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Cross-Talk between Physiological and Metabolic Adjustments Adopted by *Quercus cerris* to Mitigate the Effects of Severe Drought and Realistic Future Ozone Concentrations

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Abstract: Global climate change represents a moving target for plant acclimation and/or adaptation, especially in the Mediterranean basin. In this study, the interactions of severe drought (20% of the effective daily evapotranspiration) and O_3 fumigation (80 ppb, 5 h day^{-1}, for 28 consecutive days) on (i) photosynthetic performance, (ii) cell membrane stability, (iii) hydric relations, (iv) accumulation of compatible solutes, and (v) lipophilic antioxidant compounds were investigated in young *Quercus cerris* plants. In addition to the typical drought-induced stomatal closure, imposition of water withholding dramatically influenced the profile of stress-associated metabolites, i.e., abscisic acid (ABA), proline, and lipophilic antioxidants. However, plants were not able to delay or prevent the negative effects of water deficit, the greatest impacting factor in this study. This translated into a steep decline of photosynthetic efficiency, leaf hydration, and membrane fluidity and permeability. When water stress was coupled with O_3, plants orchestrated cross-talk among ABA, proline, and sugar in fully-expanded mature leaves, partially leading to a premature senescence.

Keywords: air pollution; global climate change; oxidative stress; Turkey oak; photosynthesis; stressors interaction

1. Introduction

Climate change, encompassing shifts in temperature, precipitation, and atmospheric composition, represents a moving target for plant acclimation and/or adaptation [1]. The Mediterranean basin is considered a global biodiversity hotspot [2], even though natural (i.e., increase in the average temperature, heat waves, drought), as well as anthropogenic factors, such as increased tropospheric ozone (O_3), are expected to be harsher in this area in the near future [3].

Mediterranean plants have already adapted to climate change through morpho-anatomical, physiological, and molecular responses [4]. In contrast to the past situation, modern climate change is a multifactorial-driven event. Its complexity leads to the fact that the information on the possible future responses of Mediterranean plants are still too fragmentary and sometimes contradictory [5]. To date, progress has been made in understanding the effects of single stress factors on tree performance (e.g., drought and O_3). However, much remains to be elucidated on combined stresses, whose overall effect is far from being deducible from the combination of unifactorial plant responses [6].

Both drought and O_3 have the potential to cause (i) a reduction of plant growth and photosynthesis, (ii) stomatal closure, (iii) cell dehydration, (iv) excess of excitation energy with massive production of reactive oxygen species (ROS), and, eventually, (v) leaf necrosis [6]. However, the simultaneous imposition of O_3 and drought can induce responses considerably different from those observed when each stressor is applied independently. The simultaneous effect of drought and O_3 can affect the plant performances stimulating antagonistic [1], additive, or synergistic responses [7]. The effect of O_3 under conditions of low water availability has been poorly investigated for tree species. More consistent is the information on conifers (e.g., Reference [8]) whereas reports about species of the Mediterranean area are scarce (e.g., Reference [9]). The assumption for which drought-induced stomatal closure would limit O_3 entering the leaf, and the consequent O_3-triggered damage [10], seems not to be universally acceptable. In some cases, drought does not protect trees from the effects of O_3 but, conversely, further exacerbates O_3 damage [11]. In addition to the physiological status of plants under stress, analyses of cellular and metabolic rearrangements adopted by plants may provide complementing evidence to describe the role(s) of several metabolites (such as compatible solutes, osmoprotectants, low-molecular weight proteins, and antioxidants) in the adaptation/acclimation of plants to harsh environmental conditions, such as the intricate interactions between drought and O_3 [12].

Turkey oak (*Quercus cerris* L., Fagaceae) is a Euro-Mediterranean species growing in acidic soils with good water availability [13]. This winter deciduous tree has been long considered a low water-saving species [14,15]. However, studies at different spatial and temporal scales showed conflicting results. Some authors reported a significant change in the diurnal patterns of the photosynthetic rate and stomatal conductance in sunlit leaves of the upper canopy of a coppice in central Italy [16] which suggests that it expressed less stomatal control than xeric-adapted species (e.g., *Fraxinus ornus*) [17]. By contrast, Manes et al. [18] observed that three-years-old seedlings are able to cope with short-term water stress through stomata regulation. Discrepancies in the literature are probably due to the different conditions adopted in the experiments. Most of our knowledge comes from studies of adult trees subjected to severe drought stress where water withholding is applied until plant wilting and mortality [19–21]. However, it should be noted that responses of young trees growing in natural and anthropic conditions can differ significantly [22]. This is particularly relevant for *Q. cerris*, for which scarce information is available on the structural and functional leaf traits that can predict its behavior under unfavorable environmental conditions.

