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Visible Foliar Injury and Ecophysiological Responses to Ozone and Drought in Oak Seedlings

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Abstract: To verify the responses of visible foliar injury (VFI), we exposed seedlings of three oak species for 4.5 months in an open air facility, using differing ozone (O_3) and drought treatments: O_3 (three levels from ambient to ×1.4 ambient), and drought (three levels of irrigation from 40% to 100% field capacity). We related the accumulated phytotoxic O_3 dose (POD_1) and cumulative drought index (CDI) to the O_3 and drought VFI and assessed growth increment (height, diameter, leaf number), biomass (of all organs), and physiological parameters: net photosynthesis per plant (P_n), photosynthetic nitrogen (PNUE) and phosphorus use efficiency (PPUE)). The results indicated that an increase in POD_1 promoted O_3 VFI in *Quercus robur* and *Quercus pubescens*, while *Quercus ilex* was asymptomatic. The POD_1-based critical level at the onset of O_3 VFI was lower for *Q. robur* than for *Q. pubescens* (12.2 vs. 15.6 mmol m^{-2} POD_1). Interestingly, drought reduced O_3 VFI in *Q. robur* but increased it in *Q. pubescens*. Both O_3 and drought were detrimental to the plant biomass. However, *Q. robur* and *Q. pubescens* invested more in shoots than in roots, while *Q. ilex* invested more in roots, which might be related to a hormetic mechanism. P_n, PNUE and PPUE decreased in all species under drought, and only in the sensitive *Q. robur* (PPUE) and *Q. pubescens* (PNUE) under O_3. This study confirms that POD_1 is a good indicator to explain the development of O_3 VFI and helps a differential diagnosis of co-occurring drought and O_3 VFI in oak forests.

Keywords: tropospheric ozone; leaf symptoms; PODy; water stress; risk assessment

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1. Introduction

Tropospheric ozone (O_3) is an oxidative pollutant harmful to plants [1]. Ozone enters the leaves through the stomata, reacts in the mesophyll, and triggers the formation of reactive oxidative species (ROS) with a cascade of events eventually promoting cell death and, finally, the appearance of visible foliar injury (VFI), physiological impairment, and growth reduction [2–4]. Furthermore, O_3 inhibits the efficient use of nutrients such as nitrogen (N) and phosphorus (P) and thereby causes a reduction of photosynthetic N and P use efficiency (PNUE and PPUE, respectively) [5,6]. Therefore, critical levels (CL) have been investigated to assess the O_3 negative impacts on several plant species, especially those related to biomass loss [7,8]. CLs are based on cumulative O_3 indexes, e.g., AOT40, defined as the accumulated exposure over 40 ppb hourly concentrations, and PODy, defined as the phytotoxic O_3 dose above an hourly threshold y of stomatal O_3 uptake [9]. PODy is considered the most realistic index with a high correlation with the detrimental effects of O_3 [10,11]. Ozone VFI is a forest-health indicator in forest monitoring programs [12].

The estimation of CL based on O_3 VFI has been proposed as a not destructive and easily repeated observation over long-term monitoring studies [13,14].

Ozone alone can affect plant growth and development, but its effect usually occurs in combination with other factors, such as drought, which is known as the most critical environmental factor limiting plant productivity worldwide [15,16]. The adverse effects of drought are progressive and, thus, are often evaluated by the cumulative drought index (CDI), defined as the accumulated difference of soil moisture relative to field capacity [17]. Drought stress also promotes the formation of specific VFI, which can be distinguished from O_3-induced foliar injury. While O_3 VFI is usually indicated by interveinal, irregular-border, yellow to dark-brown stippling [18,19], drought VFI consists in gradients of leaf margin necrosis increasing in severity from the base to the top of a plant [20], with the injury co-occurring when plants are exposed to a combination of these stress factors.

Both O_3 and drought can limit plant carbon fixation, and the effect of both stress factors has been reported as the cause of biomass loss for *Quercus* species [21,22], which are significant components of temperate forests. Previous papers from the same experiment presented here showed that the interacting factorial impacts of O_3 and drought were species-specific, and the order of O_3 sensitivity was *Q. robur* > *Q. pubescens* > *Q. ilex* from the point of view of total biomass [22] and leaf gas exchange [23,24]. Although physiological acclimations to O_3 and drought are not fully elucidated, diverse adaptation strategies were observed for tolerating stress in different oak species. One of the reasons for the variability of strategies is related to gas exchange regulation depending on their water use strategy (isohydric and anisohydric) [24]. Under elevated O_3 with sufficient water availability, the isohydric *Q. robur* limited O_3 uptake by stomatal closure, while the anisohydric *Q. ilex* and the intermediate *Q. pubescens* activated tolerance mechanisms and did not actively show a closing response of stomata. In particular, Pellegrini et al. [25] found that *Q. ilex* had a well-regulated antioxidative defense system through phenylpropanoid pathways. However, in the combination of O_3 and drought, the anisohydric *Q. ilex* and the intermediate *Q. pubescens* exhibited stomatal closure to prevent severe oxidative damage due to excess generation of ROS.

The present study aimed to characterize the VFI induced by O_3, drought, and their combination and assess their related effects on biomass, biometry, and physiological parameters. The results will help a differential diagnosis of co-occurring drought and O_3 VFI in oak forests. In detail, we addressed the following hypotheses: (1) the development of O_3 VFI may be better explained by PODy than by AOT40, (2) the reduction in soil water availability may reduce or exacerbate the negative impacts of O_3 on VFI, and (3) O_3 VFI may be an indicator to explain biomass reduction or physiological damage in Mediterranean oaks. We postulated that the effects on the development of VFI are modulated by the plant species-specific sensitivity to oxidative stressors.

