Isotopic and Water Relation Responses to Ozone and Water Stress in Seedlings of Three Oak Species with Different Adaptation Strategies

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Abstract: The impact of global changes on forest ecosystem processes is based on the species-specific responses of trees to the combined effect of multiple stressors and the capacity of each species to acclimate and cope with the environment modification. Combined environmental constraints can severely affect plant and ecological processes involved in plant functionality. This study provides novel insights into the impact of a simultaneous pairing of abiotic stresses (i.e., water and ozone (O_3) stress) on the responses of oak species. Water stress (using 40 and 100% of soil water content at field capacity—WS and WW treatments, respectively) and O_3 exposure (1.0, 1.2, and 1.4 times the ambient concentration—AA, 1.2AA, and 1.4AA, respectively) were carried out on *Quercus robur* L., *Quercus ilex* L., and *Quercus pubescens* Willd. seedlings, to study physiological traits (1. isotope signature [$\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$], 2. water relation [leaf water potential, leaf water content], 3. leaf gas exchange [light-saturated net photosynthesis, A_{sat}, and stomatal conductance, g_s]) for adaptation strategies in a Free-Air Controlled Exposure (FACE) experiment. Ozone decreased A_{sat} in *Q. robur* and *Q. pubescens* while water stress decreased it in all three oak species. Ozone did not affect $\delta^{13}C$, whereas $\delta^{18}O$ was influenced by O_3 especially in *Q. robur*. This may reflect a reduction of g_s with the concomitant reduction in photosynthetic capacity. However, the effect of elevated O_3 on leaf gas exchange as indicated by the combined analysis of stable isotopes was much lower than that of water stress. Water stress was detectable by $\delta^{13}C$ and by $\delta^{18}O$ in all three oak species, while $\delta^{15}N$ did not define plant response to stress conditions in any species. The $\delta^{13}C$ signal was correlated to leaf water content (LWC) in *Q. robur* and *Q. ilex*, showing isohydric and anisohydric strategy, respectively, at increasing stress intensity (low value of LWC). No interactive effect of water stress and O_3 exposure on the isotopic responses was found, suggesting no cross-protection on seasonal carbon assimilation independently on the species adaptation strategy.

Keywords: $\delta^{13}C$; $\delta^{18}O$; $\delta^{15}N$; ozone; gas exchange; English oak; holm oak; downy oak

1. Introduction

Oaks are widely distributed in the Mediterranean basin where drought and air pollution such as tropospheric ozone (O_3) are the main limiting factors for productivity [1–3]. The diversity of oak species is the result of the adaptation to the environment [4]. A wide phenotypic plasticity helps the

plants to adapt to chronic environmental stress through the change of morphological and physiological traits [5].

Water shortage affects tree growth through the alteration of metabolism and physiology of primary and secondary meristems [6–8]. Reduced photosynthetic carbon assimilation is often reported as a result of stomatal closure under reduced water availability [9–11]. In contrast, O_3 impairs ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and photosynthesis, thus inducing stomatal closure [12,13]. Although the individual effects of water deficit and O_3 pollution are well documented, the interaction has been less studied. A drought-induced stomatal closure may limit O_3 uptake thus reducing O_3 injury to plants [14]. However, higher effects of drought were found than long-term O_3 exposure in seedlings of *Q. ilex*, where stomata closure induced by drought did not protect from O_3 damage [15]. Variations in stable isotope ratios of plant materials allow assessing long-term stress conditions, seasonally, in relation to environmental constraints. Elevated foliar carbon isotope ratio ($\delta^{13}C$) is a proxy of high water use efficiency [16,17]. $\delta^{18}O$ values are negatively correlated with stomatal conductance (g_s) thus reflecting plant water status [18–22]. $\delta^{15}N$ significantly decreases as mean annual rainfall increases [23].

Forecasting the impact of global changes on forest ecosystem processes, such as productivity and community composition, is based on the trees' species-specific responses to the combined effect of multiple stressors and the capacity of each species to acclimate and cope with the environmental modification. Therefore, combined constraints are highly relevant in the assessment of tree responses in forest ecosystems. The relationship between physiological responses (photosynthetic and stomatal activity) and stable isotope ratios (carbon, oxygen, and nitrogen) allows identifying distinct phenotypic acclimation strategies.

Among the genus *Quercus*, a wide range of sensitivity to drought exists [24]. *Q. robur*, deciduous oak, is considered sensitive to drought and some mortality events were reported in the past following dry seasons [25]. *Q. ilex*, evergreen oak, avoids desiccation by regulating stomatal conductance in response to the increasing of water deficit intensity [26], making this species highly tolerant to drought and well adapted to Mediterranean environments. The drought tolerance of *Q. pubescens*, deciduous oak, was favored by isohydric stomatal behavior [27] and a less vulnerable hydraulic structure of leaf petioles than *Q. robur* [24]. Deciduous oaks are usually considered more O_3-sensitive than evergreen oaks in responses to O_3 [28].

The present study aimed to define the responses of *Q. robur*, *Q. ilex*, and *Q. pubescens* to three levels of O_3 (1.0, 1.2, and 1.4 times the ambient concentration in an open-air facility) and two levels of irrigation (40 and 100% of soil water content at field capacity) through the stable isotope signatures of C, O, and N, and gas exchange. The main hypothesis was that oak species respond differently to O_3 and irrigation regimes, by regulating photosynthetic rate and stomatal conductance to preserve leaf water potential and content in response to combined stresses. The results of this study provide novel insights into the impact of a simultaneous pairing of abiotic stresses (i.e., water and O_3 stress) on the water and carbon responses of oak species in leaves.