In a previous experiment [6], the behavior of *Q. cerris* saplings exposed to moderate drought and/or O_3 throughout the whole summer season was evaluated. The tolerance of the Mediterranean sympatric *Q. ilex* (evergreen), *Q. pubescens* (deciduous), and *Q. cerris* (deciduous) species to these environmental conditions was compared, focusing on their different phenotypic plasticity. The present work was performed to test if a shorter and severe drought might further limit O_3 uptake, thereby inducing different responses of *Q. cerris*, the species that had shown to be the least drought tolerant [6]. In particular, here we would like to analyze, in greater depth, the leaf-intrinsic and osmotic adjustments adopted by *Q. cerris*.

These experiments highlight, for the first time, the differential response of the same tree species under short and harsh drought, or longer and less severe water withholding, both in combination with O_3. This poses the bases for understanding the differences which occur in *Q. cerris* between acclimation (short and severe drought) and early adaptation (longer, but less severe, drought) to those stressors which can be considered a key issue for plant physiology/ecology in the era of global change.

2. Materials and Methods

2.1. Plant Material and Experimental Design

One hundred three-year-old saplings of *Q. cerris* from a forest nursery located in Gubbio (43°19′43″ N, 12°33′10″ E, 431 m above sea level (a.s.l.), Perugia, Italy), were transported to the field-station of San Piero a Grado (43°40′48″ N, 10°20′46″ E, 2 m a.s.l.), Pisa, Italy, at the beginning

of spring 2014, inside 3-L pots, where they had been grown until then. The following day, plants were transferred into 6.5-L pots filled with a growing medium containing a mixture of standard soil Einhetserde Topfsubstrat ED 63 T grob (Sinntal-Altengronau, Sinntal, Germany; peat and clay, 34% organic C, 0.2% organic N and pH of 5.8–6.8) and sand (3.5:1 in volume). Potted plants were placed into a greenhouse under controlled irrigation for two months in an air filtered environment using active charcoal where O_3 concentration was negligible (below 5 ppb), as measured by a photometric analyzer (Monitor Labs, mod. 8810, San Diego, CA, USA).

Half of the plants (WW) were kept at field water capacity for the entire period. The other half of the plants (WS) were watered with 20% of their effective daily evapotranspiration (calculated as the average of 24-h weight loss of five well-watered plants). When net CO_2 assimilation (A) of WS plants decreased by 50%, in comparison to WW plants (14 days), the O_3 treatment was started (80 $\pm$ 13 ppb for 5 h day^{-1} for 28 consecutive days, in the form of a square wave between 10:00 and 15:00). O_3 target concentration was established by doubling the average concentrations recorded between 1 April 2012 and 30 September 2012 (this period was characterized by high O_3 concentrations due to favorable conditions for its formation as high irradiance and temperature [23]) in 14 automatic monitoring stations managed by ARPAT (Regional Agency for the Environment of Tuscany), in order to simulate a future climate scenario. The O_3 concentration applied in this experiment was known to induce changes in the main photosynthetic parameters in Turkey oak plants subjected to prolonged exposure [6]. The entire methodology of O_3 exposure has been performed according to Lorenzini et al. [24]. Eighty uniformly-sized plants were transferred into four controlled environment fumigation facilities (20 plants per box), which were ventilated with charcoal-filtered air (two boxes of WW/O_3 − (control) and WS/O_3 − (drought)), or treated with O_3 (two boxes of WW/O_3 + (ozone) and WS/O_3 + (drought × ozone)). Plant position was changed twice a week in each chamber, and plants were rotated once a week (at the same time of ecophysiological measurements) between the pairs of boxes with the same treatment in order to minimize possible chamber effects. Throughout the whole experimental period midday photosynthetic photon flux density (PPFD) averaged 1644 µmol photons m^{-2} s^{-1}. Minimum/maximum air temperatures and relative humidity (measured daily with Tinytag Ultra 2 data loggers, Gemini Dataloggers, West Sussex, UK) were 19.7, 33.5 °C, and 67%, respectively. The experiment was based on a completely randomized design and the experimental unit consisted of one plant per container.