2. Materials and Methods

2.1. Plant Material and Experimental Setting

The experiment was conducted in an O_3 Free-Air Controlled Exposure (FACE) facility at Sesto Fiorentino, Italy (43°48′59″ N, 11°12′01″ E, 55 m a.s.l.). Two-year-old plants of *Q. robur* L., *Q. pubescens* Willd., and *Q. ilex* L. were obtained from nurseries and transplanted into 10-L plastic pots. They were exposed to three levels of O_3 (1.0, 1.2, and 1.4 times the ambient air concentration, denoted as AA, ×1.2, and ×1.4, respectively: 24-h averaged concentration, AA = 35.2 ppb, ×1.2 = 42.9 ppb, ×1.4 = 48.9 ppb) and three levels of water irrigation [100, 80, and 40% of field capacity (0.295 m^3 m^{-3}, Paoletti et al., 2017) on average, denoted as WW-treated (well-watered), MD-treated (moderate drought) and SD-treated (severe drought), respectively]. Three replicated plots were assigned to each treatment, with three plants per combination of species, drought, and O_3. The experiment lasted for 4.5 months, from 1 June to 15 October.

The details of the FACE facility are described in Paoletti et al. [26], and the details of the experimental design are published in Hoshika et al. [27].

2.2. Evaluation of O_3 and Drought Visible Foliar Injury

Two well-trained observers evaluated the presence of O_3 and drought VFI during the experimental period for all plants for a total of 6 evaluation dates (Table 1). We applied photo guides to verify whether O_3 and/or drought VFI was present [28–30]. VFI incidence (INC = number of injured plants/total number of plants $\times$ 100) was calculated according to Chappelka et al. [31]. POD_1-based CLs and CDI-based CLs were calculated for the corresponding day when O_3 and drought VFI onset was observed.

Table 1. Phytotoxic O_3 dose (POD_1, mmol m^{-2}) and accumulated exposure over 40 ppb hourly concentrations (AOT40, ppm h) calculated at the O_3 visible foliar injury onset, and Cumulative Drought Index (CDI) calculated at the drought visible foliar injury onset for *Q. robur* and *Q. pubescens*.

Water Regime	O_3 Treat.	Onset O_3 Injury POD_1		Onset O_3 Injury AOT40		Onset Drought Injury CDI	
		Q. robur	*Q. pubescens*	*Q. robur*	*Q. pubescens*	*Q. robur*	*Q. pubescens*
WW-treated	AA	12.07	Asymp.	17.78	Asymp.	Asymp.	Asymp.
	$\times$1.2	12.40	15.69	16.41	23.77	Asymp.	Asymp.
	$\times$1.4	11.62	20.46	15.15	33.38	Asymp.	Asymp.
MD-treated	AA	13.02	Asymp.	21.74	Asymp.	6.10	6.52
	$\times$1.2	12.13	18.41	16.41	30.43	6.10	4.04
	$\times$1.4	13.07	20.04	21.59	33.38	4.96	6.10
SD-treated	AA	Asymp.	Asymp.	Asymp.	Asymp.	10.20	10.20
	$\times$1.2	Asymp.	13.13	Asymp.	30.43	10.20	10.20
	$\times$1.4	10.72	12.81	26.23	26.23	10.20	10.20

2.3. Measure of Growth Parameters

The assessment of total annual biomass production during the experiment was performed based on dry weight per plant (DW) as described in Hoshika et al. [22], additionally discriminating the below-(roots) and above-ground biomass (stem and leaves) to calculate the ratio of root to shoot biomass (Ratio R/S). Furthermore, the total number of leaves, plant height increment (measured with a metric tape) and stem caliber increment (measured just above soil level) were expressed as the absolute values relative to the values at the beginning and end of the experiment.

2.4. Assessment of Photosynthetic Parameters

The net photosynthetic rate (P_n) was previously reported for mid-summer (July: [24]) and early and late summer and autumn (June, August, and October: [23]). Here, these published data of P_n were re-analyzed to address the cumulative effects of O_3 and drought on the photosynthetic activity. The target leaves were fully sun-exposed leaves (4–6th from the shoot tip) of the plant main shoot (one representative leaf per plant, 1 to 3 plants per replicated plot per each O_3 and W treatment). Measurements were made under light-saturated conditions (1500 µmol m^{-2} s^{-1} PPFD [photosynthetic photon flux density]) with constant CO_2 concentration (400 µmol mol^{-1}), relative humidity (40 to 50%), and leaf temperature (25 °C) using a commercial gas exchange system (CIRAS-2 PP Systems, Herts, UK). Measurements were carried out in two campaigns (8–10 June and 27 September–6 October) for all O_3 treatments and an additional campaign (6–9 August) for two O_3 levels (1.2, and $\times$1.4) on days with clear sky between 9:00 and 12:00 a.m. CET. The other detailed specifications for the photosynthetic measurements were described in our previous studies [23,24].

After the measurement of P_n in August and October, leaves were collected to examine the nitrogen (N) content. Nitrogen content per unit mass (N_{mass}) was determined by the dry combustion method using a LECO TruSpec C/N analyzer (Leco Corporation, St. Joseph, MI, USA). In October, the foliar phosphorus (P) content was also determined. Phosphorus content per unit mass (P_{mass}) was examined by an inductively coupled plasma-optical emission spectroscopy (ICP-OES) (iCAP7000, Thermo Fisher Scientific, Waltham, MA, USA). We calculated photosynthetic N use efficiency (PNUE) as the product of N_{mass} and mass-based net photosynthetic rate and photosynthetic P use efficiency (PPUE) as the product of P_{mass} and mass-based net photosynthetic rate.

2.5. Calculation of Accumulated Drought and Ozone Indexes

The accumulated drought index (CDI) was calculated from the beginning of the experimental period to the date of observation as follows:

$$\mathrm{CDI} = \sum |Sm - Fc|$$

where, *Sm* is soil moisture, and *Fc* is field capacity (0.295 m^3 m^{-3}) [26]; drought stress is considered severe when *Sm* values are lower than *Fc*.