2. Materials and Methods

2.1. Plant Material and Experimental Design

Two-year-old seedlings of potted *Q. ilex* L., *Q. robur* L., and *Q. pubescens* Willd. obtained from nearby nurseries (origins of seedlings: *see* [29]) were transferred to 10 L pots in autumn 2014 and then raised at the experimental site in Sesto Fiorentino in central Italy (43°48′59″ N, 11°12′01″ E, 55 m a.s.l.), where the experiment was performed from June to October 2015. The seedlings were grown under the combination of three levels of O_3 (1.0, 1.2, and 1.4 times the ambient concentration, denoted as AA, 1.2AA, 1.4AA, respectively) and two levels of water irrigation (100% and 40% of soil water content at field capacity, denoted as well-watered, WW, and water-stressed, WS, respectively). During rainfalls, the pots were covered by a plastic cover at 1 cm above the pot, so that evapotranspiration from the

soil was not affected (data not shown). The volumetric soil water content (SWC) was measured in the root layer (5 cm depth) by EC-5 soil moisture sensors equipped with an EM5b data logger (Decagon Devices, Pullman WA, USA). In WW plants, SWC was close to field capacity (i.e., 0.295 m^3 m^{-3}).

Details on the O_3 exposure systems are reported in [30]. In short, O_3 was distributed by Teflon tubes hanging down from a fixed grid above the trees. Ozone concentrations at plant height (approx. 1 m) were continuously recorded by O_3 monitors (Mod. 202, 2B Technologies, Boulder, CO, USA). The daily mean O_3 concentrations were 35.1 ± 1.0 ppb, 43.0 ± 1.2 ppb, and 49.0 ± 1.3 ppb in AA, 1.2AA, and 1.4 AA, respectively, over the experimental period [31].

Plots (5 m × 5 m × 2 m) were assigned to each O_3 treatment in three replicates, with three plants per each combination of species, water regime, and O_3 in each plot. The plant position was changed every month within each plot to eliminate positional effects [32].

Other results from the same experiment are available in [31] (risk assessment), [33] (antioxidants), and [29] (belowground responses).

2.2. Stable Isotopes

Randomized leaf samples (fully expanded sun-exposed leaves of 4–6th order from the shoot tip) of at least 75–100 mg dry weight (DW) each were gathered at the end of treatments (i.e., on October 15th). Samples were collected from each of the three plants per treatment replicate and joined in a single sample, resulting in three samples for each species per each treatment. Samples were dried and milled in a ball mill.

Stable isotope analyses were performed at the Slovenian Forestry Institute (Ljubljana, Slovenia). An aliquot of each sample (m = 0.200 ± 0.050 mg) was weighed into a tin capsule for C and N stable isotope measurements and silver capsule for O stable isotope measurements using a microanalytical balance Sartorius CPA2P and analyzed with Vario Pyro Cube Elemental Analyzer (Elementar, Germany) coupled with IsoPrime100 Isotope Ratio Mass Spectrometer (IsoPrime, UK).

Both certified reference materials (IAEA-601 and IAEA-602 for oxygen, and USGS40, USGS41, IAEA-N-1, and IAEA-N-2 for carbon and nitrogen) and in-house working standards (acetanilide, benzoide, oak leaves, and cellulose) were used to control the accuracy and precision of the measurements. The analytical precision was <0.1‰ for carbon and nitrogen, and <0.3‰ for oxygen, expressed as standard deviation of repeated measurements of in-house working standards ($n = 3$).

Stable isotope results ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$) are reported in terms of relative delta (δ) value as a difference between $^{13}C/^{12}C$, $^{15}N/^{14}N$, or $^{18}O/^{16}O$ ratio of the sample and international reference material (Vienna PeeDee Belemnite (VPDB), Atmospheric Nitrogen (AIR), and Vienna Standard Mean Ocean Water (VSMOW)), expressed in per mil (‰).

2.3. Leaf Gas Exchange

Leaf gas exchange was measured in the same fully expanded sun-exposed leaves (4–6th from the shoot tip) of three plants per replicated plot in each treatment using a portable infrared gas analyzer (CIRAS-2 PP Systems, Herts, UK) at controlled values of CO_2 concentration (380 ppm), leaf temperature (25 °C), photosynthetic photon flux density (PPFD, 1500 µmol m^{-2} s^{-1}), and relative humidity (RH, 40–60%). We determined light-saturated net photosynthetic rate (A_{sat}) and stomatal conductance (g_s). Measurements were carried out in two campaigns (June 8–10th and September 27th–October 6th) for all O_3 levels (AA, 1.2AA, and 1.4AA) and an additional campaign (August 6–9th) for two O_3 levels (AA and 1.4AA) on days with clear sky between 9:00 and 12:00 a.m. CET.

2.4. Predawn and Midday Leaf Water Potential and Leaf Water Content

Predawn and midday leaf water potential ($\Psi_{predawn}$ and Ψ_{midday}, respectively) were measured on clear-sky days in August with a pressure chamber (PMS Instruments Co., Corvallis, OR, USA) on two to three fully expanded sun-exposed leaves collected from randomly selected shoots (n = 3). Midday leaf water potential was measured between 01:00 and 03:00 a.m. CET under PPDF higher than

1200 µmol m^{-2} s^{-1}. Predawn measurements were carried out at 1–2 h before dawn. The leaf water content (LWC) was obtained through the measurement of fresh leaf weight (FW), then dried at 80 °C for 72 h (DW). The leaf water content was calculated as: LWC (%) = (FW−DW)/FW.

2.5. Statistical Analysis

The statistical unit was the single plot (*n* = 3 plots). To account for variability in the samples, a pooled standard deviation was computed from the three replicated plots from each species and experimental conditions. Normality of the population distribution was tested using the Shapiro–Wilk test. The homogeneity of variances was performed with the Levene's test. ANOVA analysis was performed to statistically test the effects of treatment (water and ozone), species, month, and their interaction (significance level, *p* < 0.05). The effect of O$_3$ and water stress on the relationship between A$_{sat}$ and g$_s$ was tested by analysis of covariance (ANCOVA). Post hoc Tukey test was carried out to define the differences among groups. Relationships between g$_s$ and A$_{sat}$, between LWC and δ^{13}C, between g$_s$ and δ^{18}O were calculated at the species level and tested for significant differences between regression coefficients of water and ozone stress (only significant relationships were shown). Statistical analysis was performed with the OriginPro 8 program (OriginLab Corporation, Northampton, UK).

