{CO}_2$  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 ( $V_{\text{cmax}}$ ) and RuBP regeneration (expressed as  $J_{\text{max}}$ ) (Figure 1A, D, H, I), suggesting that a photosynthetic downregulation was mainly caused by stomatal  $\text{CO}_2$  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<sub>2</sub> ( $g_{sCO_2}$ ) versus mesophyll conductance to CO<sub>2</sub> ( $g_{mCO_2}$ ) 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  $g_{mCO_2}$ ,  $V_{cmax}$  and  $J_{max}$  were found in WD treatments (Figure 1A, D, E, H, I). The lack of positive relationship between  $g_{sCO_2}$  and  $g_{mCO_2}$  in *Q. pubescens* may indicate that, while stomata remained open under O<sub>3</sub> fumigation, a decline in CO<sub>2</sub> uptake resulted in lower transport of CO<sub>2</sub> 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

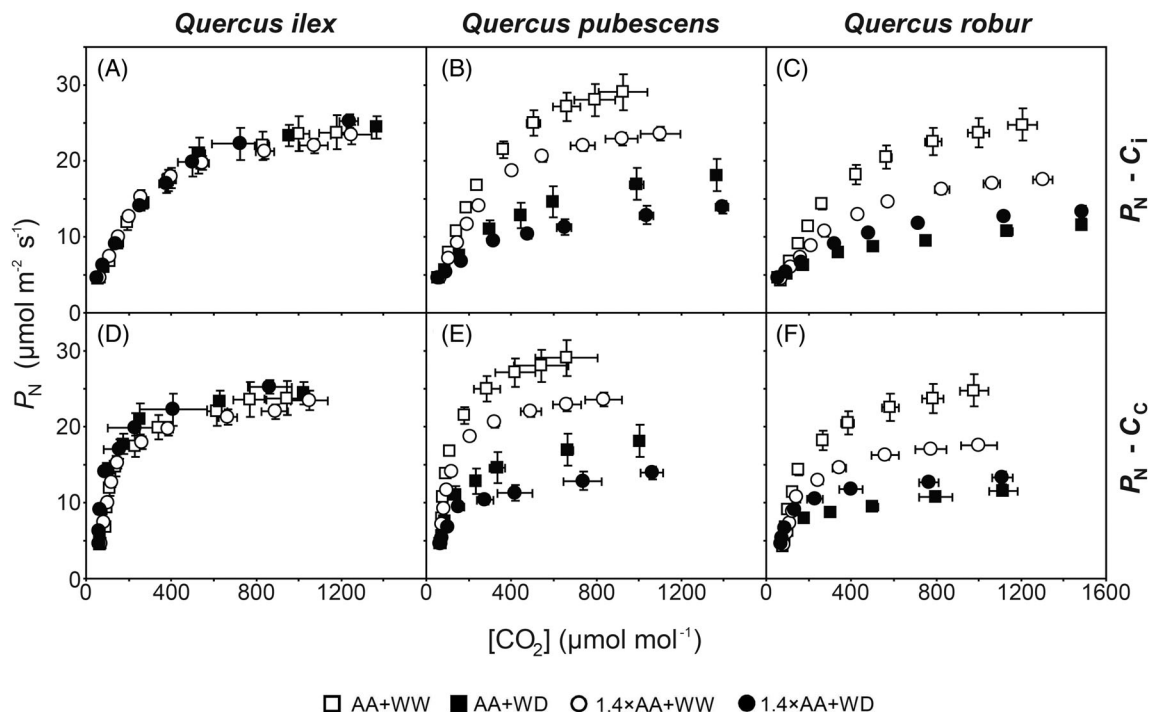
Net photosynthetic rate was significantly decreased by the combined treatment of  $1.4 \times AA O_3 + WD$  in all three oak species (Figure 1A). However, the interaction of O<sub>3</sub> and drought on photosynthetic gas exchange parameters appeared to vary with species. Previous studies suggest that drought stress may induce stomatal closure and thus might protect plants from O<sub>3</sub> damage (Khan & Soja, 2003; Tingey & Hogsett, 1985). However, WD did not prevent O<sub>3</sub> damage to the photosynthetic physiology, especially in *Q. ilex*, but rather amplified the negative effects. In fact, the negative effects on  $g_{mCO_2}$  and  $g_{totCO_2}$  were exacerbated by the combined treatment of O<sub>3</sub> and WD in *Q. ilex* (Figure 1E, F), which results in a significant decrease of CO<sub>2</sub> 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<sub>3</sub> stress singly, which may contribute to limiting the accumulation of ROS, such as hydrogen peroxide. However, the combination of O<sub>3</sub> 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 ( $g_{mCO_2}$ ) may be affected by both physical and biochemical traits (Flexas et al., 2008). We observed negative relationships between  $g_{mCO_2}$  and ABA in three oak species under O<sub>3</sub> and drought (Figure 5), while  $g_{mCO_2}$  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<sub>2</sub> 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  $g_{mCO_2}$  (Flexas et al., 2021).