AOT40 and POD_1 for each O_3 and drought treatment were calculated following the parameters applied by Hoshika et al. [22] according to the methodology designed by CLRTAP (Convention on Long-range Transboundary Air Pollution) [9].

2.6. Statistical Analysis

Multiple Linear Regression (MLR) analysis was used to estimate the relationship between the O_3 indexes (AOT40 and POD_1) and CDI versus growth (height, diameter, and N. leaves), biomass (Leaf, Shoot, Root, Total, and R/S), VFI (O_3 and Drought) and physiological parameters (P_n, PNUE, and PPUE). Two models were compared, i.e., Model 1 (POD_1 and CDI as predictor variables) and Model 2 (AOT40 and CDI as predictor variables). The statistical analyses were performed using the R software (R version 4.1.2 [32]), considering a significance of $p < 0.05$. Principal component analysis (PCA) was conducted by using OriginPro 2021b software. The PCA was applied considering VFI (O_3 and drought), growth (Height, Diameter, and N. of leaves), biomass (Leaf, Shoot, Root; Total and R/S), and physiological (Pn, PNUE, and PPUE) parameters in order to distinguish the groups of parameters better related to each symptomatic species; in this analysis, the asymptomatic *Q. ilex* species was not included.

3. Results

3.1. Visible Foliar Injury

The O_3 VFI in *Q. robur* was characterized by small homogeneously distributed dots between the primary leaf veins (Figure 1A). *Q. robur* plants from all water regimes but SD (AA and ×1.2) presented O_3 VFI (Tables 1 and 2). In fact, 11% of the SD-treated plants developed O_3 VFI at the end of the experiment, relative to 56% of the WW-treated plants (Table 2).

There were individual-specific differences on the day of VFI onset. The POD_1 values calculated for the O_3 VFI onset in *Q. robur* were similar across O_3 treatments (approximately 10.7 to 13.0 mmol m^{-2} POD_1, average = 12.1 mmol m^{-2} POD_1), while the AOT40 values corresponding to the O_3 VFI onset increased from 15-16 ppm h to 26.2 ppm h; for SD-treated plants, the O_3 VFI onset occurred only in ×1.4 (10.7 ppm h, Table 1). In addition, the MLR revealed a positive regression of O_3 VFI with POD_1 or AOT40 and a negative regression with CDI when tested with AOT40 (Model 2), but the effect was not significant when tested with POD_1 (Model 1) (Figure 1B; Table 3).

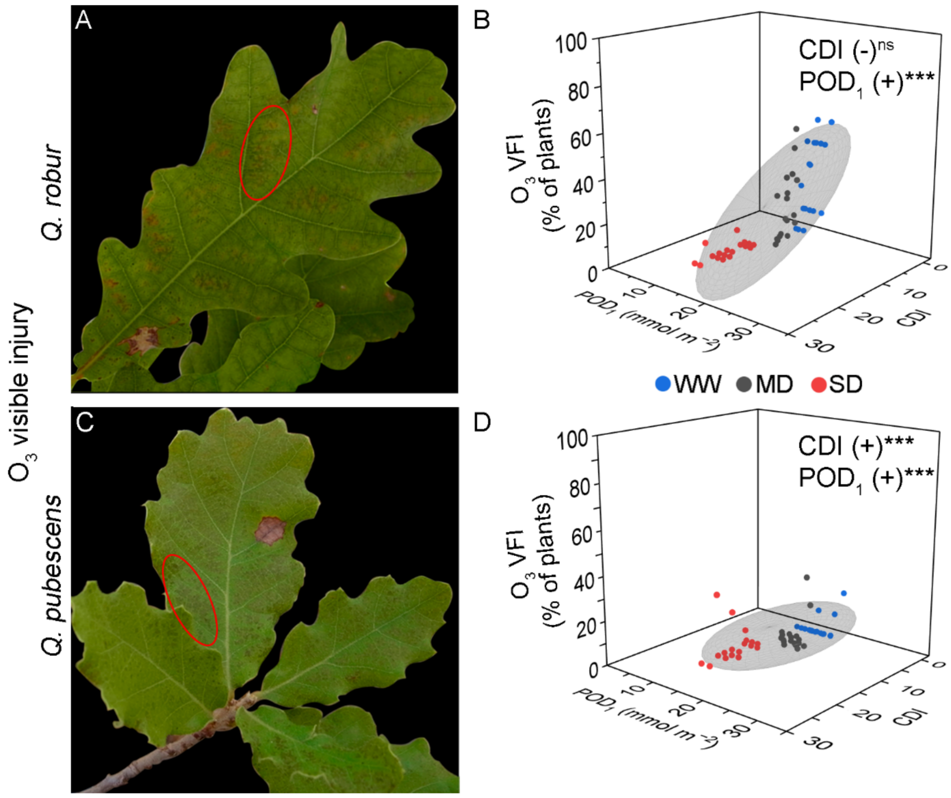

Figure 1. Illustrative examples of O_3 visible foliar injury in *Quercus robur* (**A**) and *Q. pubescens* (**C**) characterized by small homogeneously distributed dots between the primary leaf veins (ellipse). (**B**,**D**) Results from the linear multiple regression of O_3 visible foliar injury with Cumulative Drought Index (CDI) and phototoxic O_3 dose (POD_1) as predictor factors in *Quercus robur* (**B**) and *Q. pubescens* (**D**). Colored dots represent well-watered (WW-blue), moderate drought (MD-grey), and severe drought (SD-red). The grey ellipsoid represents a confidence level of 75%. (+) positive regression, *** = $p < 0.001$, ns = not significant.