3. Results

The seasonal course of A$_{sat}$ and g$_s$ differed significantly from June to September–October in each species, with higher values in early summer than in autumn in the deciduous oaks (Figure 1). δ^{13}C, δ^{18}O, and δ^{15}N were statistically different among species (Table 1), with higher δ^{13}C and δ^{18}O values in WS than WW plants of *Q. robur* and *Q. pubescens* (Figure 2). δ^{13}C and δ^{18}O increased in WS seedlings of all species relative to WW seedlings (Table 1, Figure 2). δ^{18}O was significantly affected by ozone (in *Q. pubescens*) and by water stress (in three oak species), while δ^{13}C was significantly affected only by water stress (in *Q. robur*). δ^{15}N did not change among treated plants. *Q. robur* showed significant differences of δ^{13}C and δ^{18}O between WW and WS in 1.2AA- and AA-treated plants, respectively (Table S1). Plants of *Q. ilex* showed differences of δ^{18}O between WW and WS AA-treated plants (Table S1). In *Q. pubescens*, δ^{18}O was different between WW and WS in all ozone-level-treated plants, as well as between AA and 1.2AA and 1.4AA in WS plants (Table S1).

The combination of species, water, and O$_3$, and of O$_3$ and water stress resulted in significant effects only for A$_{sat}$ (Table 1). A$_{sat}$ and g$_s$ changed between months differently within the three oak species (Figure 1). Light-saturated net photosynthetic rate (A$_{sat}$) of *Q. robur* decreased with season whereas elevated O$_3$ and drought reduced it in August and September–October; A$_{sat}$ of *Q. ilex* did not show a clear seasonal change during the experimental period, whereas it was lower in WS than WW plants in September–October; A$_{sat}$ of *Q. pubescens* reached lower values in WS plants in August and September–October whereas O$_3$ decreased it in autumn. Stomatal conductance showed lower values in WW + 1.4AA than in WW + AA in August in *Q. robur* while the WS treatment significantly decreased it in August, although such stomatal closure was not observed in September–October; g$_s$ did not show differences between water and ozone plants of *Q. ilex* (Table S2); in *Q. pubescens*, g$_s$ was lower in WS than WW leaves in August while O$_3$ did not decrease g$_s$ in this species.
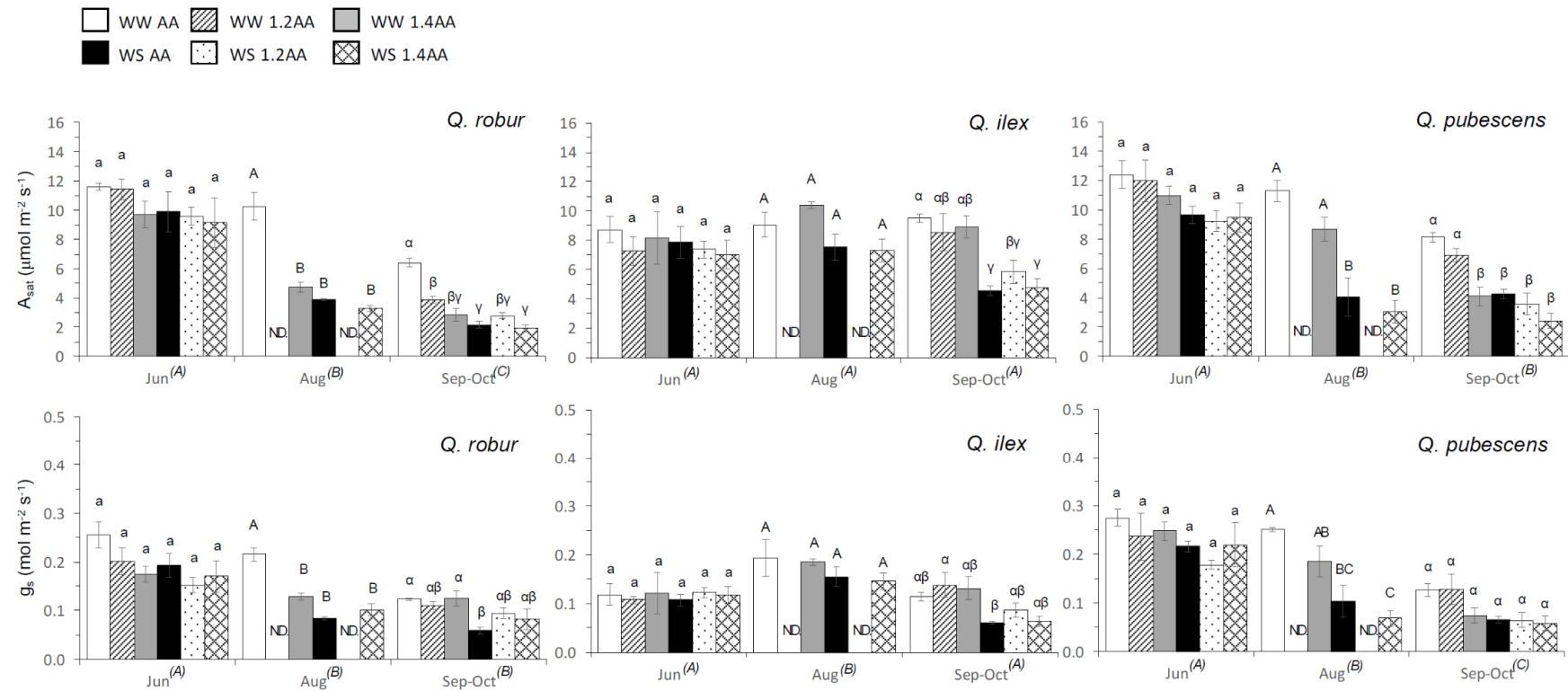
Figure 1. Light-saturated net photosynthetic rates (A_{sat}) and stomatal conductance (g_s) of leaves of *Q. robur*, *Q. ilex*, and *Q. pubescens* exposed to three levels of O_3 (1.0, 1.2, and 1.4 times the ambient concentration, denoted as AA, 1.2AA, 1.4AA, respectively) and two levels of water irrigation (treatment: WW, 100% field capacity; WS, 40% field capacity) in June, August, and September–October. The bars represent mean ± S.E. (n = 3 plots). Different letters show significant differences among treatments ($p < 0.05$, Tukey test) within each month for each species. In addition, different capital letters inside parentheses on the x axis denote significant differences among measured months ($p < 0.05$, Tukey test) in each species. N.D. denotes no data available.
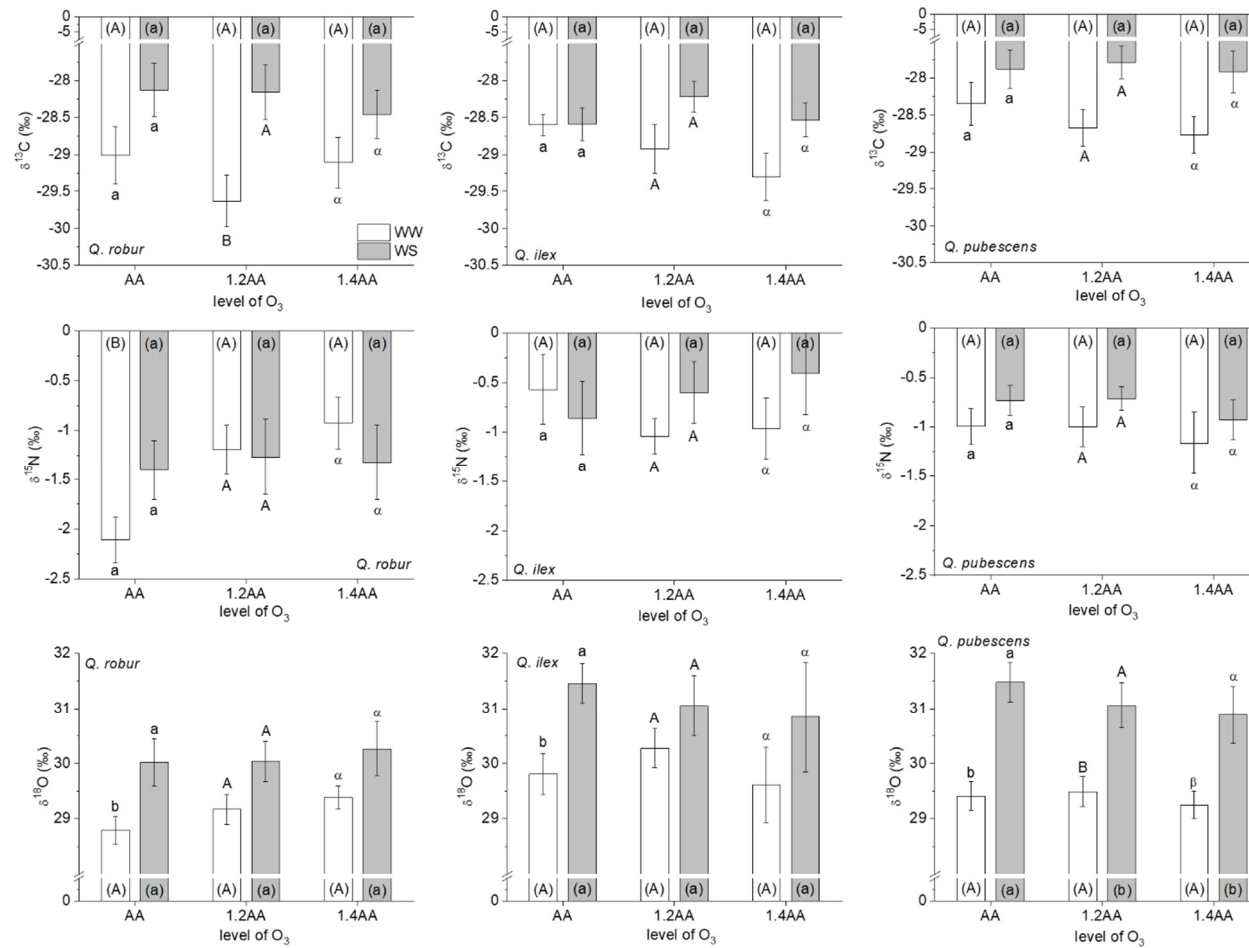
Figure 2. Leaf δ^{13}C, δ^{18}O, and δ^{15}N of *Q. robur*, *Q. ilex*, and *Q. pubescens* exposed to three levels of O$_3$ (1.0, 1.2, and 1.4 times the ambient concentration, denoted as AA, 1.2AA, 1.4AA, respectively) and two levels of water irrigation (treatment: WW, 100% field capacity, black squares, solid line; WS, 40% field capacity, white squares, dashed line). Different letters show significant differences between water levels at same O$_3$ level, different letters inside parentheses show significant differences between ozone levels at same water treatment ($p < 0.05$, Tukey test, $n = 3$ plots) in each species.

