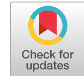
Science of the Total Environment 657 (2019) 379–390

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

When “thirsty” means “less able to activate the signalling wave trigged by a pulse of ozone”: A case of study in two Mediterranean deciduous oak species with different drought sensitivity



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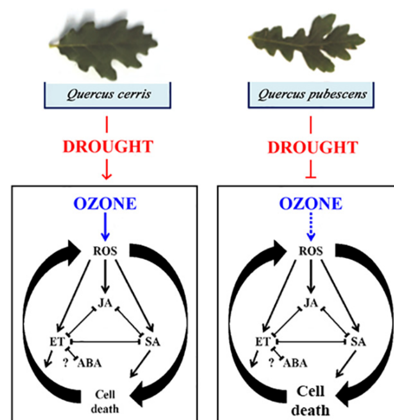
HIGHLIGHTS

- Possible alteration of O_3-signalling wave by drought was investigated in oak species.
- Two Mediterranean deciduous species with different water requirements were compared.
- Gas exchange, chlorophyll *a* fluorescence and main signalling molecules were assessed.
- Drought alters profoundly the typical O_3-triggered reaction cascade.
- Prediction of future plant behavior requires the deepening of signalling pattern.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 August 2018
Received in revised form 21 November 2018
Accepted 2 December 2018
Available online 4 December 2018

Editor: Alessandra De Marco

Keywords:
Drought
Oak species
Phytohormones
Programmed cell death
Reactive oxygen species
Signalling network

ABSTRACT

There is a lack of knowledge about the possibility that plants facing abiotic stressors, such as drought, have an altered perception of a pulse of O_3 and incur in alterations of their signalling network. This poses some concerns as to whether defensive strategy to cope episodic O_3 peaks in healthy plants may fail under stress. In this study, a set of saplings of two Mediterranean deciduous species, *Quercus cerris* and *Q. pubescens*, was subjected to water withholding (20% of daily evapotranspiration for 15 days) while another set was kept well-watered. Saplings were then subjected to a pulse of O_3 (200 nl l^{-1} for 5 h) or maintained in filtered air. *Q. pubescens* had a more severe decline of photosynthesis and leaf PDΨ_w (about −65% and 5-fold lower than in well-watered ones) and events of cell death were observed under drought when compared to *Q. cerris*, which is supportive for a higher sensitivity to drought exhibited by this species. When O_3 was applied after drought, patterns of signalling compounds were altered in both species. Only in *Q. pubescens*, the typical O_3-induced accumulation of apoplastic reactive oxygen species, which is the first necessary step for the activation of signalling cascade, was completely lost. In *Q. cerris* the most frequent changes encompassed the weakening of peaks of key signalling molecules (ethylene and salicylic acid), whereas in *Q. pubescens* both delayed (salicylic and jasmonic acid) or weakened (ethylene and

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https://doi.org/10.1016/j.scitotenv.2018.12.012
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salicylic acid) peaks were observed. This is translated to a higher ability of *Q. cerris* to maintain a prompt activation of defensive reaction to counteract oxidative damage due to the pollutant. Our results reveal the complexity of the signalling network in plants facing multiple stresses and highlight the need to further investigate possible alteration of defensive mechanism of tree species to predict their behavior.

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1. Introduction

The Mediterranean basin is one of the harshest environments for plants to inhabit due to the concomitance and fluctuation of several abiotic stressors, which include prolonged periods of summer drought and ground-level ozone (O_3) fluctuations (Paoletti, 2006; Harmens et al., 2018). Though physiological and biochemical effects of drought and O_3 alone have been exhaustively investigated (Wilkinson and Davies, 2010; Bohler et al., 2015), and controversial results are available on their interactive effect. There is no consensus as to whether drought and O_3 act synergistically (Alonso et al., 2014; Pollastrini et al., 2014) or antagonistically (Panek et al., 2002; Wittig et al., 2009). For example, the possibility that drought-stressed plants exhibit an altered ability to respond to O_3 episodes, and that water stress modifies the perception of the O_3-triggered signalling pathway have been scarcely investigated (Cotrozzi et al., 2018). The cross-talk between hydraulic and chemical signals is utilized for sensing water stress conditions at the whole plant level (Wilkinson and Davies, 2010). Therefore, the effects of a possible interaction between drought and a pulse of O_3 in terms of signalling compounds and cell death should be deepened to improve predictions of plant acclimation/adaptation strategies to climate change factors (Carmody et al., 2016; Waszczak et al., 2018).

Short exposure of plants to relatively high levels of O_3 induces physiological and molecular changes in organs/tissues. In most cases, these changes resemble those observed in programmed cell death (PCD) and hypersensitivity response (HR) in response to pathogen attack, which undergo the systemic acquired acclimation (SAA) and systemic acquired resistance (SAR) (reviewed by Pellegrini et al., 2016). In both cases (O_3 or pathogen attack), it has been observed that a biphasic accumulation of apoplastic reactive oxygen species (ROS) represents the first step of the "signalling wave" in plants. After that, ROS promote an orchestrated and tightly regulated process which involves, among others, different phytohormones, such as salicylic acid (SA), required for PCD initiation (Overmyer et al., 2005), ethylene (ET), necessary for signalling propagation (Rao et al., 2002; Tuominen et al., 2004) and jasmonic acid (JA), required to contain the spread of PCD by antagonizing SA and ET signalling (Tamaoki et al., 2003). These molecular events lead to O_3-induced HR-mimicking foliar symptoms (Overmyer et al., 2003, 2005; Kangasjärvi et al., 2005). The similarities between plant reactions to pathogens and O_3 make therefore the pollutant a useful non-invasive tool to elicit and study the signalling wave which can occur as a cross response to both abiotic and biotic apoplastic-ROS-promoting stresses (Kangasjärvi et al., 1994; Rao et al., 2000; Vainonen and Kangasjärvi, 2015).

Previously, Cotrozzi et al. (2017a) demonstrated that drought-stressed saplings of *Quercus ilex*, a Mediterranean evergreen species, had a modified ability to perceive a peak of O_3 of 200 nl l^{-1} for 5 h and to activate the metabolic responses aimed at counteracting the spread of oxidative damage. However, there is a wide range of morpho-anatomical, physiological and biochemical plasticity among species belonging to the genus *Quercus* (Cotrozzi et al., 2016) that can be on the bases of different plant responses when drought and O_3 are applied simultaneously. The present work was conducted in two co-habiting deciduous oak species, namely *Quercus cerris* and *Q. pubescens* with the attempt to test whether the higher physiological plasticity which was on the bases of the higher tolerance of *Q. pubescens* than *Q. cerris* to chronic drought and drought × O_3 (Cotrozzi et al., 2016) results a positive feature against a peak of O_3 when plants are previously subjected to prolonged drought or, alternatively, the higher morpho-anatomical plasticity of *Q. cerris* is more effective. In addition, the present experiment can offer new evidences if some drought-promoted changes in the signalling pathway can impact and alter the activation of HR and SAR by the plant when facing abrupt O_3 episodes.

2. Materials and methods

2.1. Plant material and experimental design

Three-year-old saplings of *Q. cerris* and *Q. pubescens* grown from seed under field conditions were transferred into 6.5-l pots containing a mixture of standard soil (Einhetserde Topfsubstrat ED 63, Sinntal-Alterngronau, Germany) and sand (3.5:1, in volume). Uniform-size individuals were selected and exposed to two levels of water availability: well-watered (WW) plants were kept at field capacity, whereas water-stressed (WS) plants received every day 20% of their effective daily evapotranspiration. After 7 days of differentiated water treatment, plants were equally subdivided into four sets, named WW/O_3^-, WS/O_3^-, WW/O_3^+ and WS/O_3^+, and transferred into four controlled fumigation facilities for acclimation. After 15 days of differentiated water treatment, WW/O_3^+ and WS/O_3^+ sets were exposed to a pulse of O_3 into two fumigation facilities (200 nl l^{-1} 5 h, in form of a square wave), whereas WW/O_3^-, WS/O_3^- plants were maintained under charcoal-filtered air at a negligible O_3 concentration (<5 nl l^{-1}), into two fumigation facilities. Further details of experimental design and O_3 exposure methodology are reported in Cotrozzi et al. (2017a). The water status of plants was determined before the pulse of O_3. Photosynthetic parameters were measured at 0, 5, 24 and 48 h from the beginning of the O_3 exposure (FBE). Microscopic determinations were performed at the end of the pulse of O_3 (i.e., 5 h FBE). At 0, 1, 2, 5, 8 and 24 h FBE, fully expanded leaves were harvested, immediately frozen in liquid nitrogen, and then freeze-dried and stored at −20 °C until biochemical analyses (see below).