Before the beginning of the differentiation of the irrigation regime, mature fully-expanded leaves were marked and, later, the same leaves were used for physiological measurements throughout the whole experimental period. At the end of the experiment, only marked leaves (which reached their maturity during the experimental trial) were collected for biochemical analyses, predawn leaf water potential (PDΨ_w, measured between 05:00 and 06:00), relative water content (RWC), and electrolytic leakage (EL). Visible foliar injury was assessed every week on the marked leaves of each plant in order to detect the time of onset of the first visible symptoms. During the exposure, leaf gas exchange and chlorophyll *a* fluorescence measurements were conducted at 7, 14, 21, and 28 days from the beginning of the exposure (FBE), from 11:00 to 12:00. Furthermore, diurnal profiles of leaf gas exchange and chlorophyll *a* fluorescence were evaluated at the end of the experiment (28 days), performing these analyses every 2 h between 06:00 and 18:00 under ambient light and CO_2 concentration (see Section 2.2. for methodological details). On the same day, PDΨ_w, RWC, and EL were measured, and for each replicate five fully-expanded mature leaves per plant per treatment were mixed and divided into aliquots. Aliquots for the measurement of leaf osmotic potential (Ψ_π) were stored at –20 °C until they were analyzed. Conversely, the aliquots (ten fully-expanded mature leaves per plant) for biochemical analyses were frozen in liquid nitrogen and subsequently lyophilized.

2.2. Ecophysiological Analyses

The PDΨ_w was measured on one fully expanded mature leaf of three plants using a Scholander-type pressure chamber (model 600, PMS Instrument, Albany, OR, USA) and N_2 for the

application of pressure, following the precautions suggested by Turner and Long [25]. To determine the Ψ_π, aliquots of four frozen leaves (major veins were excised) were thawed for 30 s, and 10 µL of sap were squeezed out for the determination of solute concentration with a vapor pressure osmometer (Wescor 5500, South Logan, UT, USA). Each aliquot was measured in triplicate and three replications were taken for every treatment [26]. RWC and EL were determined on the leaves previously used for leaf gas exchange and chlorophyll *a* fluorescence measurements following standard methodologies [27].

Leaf gas exchanges and chlorophyll *a* fluorescence were measured on two fully-expanded mature leaves of three plants per treatment. Values of A, stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were determined using a LI-6400 portable photosynthesis system equipped with a 2 × 3 cm chamber and 6400-02B LED light source (Li-Cor Inc., Lincoln, NE, USA), operating at 390 ppm ambient CO_2 concentration and saturating light conditions (PPFD of about 1200 µmol photons m^{-2} s^{-1}).

Modulated chlorophyll *a* fluorescence and the status of the electron transport of photosystem II (PSII) were measured with a PAM-2000 fluorometer (Walz, Effeltrich, Germany) on the same leaves used for gas exchange after dark-adapting for 40 min using a dark leaf-clip. The maximum efficiency of PSII photochemistry was calculated as $F_v/F_m = (F_m - F_0)/F_m$, where F_v is the variable fluorescence and F_0 is the minimum fluorescence of dark-adapted leaves. Maximal fluorescence, F_m, when all PSII reaction centres were closed, was determined by applying a saturating light pulse (0.8 s) at 8000 µmol m^{-2} s^{-1} in dark-adapted leaves. Fluorescence was induced with actinic light (about 480 µmol m^{-2} s^{-1}), superimposed with 800 ms saturating pulses (10,000 µmol m^{-2} s^{-1}) at 20 s intervals to determine the maximal fluorescence in the light-adapted state (F'_m). Minimal fluorescence in the light-adapted state (F'_0) was determined immediately after turning off the actinic source in the presence of a far-red (>710 nm) background for 10 s to ensure maximal oxidation of PSII electron acceptors. The saturation pulse method was used for analyzing the quenching components, as described by Schreiber et al. [28].

2.3. Biochemical Analyses

Oxidative damage was estimated in terms of lipid peroxidation determining the malondialdehyde (MDA) by-product accumulation, which takes into account the possible influence of interfering compounds in the assay (such as phenols) for the thiobarbituric acid reactive substances. Lipid peroxidation was determined according to Döring et al. [29].

Proline content was determined according to the method of Bates et al. [30], with minor modifications as reported in Cotrozzi et al. [6].

Abscisic acid (ABA) content was measured after extracting 80 mg of lyophilized leaves in 0.8 mL of HPLC-grade water overnight at 4 °C. ABA was then determined by High Performance Liquid Chromatography (HPLC; P680 Pump, UVD170U UV-VIS detector, Dionex, Sunnyvale, CA, USA) at room temperature with a reverse-phase Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter × 150 mm length) according to Perata et al. [31], with some modifications, as reported here. ABA was eluted at a flow rate of 1 mL min^{-1} using different proportions of HPLC-grade water (added with 0.05 M acetic acid, solvent A) and 100% HPLC-grade methanol (solvent B): 70% of A and 30% of B for 6 min, 2 min linear gradient to 50% of A and 50% of B, 50% of A and 50% of B for 18 min, 2 min linear gradient to 100% B, 100% B for 2 min and, finally, 2 min linear gradient to 70% A and 30% B. ABA was detected by its absorbance at 254 nm. To quantify the ABA content, known amounts of pure standard were injected into the HPLC system and an equation, correlating the peak area to ABA concentration, was formulated. Data were extrapolated by Chromeleon software version 6.60 (Dionex Corporation, Sunnyvale, CA, USA).