Table 1. ANOVA of A_{sat} and g_s (four-way analysis), $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$, $\Psi_{predawn}$, Ψ_{midday} and LWC (three-way analysis), of *Q. robur*, *Q. ilex*, and *Q. pubescens* exposed to O_3 and water treatments is reported (*p*-level: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant, $p \geq 0.05$).

	p-Level							
	A_{sat}	g_s	$\delta^{13}C$	$\delta^{18}O$	$\delta^{15}N$	$\Psi_{predawn}$	Ψ_{midday}	LWC
species	***	**	***	***	***	ns	ns	***
O_3	***	*	ns	**	ns	ns	*	ns
water	***	***	***	***	ns	***	ns	ns
species X O_3	*	ns	ns	ns	ns	ns	ns	***
O_3 X water	*	ns	ns	ns	ns	ns	ns	ns
species X water	**	*	ns	ns	ns	ns	ns	ns
species X O_3 X water	*	ns	ns	ns	ns	ns	ns	ns
month	***	***						
species X month	***	***						
month X O_3	ns	*						
month X water	***	**						
species X month X O_3	ns	ns						
species X month X water	**	ns						
month X O_3 X water	ns	ns						
species X month X O_3 X water	ns	ns						

Predawn water potential was higher in WW than WS plants of the three oak species (Figure 3; Table S1). Midday water potential of *Q. ilex* reached higher values in 1.2AA plants than AA and 1.4AA plants (Figure 3; Table S1). *Q. pubescens* showed that Ψ_{midday} was higher in 1.2AA plants than others, it was different between AA and 1.2AA of WW and WS plants, and between 1.2AA and 1.4AA of WW plants (Figure 3; Table S1). LWC of *Q. ilex* reached higher values in AA and 1.2AA plants than in 1.4AA (equally in WW and WS) plants (Figure 3); in detail it was different between AA and 1.4AA, and 1.2AA and 1.4AA in both WW- and WS-treated plants (Table S1). In *Q. robur*, LWC was lower in WS of 1.4AA plants than other conditions (Table S1). LWC of *Q. pubescens* was lower in WS plants, both 1.2AA and 1.4AA, than WW plants, and differences were found between AA and 1.2AA in WS, and between AA and 1.4AA in WW plants (Figure 3, Table S1).

The slope of the relationship between A_{sat} and g_s was significantly affected by the combined treatments of ozone and water irrigation in the three oak species (Figure 4, Table S3). Water irrigation increased the slope especially in AA for all three species. On the other hand, $1.4 \times AA$ O_3 exposure increased the slope just in WW-treated *Q. pubescens* leaves.