2.2. Water status determination

The water status was evaluated by determining the pre-dawn leaf water potential ($PD\Psi_w$) with a PMS 600 pressure bomb (PMS Instrument Company, Albany, OR, USA) and the relative water content (RWC), calculated as (FW − DW) / (TW − DW) × 100, where FW, DW and TW are the fresh, dry, and turgor weights, respectively. For further details of water status determination see Cotrozzi et al. (2017a).

2.3. Gas exchange and chlorophyll *a* fluorescence measurement

Gas exchange and chlorophyll *a* fluorescence measurements were determined between 10:00 and 13:00 (solar time) on one fully expanded mature leaf per plant, on three plants per treatment. CO_2 assimilation rate (A) and stomatal conductance to water vapor (g_s) in light-saturated conditions and ambient CO_2 concentration (photosynthetic active radiation of about 1200 μmol photons m^{-2} s^{-1} and 390 μmol mol^{-1}, respectively) were measured using an Infrared Gas Analyzer (LI-COR Inc., Lincoln, NE, USA). Modulated chlorophyll *a* fluorescence parameters were measured with a PAM-2000 fluorometer

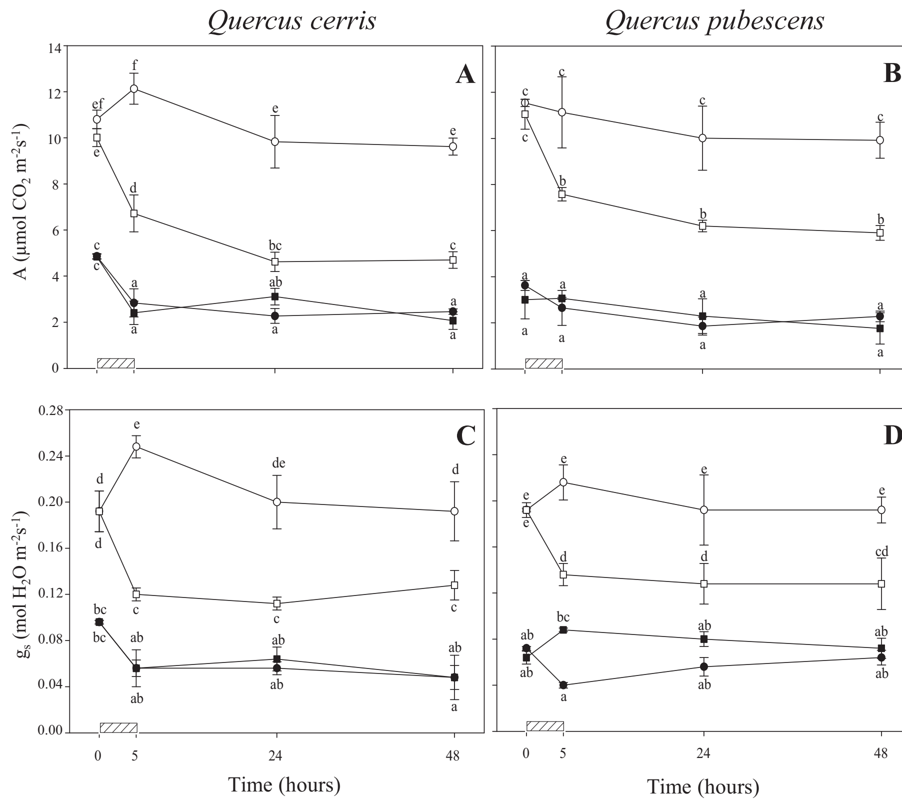

Fig. 1. Time course of leaf gas exchange parameters in *Quercus cerris* (A–C) and *Q. pubescens* (B–D) plants well-watered (○ and □ symbols) or water stressed (20% of the effective evapotranspiration daily for 15 days, ● and ■ symbols) and exposed to a single pulse of ozone (200 nl l^{-1} for 5 h, □ and ■) and maintained in filtered air (○ and ●). Data are shown as mean ± standard deviation. The measurements were carried out 0, 5, 24 and 48 h from the beginning of exposure. According to the two-way repeated measures ANOVA with treatment as variability factor, different letters indicate significant differences ($P \leq 0.05$). Abbreviations: A, CO_2 assimilation rate; g_s, stomatal conductance to water vapor. The thick bottom line indicates the time (5 h) of ozone exposure.

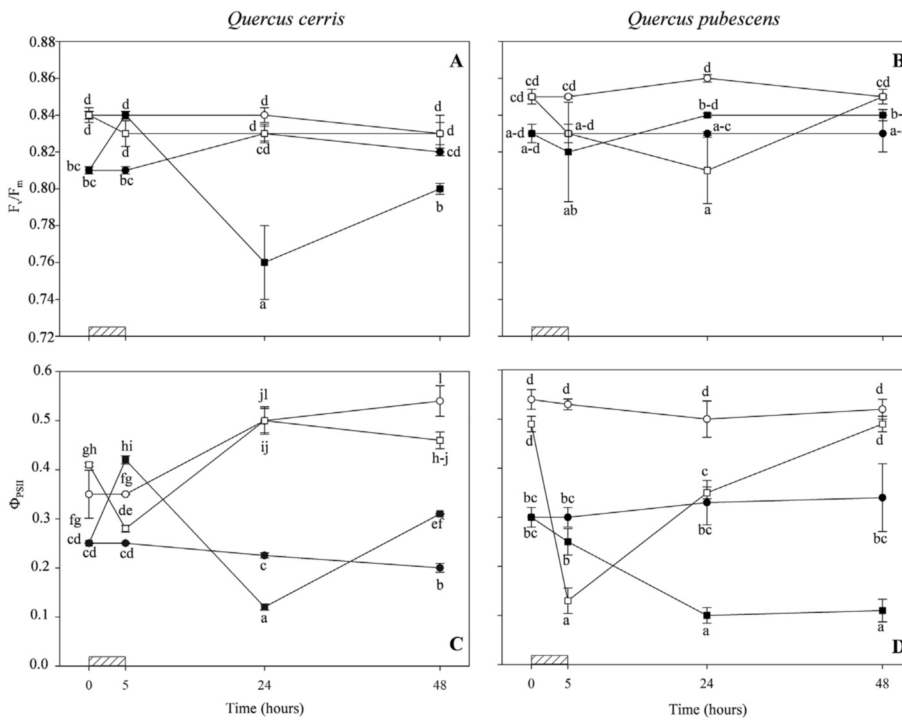

Fig. 2. Time course of leaf chlorophyll *a* fluorescence parameters in *Quercus cerris* (A–C) and *Q. pubescens* (B–D) plants well-watered (○ and □ symbols) or water stressed (20% of the effective evapotranspiration daily for 15 days, ● and ■ symbols) and exposed to a single pulse of ozone (200 nl l^{-1} for 5 h, □ and ■) and maintained in filtered air (○ and ●). Data are shown as mean ± standard deviation. The measurements were carried out 0, 5, 24 and 48 h from the beginning of exposure. According to the two-way repeated measures ANOVA with treatment as variability factor, different letters indicate significant differences ($P \leq 0.05$). Abbreviations: F_v/F_m, variable and maximal fluorescence ratio; Φ_{PSII}, photochemical efficiency in light conditions. The thick bottom line indicates the time (5 h) of ozone exposure.

(Walz, Effeltrich, Germany) on a leaf homogeneous with that used for the determination of gas exchange after 40 min of dark adaptation. The maximal PSII photochemical efficiency in the dark [$F_v/F_m = (F_m - F_0) / F_m$] and the actual photochemical efficiency in light conditions [$\Phi_{PSII} = (F_m' - F_s) / F_m'$; Genty et al., 1989] were calculated. Detailed descriptions of gas exchange and chlorophyll *a* fluorescence measurements are available in Cotrozzi et al. (2017a).

2.4. Microscopic detection of dead cells and ROS determination

Evan's blue staining was used for the detection of dead cells (Tonelli et al., 2015). H_2O_2 content was measured using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies Corp., Carlsbad, CA, USA), according to Cotrozzi et al. (2017a). After extraction with potassium-phosphate buffer (20 mM, pH 6.5), H_2O_2 was determined with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA) at 530 and 590 nm for the excitation and emission of resorufin fluorescence, respectively. After extraction with Tris-HCl buffer (50 mM, pH 7.5), O_2^- content was determined with a spectrophotometer (6505 UV–Vis, Jenway, UK), according to the method of Cotrozzi et al. (2017a). The relative number of dead and live cells was obtained by counting the number of stained (dead) and unstained (live) cells on digitalized images at 300 dpi of ten randomly selected leaf portions (15 micrographs per treatment) with an open source image processing program designed for scientific multidimensional image analyzer (ImageJ software).