Table 2. Evaluation of O_3 and drought incidence of visible foliar injury (VFI) along the experimental period for *Q. robur* and *Q. pubescens* exposed to different levels of O_3 and drought.

	Water Regime	O_3 Treat./DOY	*Q. robur*						*Q. pubescens*					
			209	218	232	240	247	266	209	218	232	240	247	266
O_3 VFI incidence	WW-treat.	AA	-	-	11	11	11	50	-	-	-	-	-	-
		×1.2	-	11	44	44	44	44	-	-	-	-	11	11
		×1.4	22	33	33	44	56	56	-	-	-	-	-	22
	MD-treat.	AA	-	-	-	11	22	44	-	-	-	-	-	-
		×1.2	-	22	22	22	44	56	-	-	-	-	-	33
		×1.4	-	-	22	33	33	33	-	-	-	-	-	22
	SD-treat.	AA	-	-	-	-	-	-	-	-	-	-	-	-
		×1.2	-	-	-	-	-	-	-	-	-	-	-	22
		×1.4	-	-	-	-	11	11	-	-	-	-	11	33
Drought VFI incidence	WW-treat.	AA	-	-	-	-	-	-	-	-	-	-	-	-
		×1.2	-	-	-	-	-	-	-	-	-	-	-	-
		×1.4	-	-	-	-	-	-	-	-	-	-	-	-
	MD-treat.	AA	-	-	33	33	50	67	-	-	-	11	11	11
		×1.2	-	-	22	58	67	67	11	11	11	22	33	33
		×1.4	-	44	33	44	50	63	-	-	22	33	33	33
	SD-treat.	AA	22	22	67	78	83	89	22	22	39	67	67	67
		×1.2	11	56	61	78	78	89	11	11	22	33	44	44
		×1.4	33	56	78	78	100	100	44	44	67	78	78	78

Table 3. Regression coefficients of the multiple linear regression for the species *Q. robur*, *Q. pubescens*, and *Q. ilex*, considering Cumulative Drought Index (CDI) and phototoxic O_3 dose (POD_1) for Model 1, and CDI and accumulated exposure over 40 ppb hourly concentrations (AOT40) for Model 2 as predictor factors, and Growth: Plant height increment (cm), Stem diameter increment (cm), and leaf number increment (N. leaves—n); Biomass: Leaf (g), Shoot (g), Root (g), Total biomass (g), and Ratio root/shoot (Ratio R/S); Visible foliar injury (O_3 and drought—% of plants); Physiological parameters: Photosynthesis (P_n—µmol $m^{-2}s^{-1}$), Photosynthetic nitrogen use efficiency (PNUE—µmol $m^{-2}s^{-1}$) and Photosynthetic phosphorus use efficiency (PPUE—µmol $m^{-2}s^{-1}$) as dependent parameters. Levels of significance (*p*), intercepts and determination coefficients (R^2) are shown.

	Parameters		Model 1 (POD_1, CDI)							Model 2 (AOT40, CDI)						
			POD_1	*p*	CDI	*p*	Intercept	*p*	R^2	AOT40	*p*	CDI	*p*	Intercept	*p*	R^2
Q. robur	Injury	O_3	6.736	***	−0.043	n.s	−64.967	***	0.717	0.002	***	−2−027	***	−20.600	***	0.738
		Drought	4.802	***	5.882	***	−68.059	***	0.887	0.001	***	4.524	***	−31.350	***	0.861
	Growth	Height	0.316	**	0.035	n.s	−1.197	n.s	0.308	0.159	***	−0.041	n.s	−0.213	n.s	0.501
		Diameter	0.032	n.s	−0.051	**	5.182	***	0.323	0.019	n.s	−0.059	***	5.214	***	0.338
		N. Leaves	0.068	n.s	−2.338	***	190.955	***	0.521	0.003	n.s	−2.351	***	191.913	***	0.521
	Biomass	Leaf	0.240	***	0.013	n.s	1.741	n.s	0.404	0.068	*	−0.040	n.s	3.743	***	0.191
		Shoot	−0.043	n.s	−0.176	***	12.517	***	0.404	−0.017	n.s	−0.166	***	12.268	***	0.404
		Root	−0.784	**	−0.568	***	40.098	***	0.508	−0.287	*	−0.391	***	35.097	***	0.476
		Total	−0.588	n.s	−0.732	***	54.357	***	0.428	−0.236	n.s	−0.597	***	51.108	***	0.427
		Ratio R/S	−0.063	***	−0.019	***	2.600	***	0.692	−0.022	***	−0.005	n.s	2.163	***	0.491
	Physiology	P_n	−0.629	***	−0.283	***	14.579	***	0.824	0.000	***	−0.152	**	11.930	***	0.738
		PNUE	−0.569	n.s	−0.307	*	14.724	*	0.558	−0.152	n.s	−0.175	*	10.032	**	0.558
		PPUE	−3.751	*	−1.560	**	82.525	**	0.916	−0.668	n.s	−0.671	*	41.067	*	0.767
Q. pubescens	Injury	O_3	1.827	***	0.645	***	−26.982	***	0.329	0.876	***	0.090	n.s	−16.377	***	0.387
		Drought	1.279	*	3.658	***	−23.673	*	0.787	0.660	*	3.259	***	−17.183	**	0.792
	Growth	Height	0.241	***	−0.001	n.s	−0.472	n.s	0.373	0.115	***	−0.060	*	1.039	n.s	0.371
		Diameter	−0.151	***	−0.052	***	8.020	***	0.485	−0.052	**	−0.017	n.s	6.595	***	0.244
		N. Leaves	−4.901	***	−1.801	**	175.047	***	0.390	−2.011	**	−0.644	n.s	136.376	***	0.283
	Biomass	Leaf	−0.019	n.s	−0.012	n.s	4.183	***	−0.070	−0.001	n.s	−0.008	n.s	3.865	***	−0.075
		Shoot	−0.205	**	−0.130	***	13.137	***	0.511	−0.091	**	−0.081	**	11.687	***	0.474
		Root	−0.054	n.s	−0.223	***	22.085	***	0.366	−0.039	n.s	−0.208	***	22.072	***	0.372
		Total	−0.278	n.s	−0.364	***	39.404	***	0.400	−0.132	n.s	−0.297	***	37.625	***	0.398
		Ratio R/S	−0.022	*	−0.017	***	2.132	***	0.316	−0.009	n.s	−0.012	**	1.955	***	0.277
	Physiology	P_n	−0.370	***	−0.255	***	13.500	***	0.795	0.000	***	−0.179	***	12.290	***	0.771
		PNUE	−0.485	*	−0.286	**	14.671	**	0.741	−0.173	*	−0.172	**	10.622	***	0.757
		PPUE	−2.385	*	−1.389	**	70.714	**	0.954	−0.575	n.s	−0.788	**	41.724	**	0.901
Q. ilex	Injury	O_3														
		Drought														
	Growth	Height	0.127	n.s	0.004	n.s	15.423	***	−0.050	0.051	n.s	−0.026	n.s	15.623	***	−0.357
		Diameter	0.153	**	0.031	*	0.799	n.s	0.305	0.042	**	−0.003	n.s	1.483	***	0.229
		N. Leaves	3.238	**	1.194	**	17.246	n.s	0.329	1.218	***	0.445	n.s	23.955	**	0.468
	Biomass	Leaf	−0.151	n.s	−0.035	n.s	5.825	***	0.022	−0.029	n.s	−0.002	n.s	4.846	***	−0.037
		Shoot	0.011	n.s	−0.053	n.s	7.619	***	0.158	−0.000	n.s	−0.055	*	7.750	***	0.157
		Root	0.453	**	0.064	n.s	7.027	***	0.230	0.072	n.s	−0.031	n.s	10.315	***	0.013
		Total	0.313	n.s	0.024	n.s	20.471	***	0.038	0.043	n.s	−0.089	n.s	22.911	***	−0.005
		Ratio R/S	0.095	*	0.012	n.s	0.158	n.s	0.294	0.022	n.s	−0.008	n.s	0.7669	n.s	0.068
	Physiology	P_n	0.094	n.s	−0.173	***	9.090	***	0.630	0.000	n.s	−0.196	***	9.380	***	0.624
		PNUE	−0.014	n.s	−0.122	**	5.768	**	0.840	−0.014	n.s	−0.118	***	5.939	***	0.846
		PPUE	−3.036	n.s	−1.870	*	96.073	*	0.814	−0.478	n.s	−1.124	*	71.158	*	0.772