Significant relationship between LWC and $\delta^{13}C$ was found in *Q. robur* and *Q. ilex* (Figure 5). *Q. robur* showed a reduction of $\delta^{13}C$ at increasing LWC, while *Q. ilex* increased $\delta^{13}C$ at increasing LWC (Figure 5). *Q. pubescens* did not show relationship between LWC and $\delta^{13}C$. A significant relationship between g_s and $\delta^{18}O$ was found in all species, and did not differ with water and ozone levels (Figure 5).
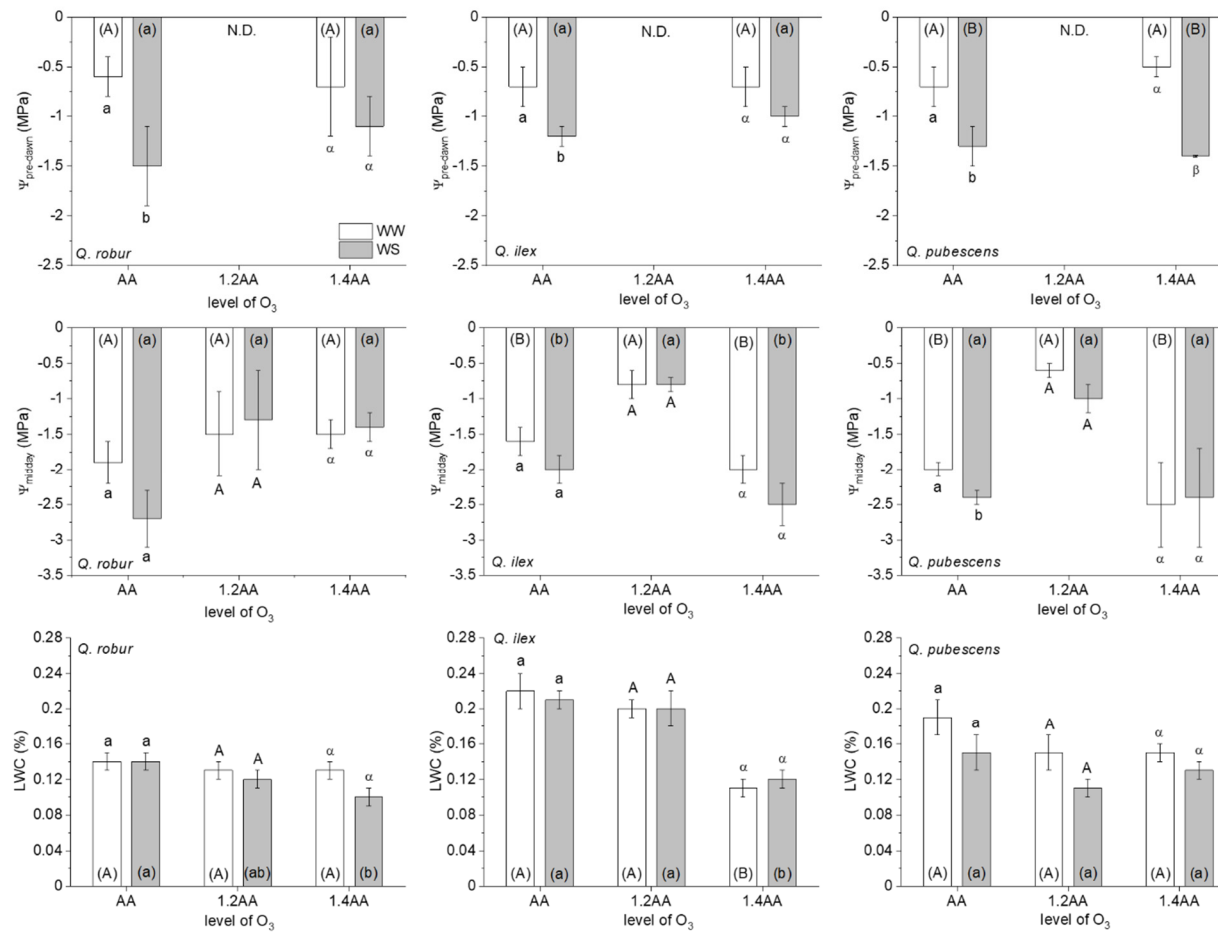
Figure 3. Predawn leaf water potential ($\Psi_{predawn}$), midday leaf water potential (Ψ_{midday}), and leaf water content (LWC) of *Q. robur*, *Q. ilex*, and *Q. pubescens* exposed to three levels of O_3 (1.0, 1.2, and 1.4 times the ambient concentration, denoted as AA, 1.2AA, 1.4AA, respectively) and two levels of water irrigation (treatment: WW, 100% field capacity, white bars; WS, 40% field capacity, grey bars). Different letters show significant differences between water levels at same O_3 level, different letters inside parentheses show significant differences between ozone levels at same water treatment ($p < 0.05$, Tukey test, $n = 3$ plots) in each species. N.D. denotes no data available.
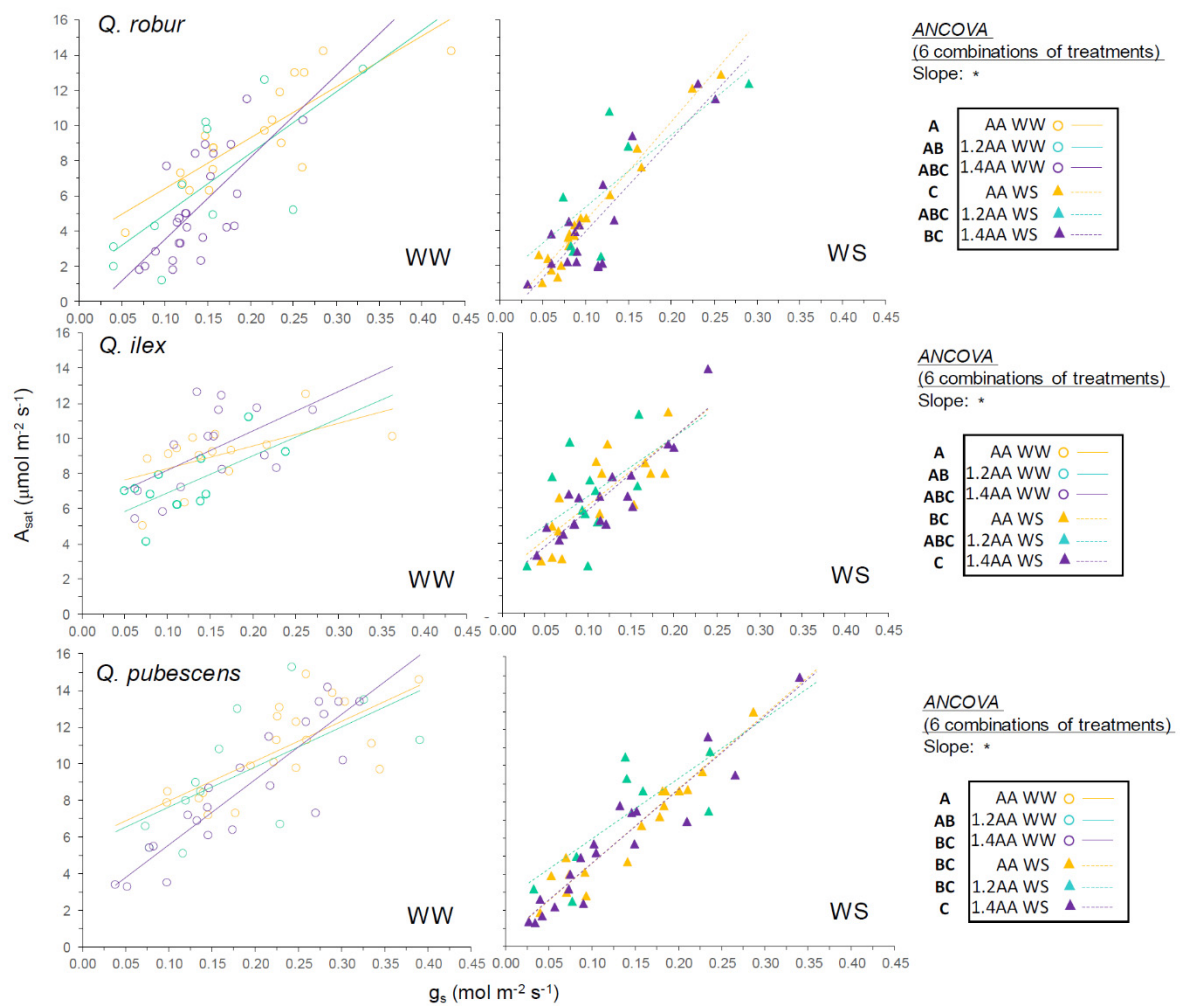