2.5. Phytohormones, osmolytes and signalling molecules bioassays

Abscisic acid (ABA) was determined by an indirect Enzyme-Linked Immunosorbent Assay (ELISA), using DBPA1 monoclonal antibody, raised against S(+)-ABA, as described by Trivellini et al. (2011). After extraction with deionized water overnight at 4 °C, the ABA determination was performed spectrophotometrically at 415 nm by an absorbance microplate reader (MDL 680, Perkin-Elmer, Waltham, MA, USA). After extraction with sulfosalicylic acid (3%, v/v), proline content was determined spectrophotometrically at 520 nm (Cotrozzi et al., 2017a). Two minutes after excision, ET production was measured by enclosing six intact leaves in air-tight glass containers. Gas samples were taken from the headspace of containers after 1 h incubation at room temperature. Separations were performed with a gas chromatograph (HP5890, Hewlett-Packard, Ramsey, MN, USA) equipped with a stainless steel column (150 mm length × 0.4 cm i.d., packed with Hysep T) and a flame ionization detector. SA was determined through a liquid chromatograph equipped with a reverse-phase column (Acclaim 120, C18 5 μm particle size, 150 mm length × 0.46 cm i.d.) and RF 2000 Fluorescence Detector provided by Dionex (Sunnyvale, CA, USA). JA was determined according to Pellegrini et al. (2013) by liquid chromatography, using the Dionex column described above and a

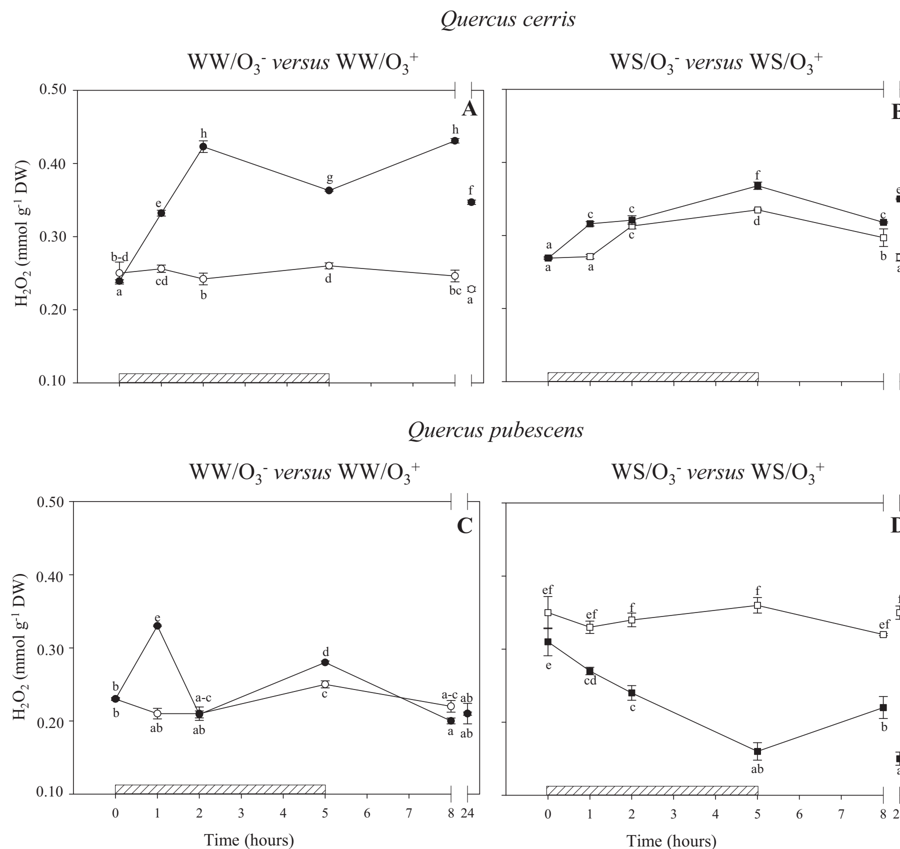

Fig. 3. Time course of hydrogen peroxide (H_2O_2) content in *Quercus cerris* (A–B) and *Q. pubescens* (C–D) plants well-watered (○ and □ symbols) or water stressed (20% of the effective evapotranspiration daily for 15 days, ● and ■ symbols) and exposed to a single pulse of ozone (200 nl l^{-1} for 5 h, □ and ■) and maintained in filtered air (○ and ●). Data are shown as mean ± standard deviation. The measurements were carried out at 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P \le 0.05$). The thick bottom line indicates the time (5 h) of ozone exposure. Abbreviations: DW, dry weight; WS/O_3^-, water stressed (20% of effective evapotranspiration daily for 15 days) and exposed to charcoal filtered air; WS/O_3^+, water stressed and exposed to a single pulse of ozone (200 nl l^{-1} for 5 h); WW/O_3^-, well-watered and exposed to charcoal filtered air; WW/O_3^+, well-watered and exposed to O_3.

UVD 170U UV/VIS Dionex detector. Further details of ET, SA and JA determinations are available in Cotrozzi et al. (2017a).

2.6. Statistical analysis

Three repeated experiments were set up following a randomized design and the experimental plot consisted of one plant per container for each species. Ecophysiological and biochemical measurements were carried out on three replicates for each treatment. The normality of data was preliminary tested by the Shapiro-Wilk test. The effects of O_3 and drought on ecophysiological parameters were tested using a two-way repeated measure analysis of variance (ANOVA) with O_3 and drought as variability factors. The effects of O_3 on biochemical parameters of WW and WS plants were evaluated by a two-way ANOVA with treatment and time as variability factors. For both ecophysiological and biochemical analyses, Fisher's LSD was used as the post-hoc test, with a significance level of $P \leq 0.05$. For each physiological/biochemical parameter, mean values of WW and WS were compared within species before the O_3 treatment using the Student's *t*-test ($P < 0.05$). Statistical analyses were performed by JMP 13 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Effects of drought on water status, physiological and biochemical parameters

The physiological and biochemical responses at the end of drought exposure are summarized in Table S1. Water withholding decreased $PD\Psi_w$ (3- and 5-fold lower than WW plants, in *Q. cerris* and *Q. pubescens*, respectively) and RWC in both species (−10 and −6%, respectively). Similarly, a significant decrease of gas exchange (around −50 and −65% of A and g_s, respectively, in comparison with their relative WW counterparts) and chlorophyll *a* fluorescence parameters (around −3 and −37% of F_v/F_m and Φ_{PSII} values, respectively) was reported in both species grown under WS conditions. In comparison to WW *Q. cerris* plants, ROS levels did not change in WS ones. By contrast, water withholding significantly led to increased levels of H_2O_2 and O_2^- in *Q. pubescens* (+52 and +65% in comparison to WW plants, respectively). A significant accumulation of proline, ABA, SA and JA was observed in *Q. cerris* (+5-fold, +61, +54 and +58%, respectively), while ET decreased (−63%). In *Q. pubescens*, water deprivation induced an increase of proline, ET and JA (+6-fold, +63 and +2-fold, respectively), did not affect ABA content, and reduced SA (−35%).

3.2. Physiological and biochemical responses induced by a pulse of O_3 in WW and WS plants

3.2.1. Gas exchange and chlorophyll fluorescence parameters

Only in saplings grown under WW conditions, the pulse of O_3 significantly decreased A and g_s in both species (Fig. 1, Table S2). Values of A in *Q. cerris* continued to decrease after the end of O_3 exposure, reaching a final reduction of 48%, compared to WW/O_3^- conditions. The reduction of A in WW/O_3^+ *Q. pubescens* was stable after the end of O_3 exposure and was of 39% at the end of the experiment. The reductions of A and g_s values due to water deprivation (Table S1) did not change throughout the entire experiment and were not further exacerbated by the O_3-exposure occurred in WS/O_3^+ plants.