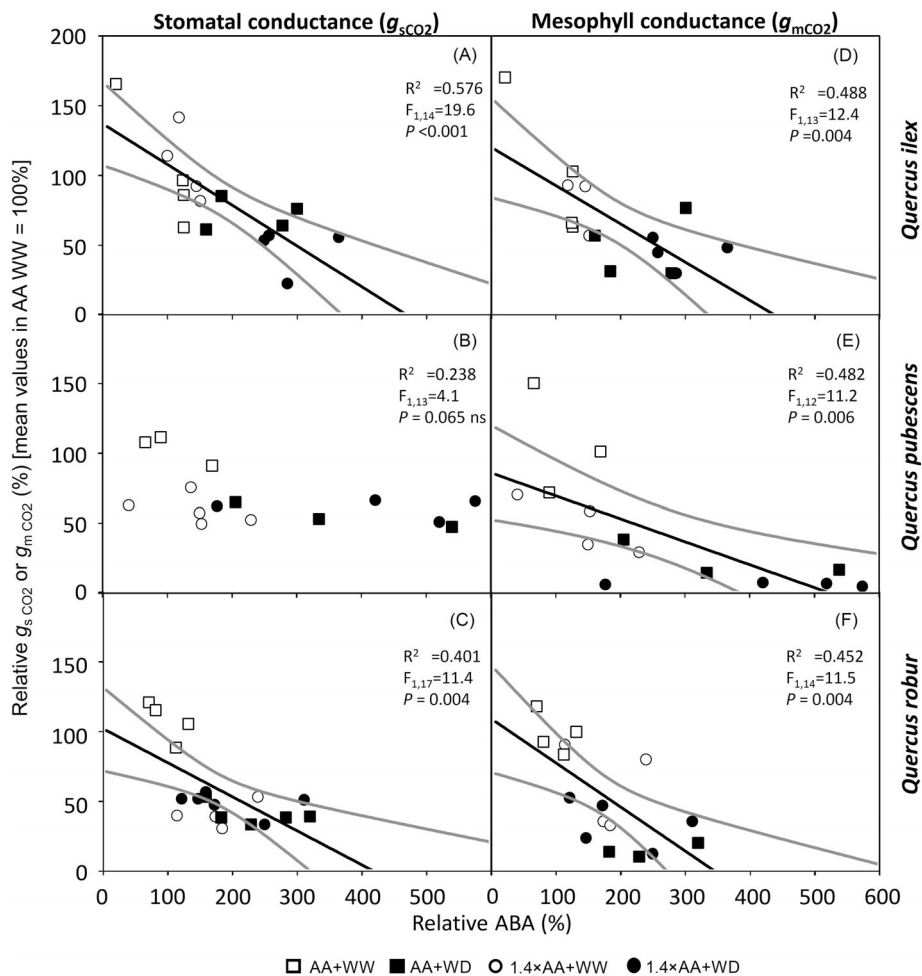
Previous studies discussed whether an ABA production could reduce  $g_{mCO_2}$ , which plays an important role in carbon uptake in abiotic-stressed plants (Brunetti et al., 2019; Mizokami et al., 2015; Sorrentino et al., 2016). However, no study has been reported about the relationship between  $g_{mCO_2}$  and ABA under elevated O<sub>3</sub> or the combination of O<sub>3</sub> and drought, although several studies have