Figure 4. Relationship between light-saturated net photosynthetic rate (A_{sat}) and stomatal conductance (g_s) of *Q. robur*, *Q. ilex*, and *Q. pubescens* exposed to three levels of O_3 (1.0, 1.2, and 1.4 times the ambient concentration, denoted as AA, 1.2AA, and 1.4AA, respectively) and two levels of water irrigation (treatment: WW, 100% field capacity; WS, 40% field capacity). Pooled data from June to September–October were used. Asterisks show the significance of an ANCOVA: * $p < 0.05$. Different capital letters denote significant differences of the slopes among the treatments ($p < 0.05$). All regressions were statistically significant ($p < 0.05$). The values of slope, y-intercept, and determination coefficient (R^2) are listed in Table S3.
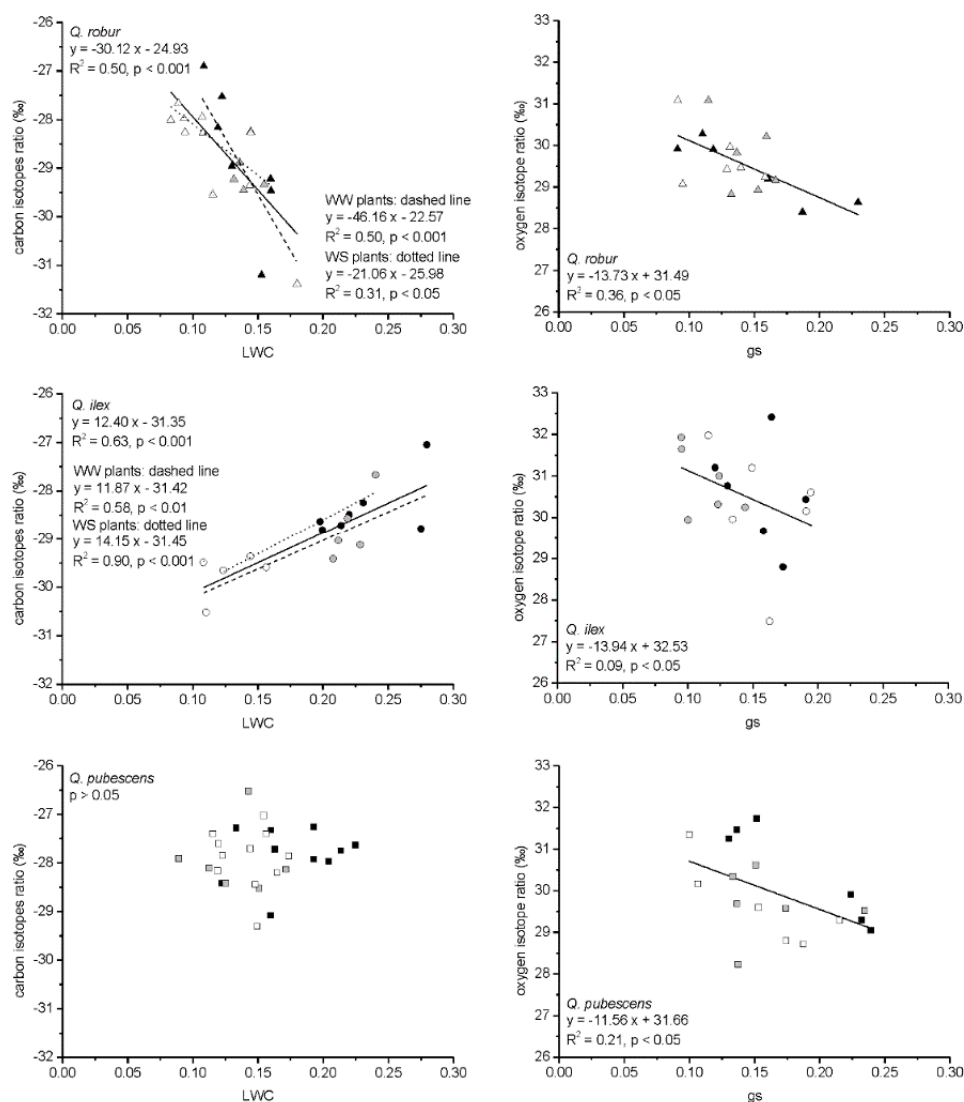
Figure 5. Relationship between leaf water content (LWC) and $\delta^{13}C$ ($n = 3$ plots), and between stomatal conductance (g_s) and $\delta^{18}O$ ($n = 3$ plots) of *Q. robur* (triangles), *Q. ilex* (circles), and *Q. pubescens* (squares) exposed to three levels of O_3 (1.0, 1.2, and 1.4 times the ambient concentration, denoted as AA, black symbols; 1.2AA, grey symbols; 1.4AA, white symbols) (regression functions are shown as solid line) and two levels of water irrigation (regression functions of WW plants are dashed line, and WS plants in dotted line) (regression functions are missing when they are not significant).