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.s = not significant.

The O_3 VFI in *Q. pubescens* was similar to that developed by *Q. robur* (Figure 1C). Independently of the water regime, plants from AA did not show O_3 VFI, while plants from ×1.2 and ×1.4 treatments presented O_3 VFI (Tables 1 and 2). The percentage of plants presenting VFI was lower than for *Q. robur,* with a maximum of 33% presenting VFI (Table 2). VFI occurred for the first time at DOY 247 or 266, i.e., around the end of the experiment. The POD_1 and AOT40 values for the O_3 VFI onset were 12.8–20.46 mmol m^{-2} POD_1 (average = 16.8 mmol m^{-2} POD_1) and 24–33 ppm h AOT40, respectively (Table 1). The MLR revealed a positive regression with the O_3 indexes (POD_1 and AOT40; Figure 1D, Table 3). Interestingly, CDI positively affected the O_3 VFI when tested with POD_1 (Model 1), but the effect was not significant when tested with AOT40 (Model 2) (Table 3).

The drought VFI of *Q. robur* was evident exclusively on the leaf edge that became dry and brownish (Figure 2A). The VFI progressively increased in MD- and SD-treated plants until the end of the experimental period, while WW-treated plants did not show any injury (Table 2). At the end of the experiment, 89–100% of the SD-treated plants showed drought VFI, relative to 63–67% of the MD-treated plants (Table 2). The CDI calculated at the drought VFI onset was the same for all SD-treated plants (CDI = 10.20 for all AA, ×1.2, ×1.4 treatments, Table 1) and similar for MD-treated plants (CDI= 4.96 to 6.10, Table 1). The MLR revealed a strong positive regression between the *Q. robur* drought VFI and CDI, although POD_1 or AOT40 also increased the extent of drought VFI (Figure 2B, Table 3).