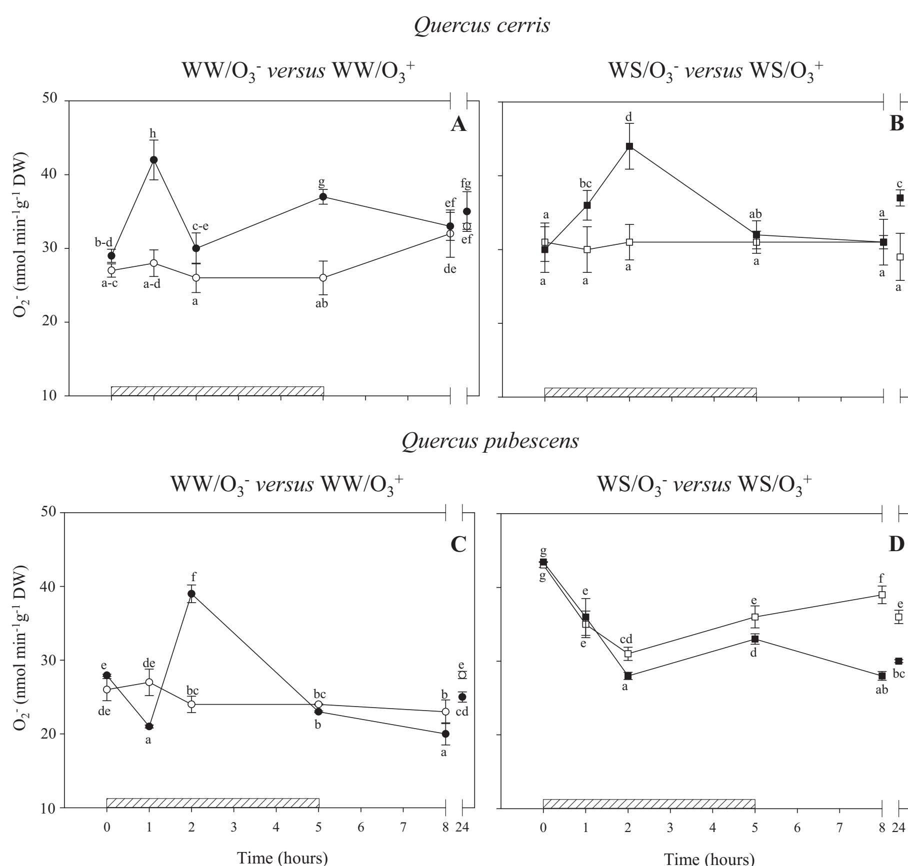

Fig. 4. Time course of anion superoxide (O_2^-) content in *Quercus cerris* (A–B) and *Q. pubescens* (C–D) plants well-watered (○ and □ symbols) or water stressed (20% of the effective evapotranspiration daily for 15 days, ● and ■ symbols) and exposed to a single pulse of ozone (200 nl l^{-1} for 5 h, □ and ■) and maintained in filtered air (○ and ●). Data are shown as mean ± standard deviation. The measurements were carried out at 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P \leq 0.05$). The thick bottom line indicates the time (5 h) of ozone exposure. For the details of abbreviations, see Fig. 3.

Independently of the treatments, F_v/F_m values were never lower than 0.80 in *Q. pubescens*, values which are typical of healthy plants (Björkman and Demming, 1987). A significant decrease of F_v/F_m was instead observed in WS/O_3^+ *Q. cerris* plants at 24 h FBE (−10%, compared with WS/O_3^-), even though F_v/F_m values recovered completely at 48 h FBE (Fig. 2A–B, Table S3). Under WW conditions, Φ_{PSII} was affected by O_3 at the end of the exposure in both species, although at a different extent: in *Q. cerris*, Φ_{PSII} dropped only by 25%, compared to WW/O_3^- plants, and completely recovered at 24 h FBE; in *Q. pubescens*, Φ_{PSII} decreased by 76% and fully recovered only at 48 h FBE. Drought stress also had a negative effect on Φ_{PSII} in both species, already at the beginning of O_3 exposure (Table S1). These lower Φ_{PSII} values were observed throughout the entire experiment. Different Φ_{PSII} patterns were instead observed between the two species under WS/O_3^+ conditions. In *Q. cerris*, the Φ_{PSII} declined in a similar way to that observed for F_v/F_m, with a strong decline at 24 h FBE, whereas in *Q. pubescens* only slight changes in Φ_{PSII}, but a strong drop of F_v/F_m at 24 h FBE were detected. In this species, values of F_v/F_m did not recover at 48 h FBE as instead occurred in *Q. cerris* (Fig. 2C–D, Table S3).

3.2.2. Reactive oxygen species

H_2O_2 production was triggered by O_3 in *Q. cerris* plants under both well-watered and drought conditions, although the timing and magnitude of induction were influenced by water availability (Fig. 3A–B, Table S3). In WW/O_3^+ plants, H_2O_2 increased at 1 h FBE and remained higher than in WW/O_3^- plants until the end of the experiment (+36%), reaching a maximum level at 2 and 8 h FBE (about 2-fold higher than WW/O_3^- plants, Fig. 3A). In WS/O_3^+ plants, H_2O_2 raised at 1 h FBE, reached its maximum at the end of O_3 exposure (+10% in comparison to WS/O_3^- ones), and remained higher than in WS/O_3^- plants during the whole recovery phase, especially at 24 h FBE (+34%, Fig. 3B). In *Q. pubescens*, the O_3-induced patterns of H_2O_2 were notably different depending on water availability (Fig. 3C–D, Table S4). In WW/O_3^+ plants, H_2O_2 exhibited a biphasic time course, being higher than in controls (i.e., WW/O_3^-) at 1 h FBE (+57%), and again at the end of O_3 exposure (+22%). During the recovery phase, H_2O_2 content did not change when compared to WW/O_3^- conditions (Fig. 3C). In WS/O_3^+ plants, where H_2O_2 values were already strongly enhanced by drought stress (0.24 ± 0.01 and 0.35 ± 0.02 under WW and WS conditions, respectively, $P \le 0.01$), O_3 significantly decreased H_2O_2 throughout the entire experiment, reaching minimum values at 5 and 24 h FBE (2-fold lower than WS/O_3^- plants, Fig. 3D).

Under both well-watered and drought conditions, O_2^- production of *Q. cerris* plants was significantly stimulated by O_3 during the entire fumigation period, except for WS/O_3^+ plants at 5 h FBE (Fig. 4A–B, Table S3). In WW/O_3^+ plants, O_2^- peaked at 1 and 5 h FBE (+50 and +42% in comparison to WW/O_3^- plants, respectively), and showed similar values than in WW/O_3^- plants at 24 h FBE (Fig. 4A). In WS/O_3^+ plants, O_2^- peaked at 2 h FBE (+44% compared to WS/O_3^- plants) and increased again at the end of the recovery phase (+28%; Fig. 4B). Similarly to H_2O_2, the effects of O_3 on O_2^- responses of *Q. pubescens* were notably affected by the plant water status (Fig. 4C–D, Table S4). In WW/O_3^+ plants, O_2^- slightly decreased at 1 h FBE (−22% in comparison to WW/O_3^- ones), reached its maximum at 2 h FBE (+63%), got back to WW/O_3^- levels at 5 and 8 h FBE, and further decreased slightly at 24 h FBE (−11%). In WS/O_3^+ plants, O_2^- decreased starting at 2 h FBE, reaching