4. Discussion

4.1. Ozone Effects

Light-saturated photosynthesis and stomatal conductance differed among oak species (Table 1), showing higher values in the two deciduous species than in the evergreen one (according to [34]), which may result in high stomatal ozone uptake and high ozone damage in deciduous trees [31,35]. Our results confirm that deciduous English oak (*Q. robur*) is more sensitive to O_3 than downy oak (*Q. pubescens*) and evergreen holm oak (*Q. ilex*) [28,31], as *Q. robur* showed lower values of A_{sat} when exposed to the highest ozone levels (1.4AA). Ozone exposure usually reduces stomatal conductance [36,37] (Figure 1). This was confirmed in WW O_3-exposed *Q. robur* leaves in August. However, in autumn, even when A_{sat} decreased, g_s remained high in *Q. robur*, implying a less efficient stomatal control after O_3 exposure (i.e., stomatal sluggishness) [38–40]. On the other hand, O_3 impact on stomatal conductance was not marked in *Q. ilex* and *Q. pubescens* throughout the experiment, suggesting a low stomatal reactivity. An

effect of ozone was also detected on Ψ_{midday}, suggesting higher water stress, especially in *Q. pubescens* at the 1.2AA ozone level (Figure 3), due to O_3 exposure in the daytime at the highest evapotranspiration demand. This response is in contrast with the observed decline of g_s under O_3, but in agreement with stomatal sluggishness (i.e., an imperfect or delayed closure of O_3-exposed stomata) [36]. In addition, O_3 may modify root anatomy, decreasing the water flux of roots in consequence with the reduced water supply to aboveground parts in the target oak species [29]. Interestingly, the overall effect of O_3 on the responses to water deficit was small in the short term (with a slight decline of g_s and Ψ_{midday}) but significant at the end of the experiment ($\delta^{18}O$ increased), while the overall effect on the carbon balance was highly significant in the short term (A_{sat} decline) but carbon isotope responses were not significant at the end of the experiment ($\delta^{13}C$). Similar findings were observed in O_3-exposed *Fagus sylvatica* leaves [41,42], highlighting a seasonal variability of O_3 effects on A_{sat} and g_s. In theory, stomatal closure may lead to a decrease in the intercellular CO_2 concentration (C_i) [43]. However, this may be compensated by the accompanying decrease in photosynthetic rate due to elevated O_3, which results in increases of C_i [36]. Even more interestingly, such responses were not species-specific in spite of the different O_3 and water deficit sensitivity, suggesting that the three oak species modulated stomatal responses to partly compensate the O_3-induced seasonal losses in carbon assimilation.