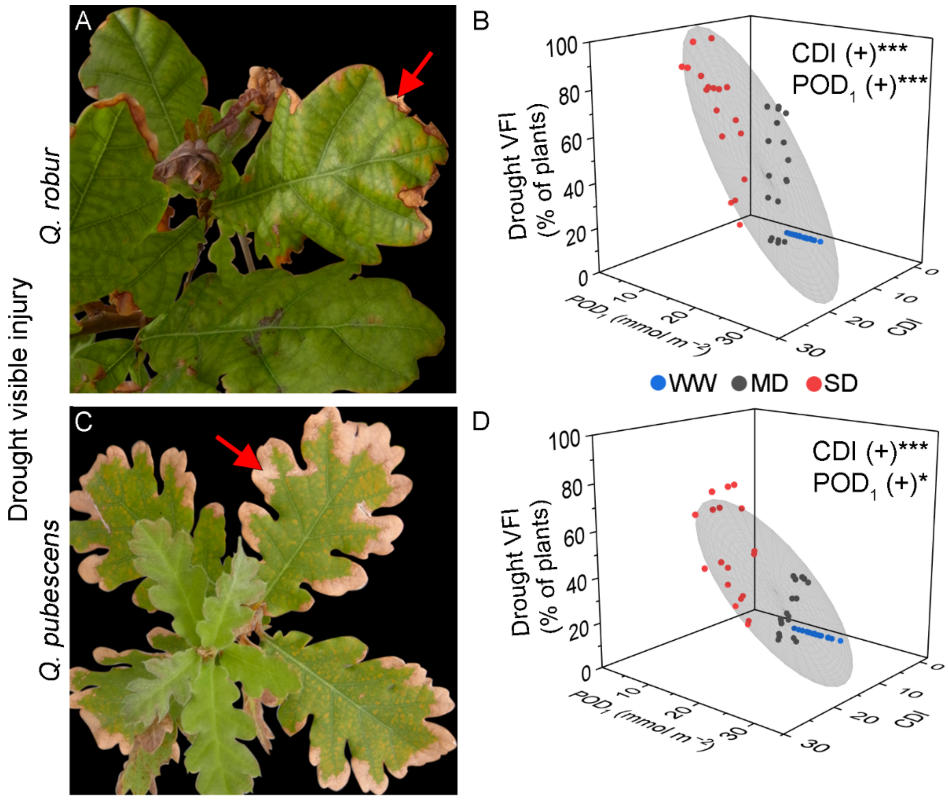

Figure 2. Illustrative examples of drought visible foliar injury in *Q. robur* (**A**) and *Q. pubescens* (**C**) characterized by dry and brownish leaf edges (arrow). (**B**,**D**) Results from the linear multiple regression of drought visible foliar injury with Cumulative Drought Index (CDI) and phototoxic O_3 dose (POD_1) as predictor factors in *Q. robur* (**B**) and *Q. pubescens* (**D**). Colored dots represent well-watered (WW—blue), moderate drought (MD—grey), and severe drought (SD—red). The grey ellipsoid represents a confidence level of 75%. (+) positive regression, * = $p < 0.05$, *** = $p < 0.001$, ns = not significant.

The drought VFI of *Q. pubescens* was similar to that developed by *Q. robur* (Figure 2C). At the end of the experimental period, *Q. pubescens* presented 44–78% of the SD-treated plants with VFI, 11–33% of the MD-treated plants, and no VFI for the WW-treated plants. As found in *Q. robur*, the CDI calculated at the drought VFI onset was the same for all SD-treated plants (CDI = 10.20 for all AA, ×1.2, ×1.4 treatments) and similar for MD-treated plants (CDI= 4.04 to 6.52, Table 1). Interestingly, the CDI values at drought VFI onset were similar in *Q. robur* and *Q. pubescens* within the same O_3 and drought treatments (Table 1). In addition, the MLR revealed a positive regression with CDI, POD_1, and AOT40 (Table 3, Figure 2D). The evergreen *Q. ilex* did not present O_3 or drought VFI.

3.2. Physiological Responses

In both *Q. robur* and *Q. pubescens*, P_n was negatively affected by POD_1 and CDI (Table 3, Figure S1B and D), but it unexpectedly increased with increasing AOT40 (Table 3). Furthermore, PNUE and PPUE were negatively related to CDI and POD_1 except for PNUE in *Q. robur* (Table 3).

For *Q. ilex*, the MLR revealed that CDI negatively affected P_n (Figure S1F), PNUE, and PPUE, with no significant relationship with the O_3 indexes (POD_1 and AOT40, Table 3).

3.3. Growth and Biomass

The MLR indicated that height increment was positively affected by POD_1 or AOT40 in *Q. robur*, while increments of diameter and number of leaves were negatively affected by CDI (Table 3). As confirmed by negative regression coefficients with CDI, most biomass parameters of *Q. robur* were reduced by drought. On the other hand, POD_1 or AOT40 positively affected leaf biomass and negatively affected root biomass, indicating a reduction of the R/S ratio under elevated O_3 exposure (Table 3, Figure S1A).

In *Q. pubescens,* the O_3 indexes (POD_1 and AOT40) were positively related to plant height increment, while CDI was negatively related to height only when tested with AOT40 (Table 3). Increments in shoot diameter and number of leaves in this species were negatively related to POD_1 and AOT40, while they were negatively related to CDI when tested with POD_1 (Model 1, Table 3). Regarding the biomass parameters, leaf biomass was not affected by any factor, while shoot biomass was negatively affected by both O_3 indexes (POD_1 and AOT40) and CDI (Table 3). Root and total biomass were negatively related to CDI, and the R/S ratio was negatively influenced by CDI and POD_1 (Table 3, Figure S1C).

In *Q. ilex,* plant height increment was not affected by any factors, while a positive relationship between diameter increments and O_3 indexes was found (Table 3). The increment in the number of leaves was positively affected by POD_1 and AOT40 and positively affected by CDI when tested together with POD_1 (Table 3), although leaf and total biomass were not significantly affected by those factors. Shoot biomass was negatively affected by CDI only when tested with AOT40, and root biomass was positively affected only by POD_1 (Table 1). The R/S ratio was positively related only to POD_1 (Table 3, Figure S1E).

The raw data off all growth parameters for the species *Q. robur*, *Q. pubescens*, and *Q. ilex* are avaible in Table S1.

3.4. Principal Component Analysis

The PCA detected separate multivariate spaces between the two symptomatic species as groups related to different growth, biomass, and physiological parameters related to O_3 or drought VFI (Figure 3).