Photosynthetic and accessory pigments were determined by HPLC according to Döring et al. [29], with some minor modifications as reported here. Fifty milligrams of lyophilized leaves were homogenized in 1 mL of 100% HPLC-grade methanol and incubated overnight at 4 °C in the dark. HPLC separation was performed at room temperature with the same instrumentation and column used for ABA determination.

Sugars were analyzed by HPLC (with the same pumps of ABA and leaf pigment determinations), according to Pellegrini et al. [32], with some minor modifications, as reported here. Sixty milligrams of leaves were homogenized in 1 mL of 100% HPLC demineralized water and heated for 60 min in a water bath at 60 °C. HPLC separation was performed with a BioRad column (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad, Segrate, MI, Italy) at 50 °C. The sum of glucose and fructose was considered as an estimation of hexoses.

2.4. Statistical Analyses

For gas exchange and chlorophyll *a* fluorescence parameters, values obtained by the two leaves belonging to the same plant were averaged as a single biological replicate. Normality of data was preliminarily tested by the Shapiro-Wilk *W* test. Data were analysed using repeated measures (in the case of the measurements carried out for more than two time-points) or two-way analysis of variance (ANOVA) with drought and O_3 stress as fixed factors. The comparisons among means were determined by the Fisher's least significant difference post-hoc test. Analyses were performed by JMP 11.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Visible Injury

At the end of the experiment, WS/O_3− and WS/O_3+ plants showed visible foliar injury in the form of bifacial and marginal yellow-brown necrosis in the youngest fully-expanded leaves, these representing typical drought-induced symptoms (Figure S1). No injury was observed due to O_3 alone, nor in WS/O_3+ plants given that the symptoms were the same to those found in the WS/O_3− leaves.

3.2. Weekly Profiles of Gas Exchanges and Chlorophyll a Fluorescence

Drought, per se, had a negative effect on A during the progression of the experiment: the strongest decrease was observed at 21 days FBE (−82%, in comparison to controls, Figure 1A). The reduction of A due to O_3 was also significant, but far less severe than that found in WS/O_3− plants (Table 1 and Figure 1A). As per A, drought constrained g_s more severely than O_3 (Figure 1B). Each independent variable had a significant effect on g_s when applied individually, but their negative impact was consistent across days (Table 1). Among the gas exchange parameters, the interaction between drought, O_3, and time was significant only for C_i (Table 1). All plants exposed to drought and/or O_3 showed different values of C_i than controls only after 14 and 28 days FBE. Noteworthy, at the end of the experiment a relevant reduction was found only in WS/O_3+ plants (−56%, Figure 1C).

According to the ANOVA test, the interaction among drought, O_3, and time was not significant for any Chl *a* fluorescence parameter (Table 1). Drought, *per se*, had a negative effect on F_v/F_m detected from 21 days FBE (−8% and −7% at 21 and 28 days FBE, respectively; Figure 1D). Differently, O_3 applied alone did not affect this parameter. Values of F_v/F_m averaged 0.75 throughout the experimental period and did not change among weeks in WS/O_3+ plants. It is worth noting that, at 28 days FBE, values of F_v/F_m were higher in WS/O_{3-} than in WS/O_3+ leaves. Similarly to F_v/F_m, drought stress alone was the major determinant for qP whose values decreased from 7 days FBE and were maintained throughout all the experimental period (except at 14 days FBE; Figure 1E). Differently, O_3 had a significant effect on qP only at 14 days FBE (+22%). For this parameter, WS/O_3+ plants showed similar values than WS/O_3− ones at the end of the experiment. Finally, either drought or O_3 had a significant effect on qNP without changing with time (Table 1). Only WS/O_3+ plants showed different values than controls at 21 and 28 days FBE (+35% and +22%, respectively, Figure 1F).