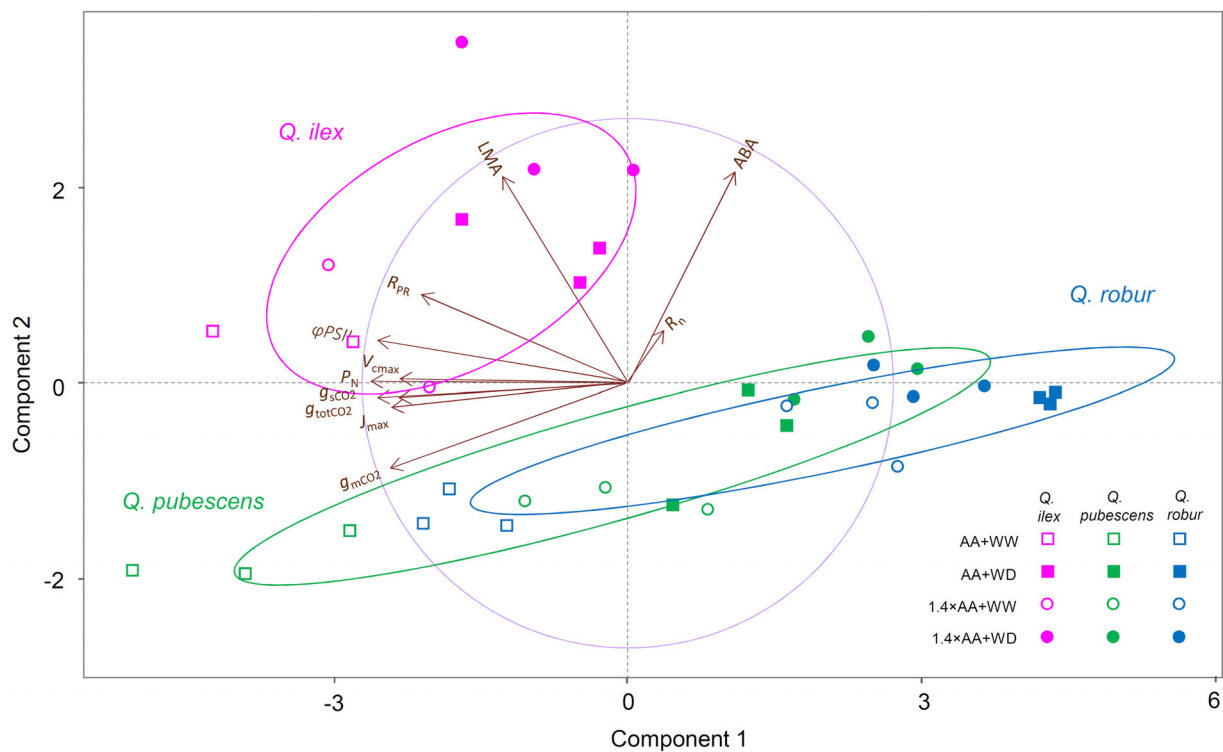




**FIGURE 4** Responses of net photosynthetic rate ( $P_N$ ) to the sub-stomatal concentration of  $\text{CO}_2$  ( $C_i$ ) (upper figures) or the concentration of  $\text{CO}_2$  inside the chloroplast envelope ( $C_c$ ) (bottom figures) for *Quercus ilex* (A, D), *Q. pubescens* (B, E) and *Q. robur* (C, F) grown under two levels of  $\text{O}_3$  treatments (AA, ambient  $\text{O}_3$  concentrations;  $1.4 \times \text{AA}$ ) and two levels of water treatments (WW, well-watered; WD, water-deficit). Each value is the mean  $\pm$  standard error ( $n = 3$  plots)