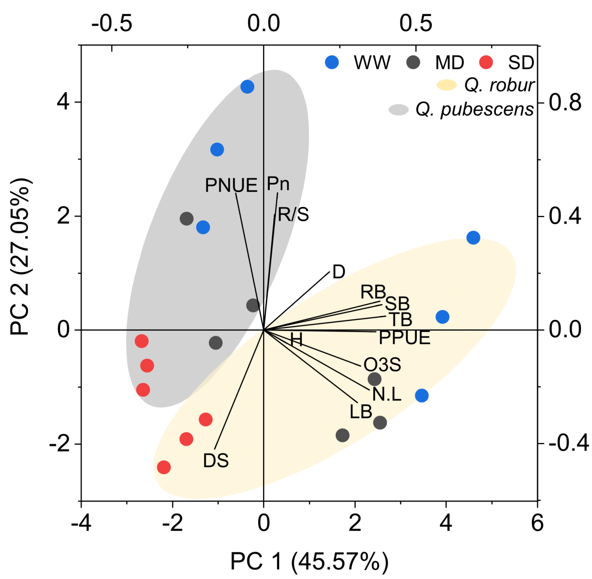

Figure 3. Bi-plot of the principal component analysis on Growth: Plant height increment (H), Stem diameter increment (S), and leaf number increment (N.L); Biomass: Leaf (L), Shoot (S), root (R), Total biomass (TB), and shoot/root ratio (R/S); Visible foliar O_3 (O_3S) and drought injury (DS); Physiological parameters: Photosynthesis (P_n), Photosynthetic nitrogen use efficiency (PNUE) and Photosynthetic phosphorus use efficiency (PPUE).

Since *Q. ilex* did not show VFI, this species was not included in the analysis. The first two components of the PCA explained 45.57 and 27.05% of the variances. The SD-treated plants of both species were grouped near the drought VFI (DS) with no other parameter following the same vector direction. The individuals of *Q. robur* (especially MD-treated plants) were grouped near the vectors of the growth parameters number of leaves and height, leaf biomass, and O_3 VFI, which presented the same direction, thus indicating that when O_3 VFI increased, these parameters also increased. The individuals of *Q. pubescens* (specially WW-treated plants) were grouped near P_n, PNUE, and R/S, with the vectors in the opposite direction of O_3 and drought VFI, thus indicating that when O_3 and drought VFI increased, these parameters decreased.

4. Discussion

4.1. Development of Visible Injury Due to Ozone and Drought Stress

The POD_1 values corresponding to the onset of O_3 VFI for the two symptomatic deciduous oaks (on average, 14.4 mmol m^{-2}) were similar to those estimated for broadleaf species under field conditions in Italy and France (10 mmol m^{-2} s^{-1}) [10]. However, the CL for the VFI onset was lower for *Q. robur* than for *Q. pubescens,* indicating its higher sensitivity to O_3, possibly related to its lower antioxidative capacity and inability to protect the cell structure [25]. Furthermore, O_3 VFI increased with increasing POD_1 in the two deciduous oaks. This suggests that PODy is a key indicator to describe the development of O_3 VFI [33] once it is well known that O_3 damage is closely related to stomatal O_3 uptake [1]. In fact, the absence of O_3 VFI in *Q. ilex* might be related to its low g_{max} (165 mmol O_3 m^{-2} s^{-1}, compared to 225 mmol O_3 m^{-2} s^{-1} and 200 mmol O_3 m^{-2} s^{-1} of *Q. pubescens* and *Q. robur,* respectively, [22] suggesting that the development of VFI might be discussed in terms of the specific-species patterns of stomatal conductance.

For both injured species (*Q. robur* and *Q. pubescens*) at the end of the experimental period, the severe drought treatment reduced POD_1 by 30 to 40% [22], which would be expected to decrease the O_3-induced VFI in plants as reported before for ecophysiological responses in poplars [34]. In *Q. robur,* the presence of O_3 VFI was decreased under drought. On the contrary, drought stress aggravated the O_3 VFI in *Q. pubescens*. Drought has been reported to have the potential to aggravate the harmful effects of O_3 [35]. Furthermore, Hoshika et al. [24] found that the combination of O_3 and drought altered the activity of the antioxidant system so that *Q. pubescens* was not protected from the severe oxidative stress resulting from the combined stress of O_3 and drought.

For the symptomatic species (*Q. robur* and *Q. pubescens*), the progression of drought VFI could be attributed to the obstruction of conducting tissue [20], conferring to both species a high sensitivity. In the asymptomatic *Q. ilex,* this phenomenon might not happen due to its capacity to increase the cell wall thickness by reinforcing the strength and rigidity of the secondary cell walls with hemicellulose and lignin deposition (data not published). Changes in lignin might function as physical desiccation tolerance and maintenance of protein integrity in drought-tolerant species [36], thus helping the photosynthetic recovery activity after re-watering from severe drought episodes [37]. The CDI threshold for the appearance of drought VFI in the two symptomatic species was higher in SD-treated than MD-treated plants, possibly due to the interaction with leaf aging, which is an important physiological and biochemical defense factor against drought stress [38]. In fact, most plants showed drought VFI in mid- or late-summer in both SD-treated and MD-treated plants when leaves were relatively old.

4.2. Effects of Ozone and Drought Stress on Growth and Biomass Parameters

For both deciduous species (*Q. robur* and *Q. pubescens*), height increment was higher when exposed to O_3 treatment. This phenomenon was verified in other species, such as *Populus* sp. [39], and it is possibly related to promoting a new leaf development as a compensative response against O_3 damage. However, the decrease in the number of leaves was eventually found to be due to O_3 exposure, which might be related to the potential O_3 phytotoxicity that triggers programmed cell death, promoting an increase in leaf senescence [40]. When combined with drought stress, the effect can be more substantial once the lack of water and nutrients promotes a decrease in new leaf development.