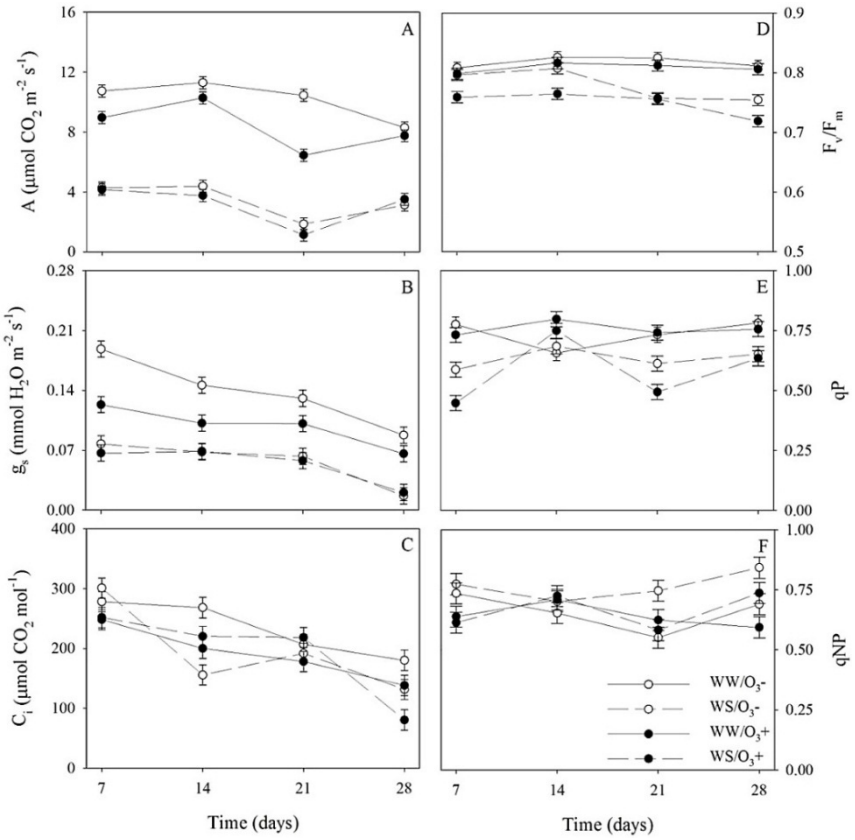

Figure 1. Profiles of CO_2 assimilation rate (**A**); stomatal conductance to water vapor (**B**); intercellular CO_2 concentration (**C**); potential PSII photochemical activity (**D**); photochemical quenching coefficient (**E**) and non-photochemical quenching coefficient (**F**) in *Quercus cerris* plants (i) regularly irrigated to maximum soil water holding capacity and exposed to charcoal-filtered air (WW/O_3-); (ii) water stressed and exposed to charcoal filtered air (WS/O_3-); (iii) regularly irrigated and O_3 fumigated (WW/O_3+); and (iv) water stressed and O_3 fumigated (WS/O_3+). Data are shown as mean ± standard error (n = 3). Abbreviations: A, CO_2 assimilation rate; g_s, stomatal conductance to water vapor; C_i, intercellular CO_2 concentration; F_v/F_m, potential PSII photochemical activity; qP, photochemical quenching coefficient; qNP, non-photochemical quenching coefficient; WS, water stressed; WW, well watered.

Table 1. *F* values of two-way repeated measures ANOVA of the effects of drought and ozone throughout time on CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), intercellular CO_2 concentration (C_i), potential (F_v/F_m), and actual PSII photochemical activity (Φ_{PSII}), photochemical (qP) and non-photochemical quenching coefficients (qNP) in *Quercus cerris* plants. Asterisks show the significance of factors/interaction: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$. d.f. represents the degrees of freedom.

Effects	d.f.	A	g_s	C_i	F_v/F_m	qP	qNP
Drought	1	1971.33 ***	92.60 ***	5.98 *	90.27 ***	40.70 ***	8.67 *
Ozone	1	60.89 ***	10.65 *	8.88 *	14.36 **	0.55 ns	6.95 *
Time	3	32.24 ***	35.00 ***	44.16 ***	7.26 **	7.53 **	3.13 *
Drought × Ozone	1	31.88 ***	8.08 *	7.06 *	3.82 ns	2.81 ns	3.69 ns
Drought × Time	3	6.36 **	2.13 ns	4.58 *	5.34 **	9.45 ***	1.97 ns
Ozone × Time	3	5.71 **	1.65 ns	2.06 ns	0.82 ns	7.24 **	2.83 ns
Drought × Ozone × Time	3	2.44 ns	0.61 ns	4.28 *	1.24 ns	0.85 ns	1.36 ns