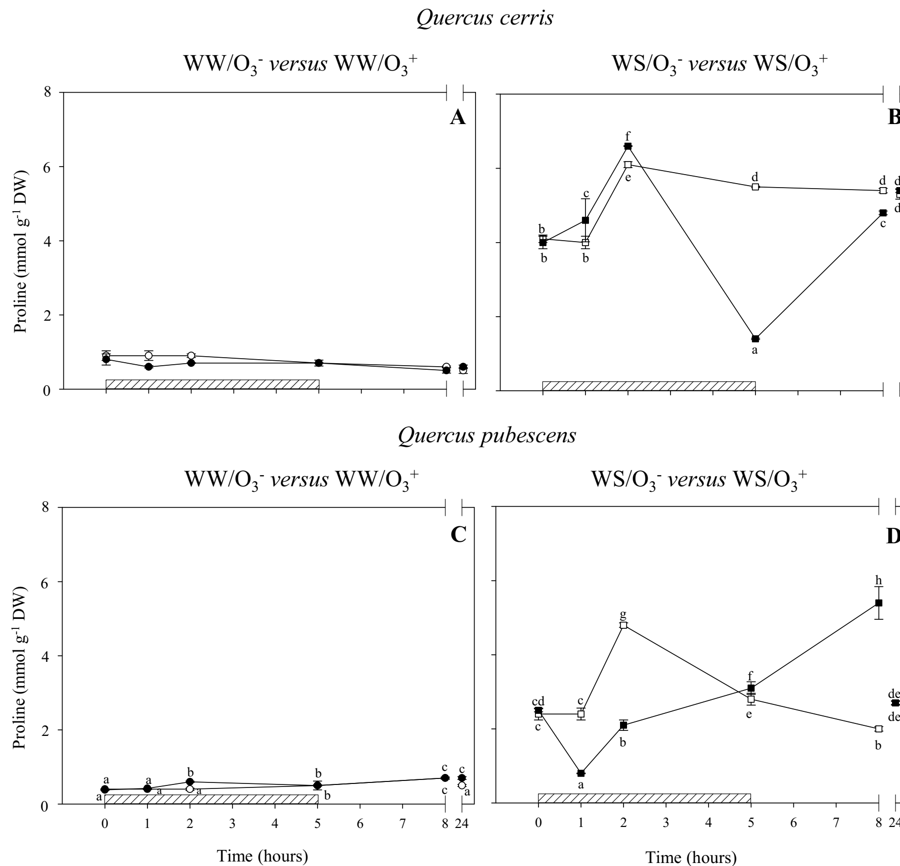

Fig. 5. Time course of proline content in *Quercus cerris* (A–B) and *Quercus pubescens* (C–D) plants well-watered (○ and □ symbols) or water stressed (20% of the effective evapotranspiration daily for 15 days, ● and ■ symbols) and exposed to a single pulse of ozone (200 nl l^{-1} for 5 h, □ and ■) and maintained in filtered air (○ and ●). Data are shown as mean ± standard deviation. The measurements were carried out at 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P \le 0.05$). The thick bottom line indicates the time (5 h) of ozone exposure. The absence of letters in A indicates not significant interaction between variability factors (see Supplementary Table S1). For the details of abbreviations, see Fig. 3.

its minimum value (−11% compared to WS/O_3^- plants), and remained lower than in WS/O_3^- plants throughout the entire experiment (Fig. 4D).

3.2.3. Osmolytes

Proline contents were affected by O_3 in *Q. cerris* plants only under water deprivation (Fig. 5A–B, Table S3). So, the levels of proline in WS/O_3^+ plants increased at 1 h FBE, reached their maximum at 2 h FBE (+8% in comparison to WS/O_3^-), drastically dropped at 5 h reaching their minimum level (about 3-fold lower that in WS/O_3^-), and then recovered after the O_3 exposure reaching the WS/O_3^- levels at 24 h FBE (Fig. 5B). Strong differences due to the plant water status were also observed in O_3-induced proline patterns of *Q. pubescens* (Fig. 5C–D, Table S4). In WW/O_3^+ plants, proline exhibited a biphasic time course, being higher than in WW/O_3^- plants at 2 h FBE (+50%) and again at the end of the recovery period (+40%, Fig. 5C). In WS/O_3^+ plants, proline values were lower than in WS/O_3^- plants at 1 and 2 h FBE, increased by the end of O_3 exposure reaching their maximum at 8 h FBE (about 3-fold higher that WS/O_3^- plants), and decreased back to WS/O_3^- levels at 24 h FBE (Fig. 5D).

ABA contents were also modified by O_3 only in WS *Q. cerris* (Fig. 6A–B, Table S3), where decreased at 1 h FBE, but then showed higher values than in WS/O_3^- plants at 2 and 5 h FBE (+95 and +43%, respectively) and even more at the end of the recovery period (about 3-fold higher than in WS/O_3^- plants, Fig. 6B). In *Q. pubescens*, the O_3-induced patterns of ABA were notably different depending on water availability of plants (Fig. 6C–D, Table S4). Under WW conditions, O_3 reduced ABA contents throughout the entire time course, except at 2 h FBE (Fig. 6C). In WS/O_3^+ plants, ABA contents decreased at 1 h FBE (−29% in comparison to WS/O_3^- plants), showed similar and slightly lower (−10%) values at 2 h FBE and at the end of O_3 exposure, respectively, increased at 8 h FBE (+55%), and finally decreased below the values of WS/O_3^- plants at the end of the recovery phase (−35%, Fig. 6D).

3.2.4. Phytohormones

The ET emissions of WW/O_3^+ *Q. cerris* decreased during the first 2 h of O_3 treatment (−53 and − 40% compared with WW/O_3^- plants, respectively), strongly peaked at 5 h FBE (about 7-fold higher than WW/O_3^- plants), and then gradually decreased reaching the WW/O_3^- levels at 24 h FBE (Fig. 7A, Table S3). Conversely, WS/O_3^+ *Q. cerris* plants exhibited minor O_3-induced ET productions at 1 and 5 h FBE, and strongly higher ET levels at the end of O_3 exposure (about 8-fold higher than WS/O_3^- ones, Fig. 7B). The ET responses to O_3 were notably different between watering conditions also in *Q. pubescens* (Fig. 7C–D, Table S4). In WW/O_3^+ plants, ET peaked at 5 h FBE and had again higher values than in WW/O_3^- plants at 24 h FBE (>2-fold higher than in WW/O_3^- ones, Fig. 7C). In WS/O_3^+ plants, ET concentrations were slightly higher than those observed in WS/O_3^- plants at 1 and 2 h FBE, peaked at the end of O_3 exposure (2-fold higher than WS/O_3^-), and showed slightly higher values at 24 h FBE (Fig. 7D).

O_3 significantly stimulated SA production in *Q. cerris* at 8 h FBE independently of water availability, although the magnitude of the peak was higher in WW/O_3^+ than in WS/O_3^+ (3- and 2-fold higher compared to WW/O_3^- and WS/O_3^-, respectively; Fig. 8A–B, Table S3). In *Q. pubescens*, the water regime affected SA responses to O_3 (Fig. 8C–D, Table S4). In WW/O_3^+ plants, SA increased during the first 2 h of O_3 treatment (+79 and +24% respectively at 1 and 2 h FBE, in comparison to WW/O_3^-), showed similar values than in WW/O_3^- plants at 5 and 8 h

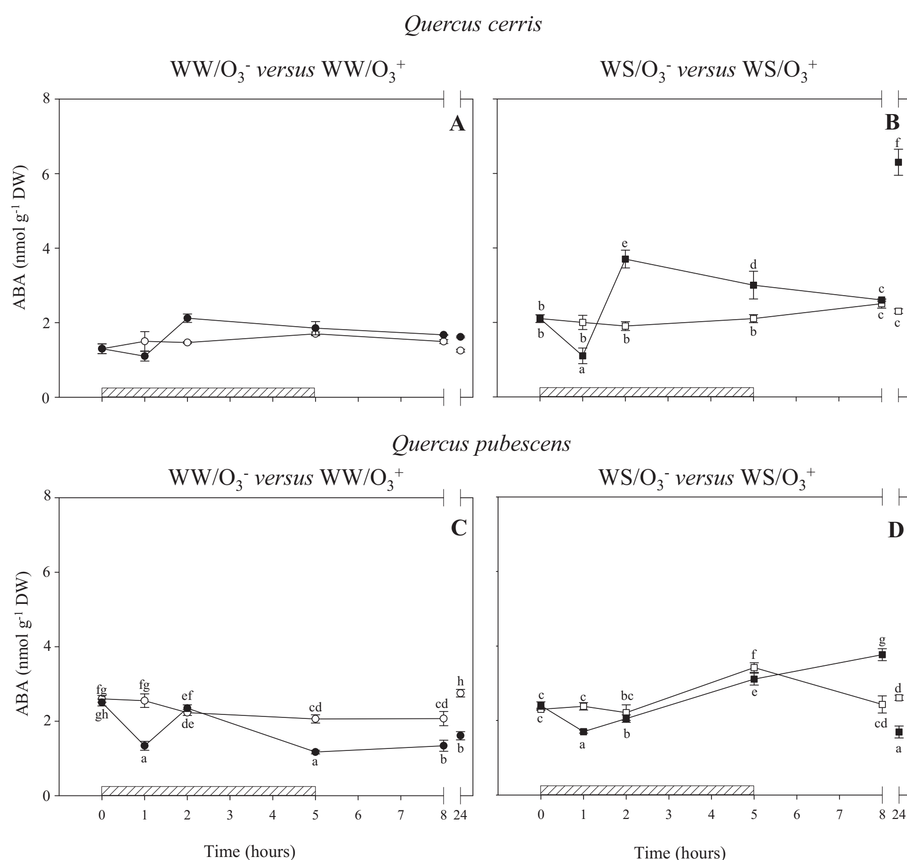

Fig. 6. Time course of abscisic acid (ABA) content in *Quercus cerris* (A–B) and *Q. pubescens* (C–D) plants well-watered (○ and □ symbols) or water stressed (20% of the effective evapotranspiration daily for 15 days, ● and ■ symbols) and exposed to single pulse of ozone (200 nl l^{-1} for 5 h, □ and ■) and maintained in filtered air (○ and ●). Data are shown as mean ± standard deviation. The measurements were carried out at 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P \leq 0.05$). The thick bottom line indicates the time (5 h) of ozone exposure. The absence of letters in A indicates not significant interaction between variability factors (see Supplementary Table S1). For the details of abbreviations, see Fig. 3.