Both O_3 and drought stresses were detrimental to the plant biomass increment in all the oak species. In fact, the reduction of biomass due to drought stress is reported for many species and is related to the reduction of water content, diminished leaf water potential and turgor loss, promotion of stomatal closure, and decreased cell enlargement growth [41,42]. As previously revealed by Alonso et al. [21], drought stress does not protect holm oak from O_3 effects when considering the whole plant response. However, differences between the species responses must be considered when comparing the species sensitivity. For example, we observed that *Q. robur* and *Q. pubescens* invested more in shoots than in roots when exposed to both stresses, while *Q. ilex* performed the opposite, which might be another strategy of *Q. ilex* indicating a hormetic mechanism of tolerance for increasing conducting tissue and maintaining the water flow. These tolerance mechanisms may be associated with morphological/anatomical adjustments, such as a versatile root system, conservative growth and carbon allocation patterns, and diverse adaptations in the leaf morphology [20,43]. This might increase the apoplastic water fraction [44] and promote the species tolerance to O_3 and drought stress.

4.3. Effects of Ozone and Drought Stress on Physiological Parameters

The O_3 and drought stress negatively affected the physiological parameters. Drought stress induced a decrease of P_n regardless of the different species, as confirmed by a negative relationship with CDI. A decrease in P_n with increasing POD_1 was verified for both sensitive species (*Q. robur* and *Q. pubescens*), while no such reduction was found in *Q. ilex*. The present discussion is based on the species responses to POD_1 once the flux-based index is more realistic [9]. In fact, AOT40 was positively related to P_n in the two deciduous oaks, which does not agree with a consensus about O_3 negatively affecting photosynthetic capacity [45]. In fact, the regression coefficient was very low (=0.000), although the regression slope was numerically significant. Even though data was generated from an underlying distribution, the significance is a rather unlikely biological sense. The data suggest that a biological-sound index such as POD_1 is superior to AOT40 for the studies of the O_3 effects on vegetations because it can consider the principal physiological cause of O_3 damage, i.e., stomatal O_3 uptake.

Drought stress decreased PNUE and PPUE for all three species, while O_3 stress negatively affected PNUE for *Q. pubescens* and PPUE for the sensitive species *Q. robur* and *Q. pubescens*. Drought stress is directly related to changes in the allocation of N and P to leaves, no matter the species sensitivity to O_3 stress. However, a reduced allocation of N and P to the photosynthetic apparatus [5,6,46] is more pronounced in O_3 sensitive species. The N-uptake efficiency and leaf N efficiency are important traits to improve growth under drought [47]; thus, the decline in root biomass might explain the decrease in PNUE and PPUE for those species, once reduced quantity of absorptive roots reduces water and nutrient uptake as verified for the same oak species in a previous study [48].

4.4. Is the Ozone Visible Injury an Indicator to Explain Biomass Reduction or Physiological Damage in Mediterranean Oaks?

The PCA biplot contains the strength of VFI, physiology, and growth relationships, along with the species-specific sensitivity to drought and O_3 stress. Relationships between O_3 VFI and biomass growth were discussed by other authors [49,50]. In the present study, we observed that the vector of O_3 VFI injury (O_3S) and total biomass (TB) were crossing at the right angle of each other, suggesting a weak association between these two parameters in Mediterranean oaks. However, the O_3S vector shows the same direction as those of leaf parameters (number of leaves [N.L] and leaf biomass [LB]) in plants presenting more O_3 VFI, which may indicate the promotion of carbon allocation to leaves as a compensation response against O_3 injury. In addition, opposite directions of the vectors were found for O_3 VFI (O_3S) and net photosynthesis (P_n), PNUE, and the R/S ratio, highlighting a negative correlation between O_3 VFI and these parameters. The results indicate that O_3 VFI was not a direct indicator of biomass reduction under elevated O_3 in these oaks but provides important insights regarding the impairment of photosynthetic capacity and biomass partitioning to roots. Mediterranean oak species generally develop taproots that grow deep into the soil, enhancing resistance to abiotic stress such as drought [51]. However, small amounts of roots due to O_3 exposure imply a loss of water and nutrient uptake, suggesting that O_3 VFI should be considered a bioindicator in forests exposed to the combination of O_3 pollution and drought.

5. Conclusions

We examined O_3- and drought-induced VFI and their effects on growth, biomass, and physiological parameters by using cumulative indexes and oak species known for showing differential sensitivity to these stressors. The increase in POD_1 promoted the development of specific O_3 VFI in the isohydric *Q. robur* and the intermediate *Q. pubescens,* while the anisohydric *Q. ilex* was asymptomatic. In *Q. robur*, the presence of O_3 VFI was decreased under drought probably because drought-induced stomatal closure reduced O_3 uptake and thus limited O_3 damage. However, drought stress aggravated O_3 VFI in *Q. pubescens*. This result indicates the importance of the protective role of antioxidant activity under the combination of O_3 and drought, which may be weakened by the combined stress factors and become a dominant factor in species that are not strictly isohydric. On the other hand, the drought VFI was clearly distinguished from the O_3-induced VFI, and it developed with increasing CDI in *Q. robur* and *Q. pubescens* but not in *Q. ilex*, suggesting a high tolerance of *Q. ilex* to drought stress. Therefore, we suggest using the specific O_3 or drought VFI as a bioindicator, especially for establishing the onset injury CL.

We also confirmed that P_n was decreased progressively with POD_1 and CDI in the two deciduous oaks, in tandem with PNUE decline, suggesting a cumulative effect of O_3 and drought on photosynthetic capacity. As a result, both stress factors showed a deleterious effect on the development of VFI and biomass growth. Interestingly, the two deciduous oaks increased the allocation to shoot growth rather than to root growth when exposed to both stresses, while an opposite result was found in *Q. ilex*. The imbalance in carbon allocation to roots may reduce the stability against strong winds and impair water uptake under the warming climate expected in future climate change [52,53].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11141836/s1, Figure S1: Results of the multiple linear regression for shoot/root (Ratio R/S) and Photosynthesis (P_n) parameters; Table S1: Raw data of growth parameters.

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