3.3. Diurnal Profiles of Gas Exchanges and Chlorophyll a Fluorescence

At the end of the experiment, gas exchange parameters did not show any significant interaction among drought, O_3, and daytime (Table S1). Drought, per se, decreased both A and g_s throughout all of the days and the lowest values were recorded early in the evening (−84% and −82% at 18:00, as compared to controls; Figure S2A,B). The reduction of A and g_s due to O_3 treatment was also significant (Table S1), but far less steep than that induced by drought (applied as a single or combined factor). By contrast, the interactions between drought or O_3 with the time of day were significant for C_i (Table S1). Throughout the whole day, all treated plants had similar or higher values of this parameter than controls, except WS/O_3+ ones at 10:00 (Figure S2C). Drought affected the F_v/F_m ratio from early morning, independent of the presence of O_3 (Figure S2D). However, in WS/O_3− and WS/O_3+ plants the Fv/Fm values at 18:00 recovered almost completely. Differently to the F_v/F_m ratio, each independent variable showed a significant effect of qP (Table S1). In addition to the negative effect of drought, O_3 also induced a decrease of qP in the early morning (−29% as compared to controls; Figure S2E). Among all of the Chl *a* fluorescence parameters, the interaction among drought, O_3, and time was significant only for qNP (Table S1). Drought, *per se*, induced a marked increase of qNP throughout the time of day. It is worth noting that WS/O_3+ plants showed values of qNP similar to those found in the WS/O_3− ones after midday (Figure S2F).

3.4. Leaf Water Status and Osmolyte Contents

At the end of the experiment, only drought had a negative effect on PDΨ_w (Table 2). The decrease of this parameter was more pronounced in WS/O_3− than in WS/O_3+ plants (−440% and −200%, as compared to controls). As in the case of PDΨ_w, drought, alone, constrained Ψ_π and EL more severely than when combined with O_3. The interaction between drought and O_3 was not significant for RWC (Table 2), whereas this parameter decreased significantly under drought (−8%). It is worth noting that drought (alone or combined) had a significant effect on MDA. The increase of this parameter was more pronounced in WS/O_3− plants than in the WS/O_3+ ones (+17% and +5%, as compared to controls).

The interaction between drought and O_3 was not significant for proline, but each single treatment had a significant effect on this metabolite (Table 3). Drought-stressed plants retained higher levels of this osmolyte than controls. Only the imposition of water withholding induced an increase of ABA that was more pronounced in WS/O_3− than in WS/O_3+ plants (+164% and +43%, as compared to controls). The combination of drought and O_3 had a significant effect on hexoses that did not vary in plants subjected to drought or O_3 alone (+22%; Table 3).

Table 2. Predawn leaf water potential (PDΨ_w), leaf osmotic potential (Ψ_π), malondialdehyde (MDA) by-products, relative water content (RWC), and electrolytic leakage (EL) estimated in *Quercus cerris* plants. For details of the treatments, see the caption of Figure 1. Data are shown as means (n = 3). Following two-way ANOVA, for each parameter different letters in each column, indicate significant differences ($p \leq 0.05$). *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$. Abbreviation: SEM, standard error of the mean.

Treatment	PDΨ_w (–MPa)	Ψ_π (–MPa)	MDA (nmol g^{-1} DW)	RWC (%)	EL (%)
WW/O_3−	0.5 ^{a}	3.6 ^{a}	119 ^{a}	79	26.2 ^{a}
WS/O_3−	2.7 ^{c}	4.8 ^{c}	139 ^{c}	73	51.4 ^{c}
WW/O_3+	0.9 ^{a}	4.0 ^{ab}	118 ^{a}	75	26.6 ^{a}
WS/O_3+	1.5 ^{b}	4.2 ^{b}	125 ^{b}	73	36.1 ^{b}
SEM	0.18	0.14	1.5	1.4	2.02
Drought	***	**	***	*	***
Ozone	*	ns	**	ns	**
Drought × Ozone	**	**	**	ns	**

Table 3. Proline, abscisic acid (ABA), and hexoses (μmol g^{-1} DW) estimated in *Quercus cerris* plants. For details of the treatments, see the caption of Figure 1. Data are shown as means (n = 3). Following two-way ANOVA, for each parameter different letters in each column, indicate significant differences ($p \leq 0.05$). *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$. Abbreviation: DW, dry weight; SEM, standard error of the mean.