FBE, and reached again their maximum level at the end of the recovery period (+75%, Fig. 8C). In WS/O_3^+ plants, SA concentrations peaked at 2 h FBE (+97% in comparison to WS/O_3^-) and again at 8 h FBE, although at a lower extent. Minor SA increases were also observed at 2 and 5 h FBE, but not at 24 h FBE (Fig. 8D).

In WW/O_3^+ *Q. cerris*, JA strongly peaked already at 1 h FBE (about 3-fold higher than in WW/O_3^- plants) and then decreased slowly until 8 h FBE, maintaining however higher values than those found in WW/O_3^- plants, levels that were maintained at 24 h FBE (Fig. 9A, Table S3). In WS/O_3^+ *Q. cerris*, JA slightly increased at 1 h FBE and remained higher than in WS/O_3^- plants throughout the entire experiment, with maximum values reached at 8 h FBE (+27% in comparison to WS/O_3^-, Fig. 9B, Table S2). In *Q. pubescens*, the O_3-induced patterns of JA were notably affected by the water status of plants (Fig. 9C–D, Table S4). In WW/O_3^+ plants, JA concentrations were higher than in WS/O_3^+ plants at every time of analysis, except at 8 h FBE, reaching maximum values at 1 h FBE (+51% in comparison to WS/O_3^-; Fig. 9C). In WS/O_3^+ plants, JA contents were lower than in WS/O_3^- plants at 2 h FBE (−9%), whereas higher at 8 h FBE (+35%), to finally decrease again at the end of the recovery period (−17%; Fig. 9D).

3.3. Macroscopic and microscopic symptoms

At the end of the exposure (i.e., 5 h FBE), although leaves were macroscopically symptomless, microscopic analysis reported the presence of dead cells in both species (Fig. 10). In *Q. cerris*, cell death events were only subordinated to O_3 imposition, independently (and to a similar extent, $P > 0.05$) to the water regime. Conversely, dead cells were also promoted by drought alone (WS/O_3^-) in *Q. pubescens* in which the highest extent of cell death between species and treatments was observed in WS/O_3^+ plants ($P \le 0.05$).

4. Discussion

Plants are frequently exposed to combinations of biotic and abiotic stresses that can strongly influence their growth, productivity and distribution. Evidence suggests that under combined stresses plants exhibit tailored physiological, biochemical and molecular responses, in addition to several cross responses as part of their stress tolerance strategy (Ramegowda and Senthil-Kumar, 2015). We recently reported that even a single episode of O_3 can dramatically modify the responses of the Mediterranean evergreen *Q. ilex* when the saplings were already facing other abiotic stresses (i.e., drought and salinity; Cotrozzi et al., 2017a; Guidi et al., 2017). In this study, we present a pioneering investigation on the perception of a single episode of O_3 in two drought-stressed Mediterranean deciduous oak species with a different tolerance to water withholding. Our dataset offers clear evidences on the role of specific cross-talks among signalling molecules in plant-environment interactions. Below, we detail how the effects of drought per se, which have been deeply investigated in tree species and do not represent the core of novelty of the present manuscript, may have serious consequences in altering the O_3-promoted signalling wave. In this attempt, we are aware that the picture of signalling network is further complicated by the fact that changes observed in pattern of some molecules can be a stress-specific response to drought. For instance, JA is activated by peroxidative processes strictly connected to drought-promoted oxidative stress, and SA can serve as mediator of cell survival by providing better antioxidant defenses (Munné-Bosch and Peñuelas, 2003;

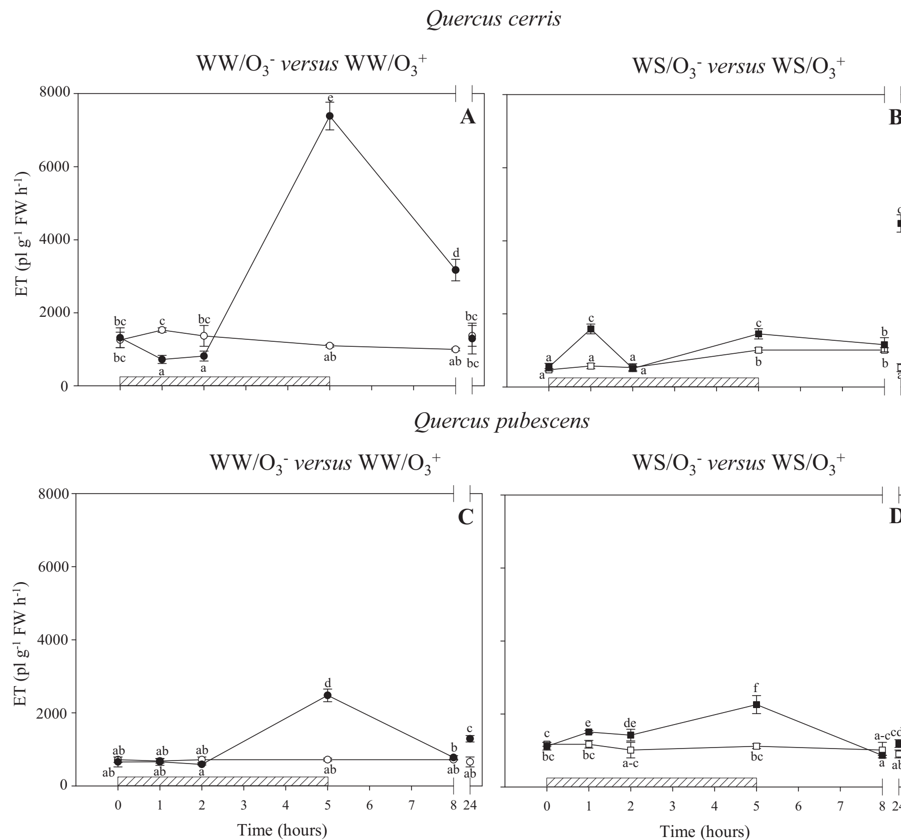

Fig. 7. Time course of ethylene (ET) content in *Quercus cerris* (A–B) and *Quercus pubescens* (C–D) plants well-watered (○ and □ symbols) or water stressed (20% of the effective evapotranspiration daily for 15 days, ● and ■ symbols) and exposed to a single pulse of ozone (200 nl l^{-1} for 5 h, □ and ■) and maintained in filtered air (○ and ●). Data are shown as mean ± standard deviation. The measurements were carried out at 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P \le 0.05$). The thick bottom line indicates the time (5 h) of ozone exposure. For the details of abbreviations, see Fig. 3 (FW, fresh weight).

Munné-Bosch et al., 2008), rather than part of the signalling pathway. We are also conscious that this topic is in its infancy and deserves intensive future research.

In accordance with our previous findings (Cotrozzi et al., 2016, 2017b, 2018), *Q. cerris* and *Q. pubescens* showed similar hydric and photosynthetic responses to the variable environments: (i) drought induced a strong decrease in PDΨ_w, but did not hardly drop the leaf water content, as confirmed by the accumulation of proline, a key osmoprotectant as well as a powerful antioxidant metabolite (Verslues and Sharma, 2010); (ii) CO_2 photo-assimilation strongly declined due to stomatal limitations induced by water withholding, both when it was applied singularly and previously to O_3, as well as by O_3 alone; and (iii) under drought, biochemical limitations also contributed to the constrained photosynthetic process as revealed, for example, by the reduction of PSII activity. Although *Q. cerris* and *Q. pubescens* showed similar stomatal reduction against water and O_3 stress, parameters derived from chlorophyll *a* fluorescence highlighted a species-specific sensitivity to a single episode of O_3, which, in turn, had different effects based on the water conditions of the plants. After drought, *Q. cerris* tolerated better the O_3 pulse as it was the only species in which the O_3-promoted photoinhibition was completely recovered at 48 h FBE. This difference between the species in terms of PSII efficiency may be related to a lower PDΨ_w of *Q. pubescens* as well as to a higher sensitivity of *Q. pubescens* PSII to O_3. Further investigations would be needed to clarify if these differential responses between the species, as well as those reported below, were due to a different O_3 uptake and drought tolerance, rather than to species-specific reactions against the interactive effects of O_3 and water stress (Gao et al., 2017).