**FIGURE 5** Relationships between relative stomatal conductance ( $g_{s\text{CO}_2}$ ) or mesophyll conductance ( $g_{m\text{CO}_2}$ ) and relative leaf abscisic acid (ABA) contents in three Mediterranean oaks (*Quercus ilex*, *Q. pubescens*, and *Q. robur*) grown under two levels of  $\text{O}_3$  treatments (AA, ambient  $\text{O}_3$  concentrations;  $1.4 \times \text{AA}$ ) and two levels of water treatments (WW, well-watered; WD, water-deficit). The mean  $g_{s\text{CO}_2}$ ,  $g_{m\text{CO}_2}$ , 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<sub>2</sub> transport ( $g_{sCO_2}$ ,  $g_{mCO_2}$ , and  $g_{totCO_2}$ ), and leaf traits (ABA, LMA) of three oak species (*Quercus ilex*, *Q. pubescens*, and *Q. robur*) grown under two levels of O<sub>3</sub> treatments (AA, ambient O<sub>3</sub> concentrations; 1.4 × 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  $g_{mCO_2}$  to drought (e.g. Brunetti et al., 2019). In our experiment, we found that WD increased leaf ABA content in three oaks, while O<sub>3</sub> increased [ABA] in *Q. robur* leaves (Table 1). As a result,  $g_{mCO_2}$  declined with an increasing concentration of ABA in leaves of the three oak species grown under elevated O<sub>3</sub> 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<sub>3</sub> and drought in the same experiment (Cocozza et al., 2020). Shrinkage of mesophyll cells due to low leaf water potential may negatively affect  $g_{mCO_2}$  by physically constraining CO<sub>2</sub> 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  $g_{sCO_2}$  and ABA was found under the combination of O<sub>3</sub> and drought (Figure 5B, C). It is known that O<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub>-induced ethylene emissions (Hoshika et al., 2019; Wilkinson & Davies, 2010). Changes

in stomatal function will influence the availability of CO<sub>2</sub> in the internal sub-stomatal leaf air-space (Killi et al., 2016). In fact, a significant increase in C<sub>i</sub> was observed in *Q. pubescens* subjected to the combined treatment of O<sub>3</sub> and WD (Table S2). This may feedback into the rate of CO<sub>2</sub> movement across the mesophyll to the chloroplast. As  $g_{mCO_2}$  is a flux-weighted function affected by the interaction of CO<sub>2</sub> availability, biochemical/physical transport constraints and CO<sub>2</sub> uptake in the chloroplast (Tholen et al., 2012), shifts in stomatal physiological function, mesophyll properties or the biochemistry of photosynthesis induced by O<sub>3</sub> fumigation are likely to be evident in rates of CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> diffusion to sub-stomatal cavities under elevated O<sub>3</sub> (Watanabe et al., 2014). However, this was not sufficient to enhance total diffusive conductance to CO<sub>2</sub> ( $g_{totCO_2}$ ) under elevated O<sub>3</sub> in the three oak species examined in this study (Figure 1F).

Another important factor influencing C<sub>i</sub> is non-uniform stomatal apertures, i.e. stomatal patchiness, which may be caused by drought or O<sub>3</sub> exposure (Omasa et al., 2002; Terashima, 1992). Stomatal patchiness may result in an overestimation of C<sub>i</sub>, thus leading to an

underestimation of  $g_{mCO_2}$ . 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  $g_{mCO_2}$  in the three oak species and it is reinforced by the close correlation between the variable  $J$  and the curve-fitting estimates of  $g_{mCO_2}$  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  $g_{sCO_2}$  and  $g_{mCO_2}$  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  $g_{mCO_2}$  was correlated with foliar [ABA] in the three oaks, while a negative correlation of  $g_{mCO_2}$  with LMA was found in *Q. pubescens*. However, in two deciduous oaks, a weak or no significant correlation between  $g_{sCO_2}$  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  $g_{mCO_2}$  rather than  $g_{sCO_2}$  in the two deciduous oaks when treated with  $O_3$  and WD factors together. The results suggest that  $g_{mCO_2}$  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.

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## DATA AVAILABILITY STATEMENT

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

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