Treatment	Proline	ABA	Hexoses
WW/O_3-	0.78	0.14 ^{a}	584 ^{a}
WS/O_3-	1.65	0.37 ^{c}	561 ^{a}
WW/O_3+	0.36	0.13 ^{a}	561 ^{a}
WS/O_3+	1.13	0.20 ^{b}	712 ^{b}
SEM	0.047	0.015	12.3
Drought	***	***	***
Ozone	***	**	***
Drought × Ozone	ns	*	***

3.5. Leaf Pigments

Drought (alone or combined) induced an increase of Car/Chl ratio that it was similar in WS/O_3- and WS/O_3+ plants (+7% and +9%, in comparison to the controls). By contrast, WW/O_3+ plants showed the lowest ratio (−6%, Table 4). Similarly to Car/Chl ratio, drought induced an increment of the VAZ/Chl ratio but a decrease was induced by O_3 alone. The combination of drought and O_3 had a significant effect on β-carotene content even though an increase was observed only in plants subjected to drought or O_3. Drought or O_3 induced a significant increase of α-tocopherol (+51% and +26%, respectively) whilst their combination led to a decrement of its foliar content (Table 4).

Table 4. Leaf pigment content in *Quercus cerris* plants. For details of the treatments, see the caption of Figure 1. Data are shown as means (n = 3). Following two-way ANOVA, for each parameter's different letters in each column, indicate significant differences ($p \leq 0.05$). *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$. Abbreviations: Car, total carotenoids; Chl, chlorophyll *a* + chlorophyll *b*; DW, dry weight; SEM, standard error of the mean; VAZ Violaxanthin + Antheraxanthin + Zeaxanthin.

Treatment	Car/Chl (μmol μmol^{-1})	VAZ/Chl (μmol μmol^{-1})	β-Carotene (μmol g^{-1} DW)	α-Tocopherol (μmol g^{-1} DW)
WW/O_3-	0.97 ^{b}	0.27	19.2 ^{a}	3.5 ^{b}
WS/O_3-	1.04 ^{c}	0.29	22.8 ^{b}	5.3 ^{d}
WW/O_3+	0.91 ^{a}	0.24	24.4 ^{b}	4.4 ^{c}
WS/O_3+	1.06 ^{c}	0.28	19.9 ^{a}	2.8 ^{a}
SEM	0.034	0.008	1.22	0.08
Drought	***	**	ns	ns
Ozone	ns	*	ns	***
Drought × Ozone	*	ns	***	***

4. Discussion

Stomatal regulation is one of the most important physiological mechanisms involved in plant acclimation/adaptation to environmental cues [33]. In the present study, weekly profiles of leaf gas exchanges confirm the main results observed with the imposition of prolonged and moderate water withholding [6]: (i) drought induced severe impairments of photosynthetic process; (ii) no further reductions were observed when O_3 was added to drought; and (iii) stomata posed the predominant limitation to CO_2 assimilation in both drought- and O_3-stress conditions, which corroborates the anisohydric behavior of *Q. cerris* [15,34]. Chlorophyll *a* fluorescence measurements reveal again that only drought stress (alone and/or combined with O_3) inhibits the photochemical efficiency of PSII.

Conversely, what was not pointed out under prolonged and mild-severe water stress are the lower values of C_i found in WS/O_3+ plants as compared to WS/O_3- ones, despite similar g_s levels.

Furthermore, the dissipation of excess excitation energy via non-photochemical mechanisms, in this case not associated to an enhancement of xanthophyll cycle, was necessary in WS/O_3+ and WS/O_3− plants. Both of these differences with the previous experiment by Cotrozzi et al. [6] can be explained by the fact that biochemical traits respond more promply to stressful conditions perceived by the plant as compared to morpho-anatomical changes, which are usually preponderant in adaptation to persistant environmental stress [35].