Plant response to a pulse of O_3, which mimics the biotic defense reactions, includes two steps: the first is a biphasic oxidative burst with a massive, rapid, and transient increase in apoplastic ROS production; the second is the ROS-promoted induction of the PCD, a physiological process that selectively targets and eliminates unwanted cells which mimics the HR occurring in incompatible plant-pathogen interactions and often precedes the acquisition of a systemic resistance by plants (Kangasjärvi et al., 2005; Vainonen and Kangasjärvi, 2015; Pellegrini et al., 2016). Therefore, ROS should not be considered as unavoidable toxic by-products of aerobic metabolism, as ROS enhancement below the cytotoxic level can play a key role in intracellular communication which triggers the acclimation ability of plants, and indirectly orchestrates PCD (Choudhury et al., 2017; Mittler, 2017; Cotrozzi et al., 2017a). Choudhury et al. (2017) recently stated that different abiotic stresses and/or different combinations of abiotic stresses cause changes in ROS signatures in plant cells. Decoding these signatures via different ROS sensors can create a stress-specific signal that tailors the acclimation response to the type of stress combination affecting the plant. In this experiment we found species- and environmental-specific ROS patterns. In *Q. cerris*, an O_3-induced oxidative burst was observed independently of the water conditions, although it was more pronounced under optimal watering than under drought, and it continued after the O_3 exposure. *Q. pubescens* showed a O_3-triggered oxidative burst only during the O_3 exposure and under well-watered conditions, as ROS levels were already enhanced when plants were subjected to water withholding. In the present experiment, the subsequent O_3 episode did not induce a further ROS accumulation, it resulted in a ROS decline, actually. It is conceivable that the lack of a ROS enhancement in drought-stressed

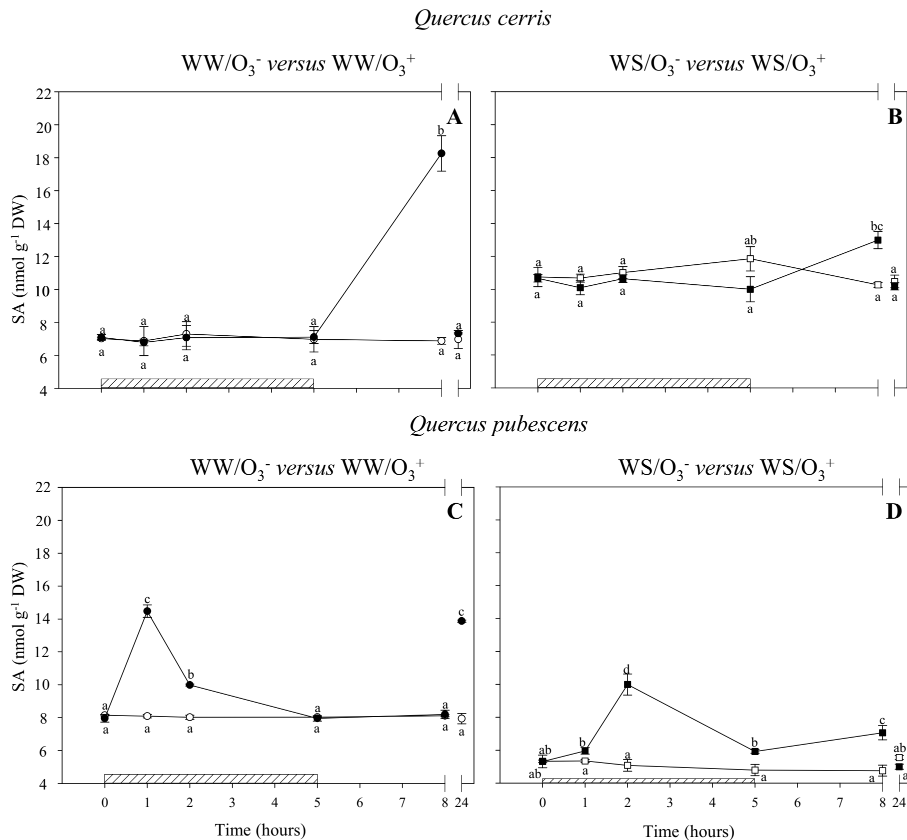

Fig. 8. Time course of salicylic acid (SA) content in *Quercus cerris* (A–B) and *Q. pubescens* (C–D) plants well-watered (○ and □ symbols) or water stressed (20% of the effective evapotranspiration daily for 15 days, ● and ■ symbols) and exposed to a single pulse of ozone (200 nl l^{-1} for 5 h, □ and ■) and maintained in filtered air (○ and ●). Data are shown as mean ± standard deviation. The measurements were carried out at 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P \leq 0.05$). The thick bottom line indicates the time (5 h) of ozone exposure. For the details of abbreviations, see Fig. 3.

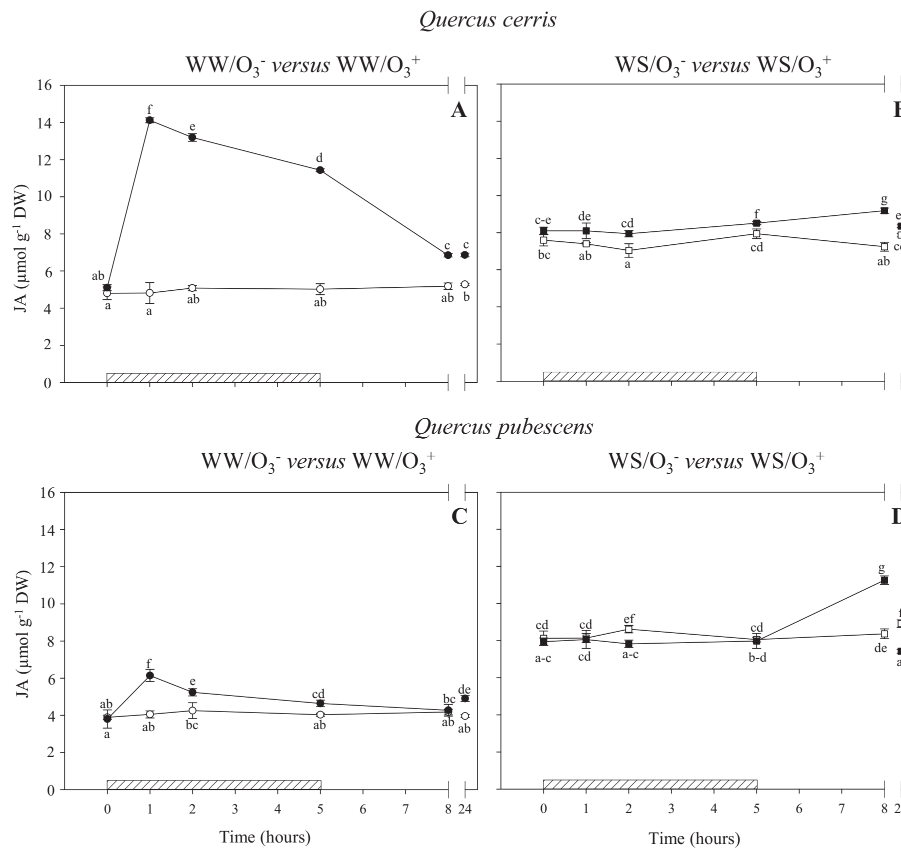

Fig. 9. Time course of jasmonic acid (JA) content in *Quercus cerris* (A–B) and *Q. pubescens* (C–D) plants well-watered (○ and □ symbols) or water stressed (20% of the effective evapotranspiration daily for 15 days, ● and ■ symbols) and exposed to a single pulse of ozone (200 nl l^{-1} for 5 h, □ and ■) and maintained in filtered air (○ and ●). Data are shown as mean ± standard deviation. The measurements were carried out at 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P \leq 0.05$). The thick bottom line indicates the time (5 h) of ozone exposure. For the details of abbreviations, see Fig. 3.

saplings of *Q. pubescens*, and consequently of a signalling induction, is likely the reason of their higher PSII sensitivity to the pulse of O_3. The amplification of ROS signals and the complete induction of defensive genes, however, seem to require an intricate interplay with other signalling molecules, including phytohormones (Choudhury et al., 2017).