4.2. Water Deficit Effects

The three oak species showed seasonally contrasting patterns in their A_{sat} response to water deficit. However, the reduction of photosynthesis of oaks was observed in drought-exposed plants in September–October, highlighting the influence of environmental constraints on tree carbon assimilation [44]. Also, the stomatal conductance was reduced by low water availability in *Q. robur* and *Q. pubescens*, whereas g_s of *Q. ilex* was maintained at the same level from June to September–October. Higher sensitivity of gas exchange to water deficit was observed in deciduous oaks than in the evergreen *Q. ilex*, which is consistent with the previous finding [45]. A significant increase of the slope in the relationship between A_{sat} and g_s across the irrigation regimes was found in three oak species, suggesting an increased intrinsic water use efficiency under water stress conditions (Figure 4). The WS-treated *Q. robur* leaves had a high slope (Table S3), indicating that water deficit intensively induced stomatal closure in this species, which results in a reduced supply of CO_2 to the carboxylation sites [46,47]. For all oak species, $\delta^{18}O$ differed between WW- and WS-treated plants exposed to AA ozone level, resulting in high $\delta^{18}O$ values from water-limiting conditions, and reduced total canopy transpiration. Water stress generally limits the uptake and transfer of N into the upper plant parts, resulting in an increased $\delta^{15}N$ [48]. However, no response of $\delta^{15}N$ in *Q. ilex* and *Q. pubescens* suggests that the responses of g_s were not severe, in contrast with what was observed in crops [49]. However, in *Q. robur*, $\delta^{15}N$ was affected by ozone exposure in well-watered plants (WW), with higher values in 1.2AA and 1.4AA than AA WW plants, indicating a sensitivity to environmental constraints other than growth conditions (Figure 2, Table S1).

4.3. Combined Responses

To the best of our knowledge, this is the first study about effects of these combined stressors (namely water deficit and ozone exposure) on isotope signature in the discrimination of adaptation strategies in three species from the same genus (*Quercus* sp.). Although stable isotope ratios are considered a reliable proxy of plant stress condition and oak species were discriminated on the basis of $\delta^{13}C$ for water stress, the combination of water and O_3 stress did not result in any significant interaction on the isotope signatures. These results confirmed the previous study where the carbon isotopic discrimination was observed in relation to water deficit, but not to ozone in seedlings of *Fagus sylvatica*, *Quercus robur*, and an ozone-sensitive *Populus* clone [50]. The only significant ozone x water interaction was on the responses of A_{sat}. It has been reported that fast-growing plant species, namely poplar, with high water requirements are more susceptible to ozone and drought stress [50]. In midsummer and autumn, water stress and O_3 negatively affected A_{sat} in *Q. robur* (Figure 1). On the other hand, A_{sat}

of *Q. pubescens* was reduced by ozone and by water treatment, although water stress had stronger negative effects than ozone stress (Figure 1). The photosynthetic rate of *Q. ilex* was reduced by water stress in autumn, showing seasonal plasticity (Figure 1). However, drought did not induce decoupling in the oak response of A_{sat} to g_s, and minor effects of ozone were observed, suggesting a resistant plant behavior to a changing environment. Such behavior might be a potential mechanism of oak species to adapt to disturbances such as drought and O_3.

The isotope signature highlighted an isohydric behavior of *Q. robur*, where $\delta^{13}C$ and $\delta^{18}O$ were high in response to water stress (Figure 2; Table S1). In detail, $\delta^{13}C$ was significantly affected by water stress, while $\delta^{18}O$ was significantly affected by ozone and by water stress (Figure 2; Table S1). The species showed natively different sensitivity of $\delta^{13}C$ to LWC in *Q. robur* (negative correlation) and *Q. ilex* (positive correlation) (Figure 5), although it did not show differences in response to water stress and ozone levels of the experiment. Therefore, the 40% reduction of irrigation in the experiment determined a mild stress to be detectable through LWC, which is representative of realistic field conditions. Overall, $\delta^{13}C$ decreased under water stress and $\delta^{18}O$ increased under ozone stress, while $\delta^{15}N$ did not respond to water and ozone stress. *Q. robur* and *Q. pubescens* exhibited a more "conservative" water use strategy, characterized by tight stomatal control of transpiration in order to maintain high water use efficiency (according to [50,51]). On the other hand, *Q. ilex* showed a low variability in stomatal conductance in comparison to the other species, suggesting an anisohydric behavior to maintain stomata open under water deficit [52].

5. Conclusions

Responses of photosynthesis to water stress were species-specific, with *Q. pubescens* and *Q. robur* more sensitive than *Q. ilex*, suggesting different adaptation strategies of deciduous and evergreen species. Effects of O_3 on physiological traits were lower than those induced by water stress. In detail, the significant interactions of plant water status and O_3 stress on photosynthesis were as expected, that is, water deficit reduced the negative impacts of O_3. Surprisingly, the effect on photosynthesis did not translate into effects on the seasonal carbon assimilation, that is, $\delta^{13}C$ did not show a significant response to the combination of water and O_3 stress, suggesting adaptation strategies to overcome the growing season. However, $\delta^{13}C$, $\delta^{18}O$, and $\delta^{15}N$ were different among species, with higher $\delta^{13}C$ and $\delta^{18}O$ values in WS than WW plants of *Q. robur* and *Q. pubescens*, whereas $\delta^{15}N$ did not change among treated plants. Different acclimation strategies to cope with stress conditions were found, through the different sensitivity of $\delta^{13}C$ to LWC in *Q. robur* (negative correlation) and *Q. ilex* (positive correlation). However, no significant isotopic responses were found to discriminate combined treatments and to differentiate response strategy across the studied oak seedlings. These results warrant further isotopic studies over longer time periods and wider ranges of plant species, in order to understand the carbon and water responses of vegetation to a changing environment and different levels and combinations of environmental constraints.

Conflicts of Interest: The authors declare no conflict of interest.

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