In addition to stomata regulations, a common response of tree species to low water availability consists on the accumulation of inorganic ions and/or the synthesis of protective solutes (e.g., active sugars, polyalcohols, and amino acids). It is known that anisohydric tree species (as *Q. cerris* [15,34]) might be more exposed to hydraulic failure rather than carbon starvation when subjected to severe water stress [36]. Specific attention has, therefore, been devoted to the strong interconnection between carbon metabolism and leaf water status [37]. According to Bartlett et al. [38], the investment of energy and carbon reserves, and the consequent osmoregulation, is a crucial process underlying plant acclimation to drought conditions and influencing the interaction of water withholding with other stressors (i.e., O_3). According to previous studies [38–42], the measurements of leaf water status and osmolyte contents show clearly that osmotic adjustments were adopted by *Q. cerris* subjected to drought stress, irrespective of the imposition of O_3. In particular, cellular water status in WS/O_3− was more compromised than in WS/O_3+ plants and this was associated with a greater increase of ABA and a concomitant accumulation of proline. However, proline seemed not to be involved in cytosolic osmotic regulation (due to the low contribution to the overall Ψ_π), but it might have been involved in the recycling of NADPH via its synthesis from glutamate and/or acting as a free radical scavenger [6,43]. In contrast, only the combination of both the stressors led plants to accumulate slightly higher levels of foliar hexoses. It is known how the regulation of soluble sugar metabolism is mediated by multiple signals, which are generated at different sites depending on environmental conditions and developmental stage [12,44]. Under stress conditions, the export of carbon from mature leaves is reduced; less sugar is loaded into the phloem because of the decreased strength of organ sinks, leading to the accumulation of hexoses in mature leaves [44]. Therefore, it is likely that only in WS/O_3+ plants show an interaction among ABA and/or proline and hexoses might occur as a result of an orchestrated signaling response. It has been demonstrated that ABA [45] can promote leaf senescence and that ABA, proline, and sugars are strictly interlinked together under drought stress conditions [46]. This orchestrated biochemical response might be a part of a premature leaf senescence process. Although premature leaf death is regarded as an inevitable (and negative) consequence of the stress perceived by the plant, it could also be considered as a form of programmed cell death and a plastic trait of leaves under stress [44]. In particular, the ability to modulate the timing of leaf senescence can maintain the overall carbon balance of plants [44]. According to Reference [47], the lower Ci found in WS/O_3+ plants observed at the end of the experiment indicates that the biochemical demand for CO_2 was downregulated in response to declining CO_2 availability, associated with stress-induced stomatal closure. This ability is acquired only when drought is applied simultaneously to O_3 and this suggests that the pollutant may have some "mitigating effects" against limited water availability, as confirmed by: (i) the lowest values of MDA by-products and EL; (ii) the smaller reduction of PDΨ_w, and (iii) the lack of the enhancement of lipophilic antioxidant systems found in WS/O_3+ plants. This orchestrated response found in *Q. cerris* leaves was not deducible from the data collected in this species in our previous work [6] and it represents the core of the novelty of the present experiment.

5. Conclusions

A low tolerance to drought stress of *Q. cerris* was confirmed, independently of the severity and duration of stresses. In particular, the leaf-intrinsic and osmotic adjustments adopted by WS/O_3− plants did not cope with short-term and severe water stress. In addition, *Q. cerris* can be classified as resistant to O_3, not only in terms of visible injury [48]. This tree species is equipped with photoprotective and lipophilic antioxidants associated with chloroplasts that protect cellular and

photosynthetic membranes from the O_3-triggered oxidative damage [49]. The simultaneous imposition of O_3 and drought affected plant performances stimulating antagonistic responses. WS/O_3+ plants partially adjusted and optimized their photosynthetic activity leading to a premature senescence of fully-developed leaves as an acclimation strategy to multiple stress conditions.Climate change will affect the global distribution patterns of vegetation, including those species that are usually considered adaptable to unfavorable environmental conditions (such as *Quercus* spp.). The abrupt global change factors pose new challenges for tree species that have already adapted to gradual climate fluctuations. Further studies may be useful to investigate the biochemical and molecular mechanisms underlying the differences which occur in *Q. cerris* between acclimation and early adaptation to those stressors (single and/or combined) and possible processes favoring or limiting metabolic adjustments.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/8/5/148/s1. Table S1: *F* values of two-way repeated measures ANOVA of the effects of drought and ozone throughout daytime on CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), intercellular CO_2 concentration (C_i), potential PSII photochemical activity (F_v/F_m), actual PSII photochemical activity (Φ_{PSII}), photochemical (qP), and non photochemical quenching (qNP) in *Quercus cerris* plants. Asterisks show the significance of factors/interaction: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p > 0.05$. d.f. represents the degrees of freedom. Figure S1: Symptoms in leaves of *Quercus cerris* water stressed and exposed to charcoal filtered air (WS/O_3−); regularly irrigated and O_3 fumigated (WW/O_3+); water stressed and O_3 fumigated (WS/O_3+). Controls were regularly irrigated to maximum soil water holding capacity and exposed to charcoal filtered air (WW/O_3−). Figure S2: Diurnal profiles of foliar leaf gas exchange and chlorophyll fluorescence parameters in *Quercus cerris* plants (i) regularly irrigated to maximum soil water holding capacity and exposed to charcoal filtered air (WW/O_3−); (ii) water stressed and exposed to charcoal filtered air (WS/O_3−); (iii) regularly irrigated and O_3 fumigated (WW/O_3+); (iv) water stressed and O_3 fumigated (WS/O_3+). Data are shown as mean ± standard error (n = 3). Abbreviations: A, CO_2 assimilation rate; g_s, stomatal conductance to water vapor; C_i, intercellular CO_2 concentration; Φ_{PSII}, actual PSII photochemical activity; qP, photochemical quenching coefficient; qNP, no photochemical quenching coefficient.

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