Phytohormones are naturally-occurring small organic molecules that are not only important for plant developmental processes, but also play a key role as signalling molecules in defense and immune responses (Shigenaga and Argueso, 2016). Keeping in mind that O_3 mimics the biotrophic pathogen-plant interactions (Shigenaga and

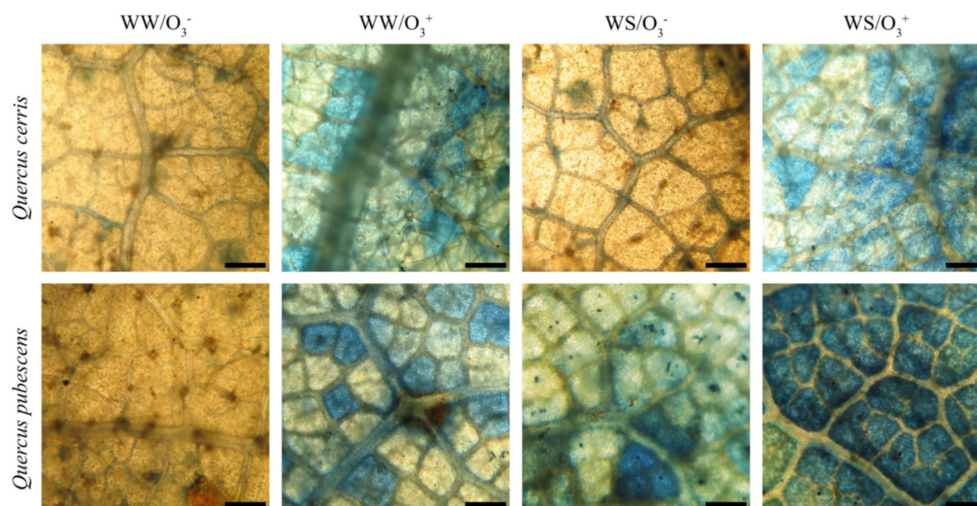

Fig. 10. Localization of dead cells visualized with Evan's blue staining in leaves of *Quercus cerris* and *Q. pubescens* plants well-watered (WW) or water stressed (WS, 20% of the effective evapotranspiration daily for 15 days) and maintained in filtered air (O_3^-) or exposed to single pulse of ozone (200 nl l^{-1} for 5 h, O_3^+). Bar: 50 µm.

Argueso, 2016), phytohormones signature (SA, ET and JA) can integrate the picture about the degree of plant sensitivity to O_3 by influencing the initiation, propagation and containment of O_3-induced cell death. ABA is another plant hormone which influences this cross-talk either in the first phase of stress by inducing stomatal closure and reducing water loss (in this phase, SA, JA and ET may not be activated and ABA can antagonize their induction) or having a subsequent direct effect on the accumulation of other phytohormones to regulate the defense reaction (I.B. Rejeb et al., 2014).

In our study, phytohormone profiles were different between the two species during and after the O_3 treatment, which is supportive for the pivotal role played by drought stress in altering O_3 responses, especially in relation to the species-specific drought sensitivity (Cotrozzi et al., 2017a). In well-watered *Q. cerris* plants, the ROS induction, observed already at the beginning of the O_3-exposure, triggered a strong accumulation of ET at the end of the O_3-exposure which was subsequently followed by a strong induction of SA at 8 h FBE. The induction of SA was likely delayed by the strong accumulation of JA occurred during the first hours of O_3 exposure, given the antagonistic action between these hormones in the regulation of defense reaction (Shigenaga and Argueso, 2016; Pellegrini et al., 2016). The chronological order of the first peaks of the signalling molecules responsible for the initiation and propagation phases of the O_3-induced response were confirmed in *Q. cerris* plants kept under drought (SA followed again ROS and ET). However, these accumulations were attenuated by water withholding and this made unnecessary a prompt and high-amplitude activation of JA to control the levels of SA and ET. The lower magnitude of phytohormone peaks observed in *Q. cerris* under drought paralleled the lower ROS induction, especially concerning the level of H_2O_2, which confirms a strict and finely-tuned interplay among these signalling molecules (Cotrozzi et al., 2017a). Characteristic feature of drought-stressed *Q. cerris* plants was, instead, the activation of ABA biosynthesis, which paralleled the trend of ET, showing a small first peak after a few hours of O_3 exposure, followed by a strong accumulation at 24 h FBE. The crucial involvement of ABA and ET in plant responses to drought is well known (Munné-Bosch and Alegre, 2004). ABA represents the most important regulator of stomatal functioning (Wilkinson and Davies, 2002), whereas ET acts as an inhibitor of shoot growth and a promoter of ripening, senescence and abscission (Abeles et al., 1992). If the prompt activation of ABA and ET could be interpreted as a first attempt by plants to control and regulate the signalling response of *Q. cerris* drought-stressed plants when facing O_3 stress, the strong accumulations of these phytohormones observed 24 h FBE were likely the adverse result of the harsh conditions induced by stress combination.

Well-watered plants of *Q. pubescens* showed a different and specific cross-talk among signalling molecules in response to the pulse of O_3. First, SA peaked at the first hours of O_3 exposure, before ET, likely due to a lower accumulation of the antagonist JA. Second, both SA and ET remained higher than controls at 24 h FBE. In *Q. pubescens*, the imposition of drought altered completely the response to the pulse of O_3. In these plants, the absence of a O_3-triggered ROS induction did not alter the timing of SA and ET response, but weakened their stimulation during the first hours of O_3-exposure. In addition, at 24 h FBE the accumulation of SA was strongly reduced in WS/O_3^+ when compared to the well-irrigated counterpart.

Proline plays several roles in plants facing both abiotic and biotic stresses and under stress its metabolism is affected by multiple and complex regulatory pathways which can deeply influence cell death (Verslues and Sharma, 2010; Zhang and Becker, 2015). Proline was affected by O_3 exposure only under drought conditions, although *Q. pubescens* plants under well-watered conditions showed a slight increase of this metabolite in concomitance of the activations of SA and JA. This outcome suggests a potential cross-talk among these molecules not only under drought but even in case of other stressors, as previously reported by K.B. Rejeb et al. (2014). Patterns of proline in the two species were completely different under drought: in *Q. cerris*, proline content dropped strongly at the end of the O_3 exposure, likely to assist H_2O_2 and O_2^- propagation waves in view of its role as ROS scavenger (Verslues and Sharma, 2010; Zhang and Becker, 2015). In saplings of *Q. pubescens*, proline content dropped in the first hours of O_3 exposure and increased at 8 h FBE, so proposing again a potential cross-talk with JA, given their similar trends. This confirms that besides the involvement of ROS and phytohormones, other metabolites, which can however have other role(s) than acting as signal compounds, may take part to the complex signalling pathway in plants which experience stress conditions and are active in regulating/modulating the response.

Microscopic analyses supported our interpretations of the species- and environmental-specific cross-talk among signalling molecules, as well as of the final effects of stress combinations on plant metabolism. Although visible symptoms were always absent in leaves of both species independently to the treatment, Evan's blue staining indicated that O_3-induced cell death occurred in both species independently of the water conditions. The fact that the highest incidence of cell death events was observed in WS/O_3^+ saplings of *Q. pubescens* is supportive for an altered perception of O_3 when this species was suffering for drought. Accordingly, only in *Q. pubescens* death cell events were observed in WS plants before the O_3 treatment, this compromising their ability to activate adequate responses to the subsequent pollutant exposure, which finally resulted in an uncontrolled and strong oxidative damage. These outputs confirm the pivotal role of phytohormones and signalling molecules in biochemical events mimicking O_3-induced HR (Overmyer et al., 2003, 2005; Kangasjärvi et al., 2005).

In conclusion, our results show that the perception of a pulse of O_3 is species-specific and can be severely altered by drought conditions. The higher ability of *Q. cerris* than *Q. pubescens* to perceive and propagate the O_3-induced ROS wave even under water limitation, allowed this species to protect better its PSII functionality when an episodic O_3 pulse occurred after a period of drought stress. Conversely, *Q. pubescens* resulted more sensitive since its strategy to cope O_3 when well-watered crashed under drought conditions, as demonstrated by further negative impacts on PSII efficiency which occurred under combined stress. These different sensitivities between the species to the variable environments were driven by specific cross-talks among signalling molecules which diverged in the molecules involved, as well as in the timing and magnitudes of their accumulations. Our study confirms the potential of using O_3 as a good tool to study the orchestrated signalling responses of plants when exposed to both biotic and abiotic apoplastic-ROS-promoting stresses (Kangasjärvi et al., 2005), but a further deep investigation on the O_3-promoted signalling network is necessary, especially in tree species for which timing and profile of signalling compounds seem to be very different to those observed in herbaceous species for which much more information is available.

Acknowledgments

This study was supported by MIUR, Rome, Italy, project PRIN "Planning the green city in the global change era: Urban tree functions and suitability for predicted future climates (TreeCity)". We would like to thank Mr Andrea Parrini for running the fumigation facilities. The authors wish to thank the two anonymous peer reviewers for their constructive comments, suggestions and criticisms, which they found useful and have helped substantially improve and clarify an earlier draft of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.12.012.

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