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Deciphering the role of low molecular weight antioxidants in the sensitivity of *Melissa officinalis* L. to realistic ozone concentrations

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ABSTRACT

To decipher the role of low molecular weight antioxidants in the sensitivity of *Melissa officinalis* L. (lemon balm, an aromatic plant, widely cultivated for pharmaceutical, food, beverage and cosmetic purposes) to realistic ozone (O_3) concentrations, plants of this species were exposed to the gaseous pollutant (80 ppb for 5 h), and investigated for their antioxidative systems and stress tolerance. Ozone treated leaves tried to cope with the increased oxidative pressure by improving the synthesis of most of the tested antioxidant compounds (e.g. superoxide dismutase, ascorbic acid, phenols and total carotenoids), whereas catalase and proline were not increased. The biosynthesis of rosmarinic acid (i.e. the dominant active phenolic compound of the investigated species) was also triggered by O_3. These cellular processes were orchestrated during and after the exposure, inducing a partial and transient control of the reactive oxygen species (i.e. hydrogen peroxide and superoxide anion) and an increase of the antioxidant capacity throughout the recovery phase (12–48 h from the beginning of the exposure). However, they were not able to protect the cell structure, as demonstrated by the macroscopic damages observed on both leaf surfaces of fumigated plants.

1. Introduction

Tropospheric ozone (O_3), produced through a series of photochemical reactions by a variety of precursors, such as nitrogen oxides and volatile organic compounds under light conditions, is a major and widespread air pollutant that can affect global environment and many biological activities (The Royal Society, 2008). Since the 20th century, many areas worldwide have frequently experienced high O_3 pollution conditions, making this problem an important issue in the environmental sciences (Lefohn et al., 2018). Background O_3 level is predicted to rise up to 42 − 84 ppb in 2100, depending on the time-space variability, with occasionally peaks exceeding 200 ppb (Yang et al., 2018). Ozone can impair humans and other animals [Bhuiyan et al., 2018; Nuvolone et al., 2018; European Union (EU) target value for the protection of human health is 120 μg m^{-3} (around 60 ppb), as maximum daily 8-h mean, not to be exceeded on more than 25 days per year, averaged over three years; EEA, 2019] as well as plant health and biodiversity (EU target value for the protection of vegetation is 18,000 μg m^{-3} h, more than 9000 ppb h, as accumulated O_3 exposure over a threshold of 40 ppb (AOT40) accumulated over May to July, averaged over five years; Krupa et al., 2001; Zhang et al., 2018; EEA, 2019).

Several experimental studies have focused on the interaction of O_3 with plants leading to some overall and widely accepted conclusions. Among them, firstly, O_3 only causes damage if a sufficient amount reaches sensitive cellular locations (e.g. intercellular spaces; Pasqualini et al., 2002; Cotrozzi et al., 2018b, c; Fernandes et al., 2019), a phenomenon thus related to the external O_3 concentrations as well as to the ability of plants to reduce O_3 influx by stomatal closure and membrane impermeability (i.e. 'avoidance', *sensu* Levitt, 1980). Secondly, O_3 injury can be counteracted if plants are able to repair and/or compensate oxidative damage (i.e. 'tolerance'; *sensu* Levitt, 1980) by the regulation of antioxidant compounds (Dizengremel et al., 2008; Pellegrini et al., 2018a; Shang et al., 2020). Ascorbic acid (AsA) is considered the first line of defence against O_3-derived reactive oxygen species (ROS) in the leaf apoplast (Bellini and De Tullio, 2019), but several other enzymatic [e.g. superoxide dismutase, (SOD), catalase (CAT) and peroxidase] and non-enzymatic (e.g. polyphenols, carotenoids and proline) antioxidants are involved in the detoxification process in a complex network (Gill and Tuteja, 2010). These antioxidant compounds can be "preformed"

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and/or "induced" molecules, the latter being synthesized by plants in response to O_3 (Nali et al., 2004; Shang et al., 2020) as well as under physical injury or infections (Treutter, 2005).

Melissa officinalis L. (lemon balm, *Lamiaceae*) is an aromatic plant, widely cultivated for the high amount of biologically active compounds (e.g. secondary metabolites) contained in leaf tissue and essential oils extracted from leaves; it represents a raw material for pharmaceutical, food, beverage and cosmetic purposes (Moradkhani et al., 2010). In a previous study (Döring et al., 2014a), we tested the sensitivity of *M. officinalis* to realistic O_3 concentrations (80 ppb for 5 h), since an evident sensitivity of this species to elevated O_3 concentrations (200 ppb for 5 h) was previously reported in terms of photosynthetic function, membrane damage, visible injury (Pellegrini et al., 2011), and cross-talk among hormones and redox signalling molecules (Pellegrini et al., 2013). Although the stomatal closure observed at the end of O_3 treatment (at 5 h from the beginning of exposure, FBE) as well as throughout the recovery period (at 12 and 24 h FBE), the rate of O_3 uptake by *M. officinalis* leaves was not reduced enough to protect the photosynthetic system, as confirmed by significant reduction in carbon dioxide fixation and carboxylation efficiency (Döring et al., 2014a). This outcome was confirmed by cytological observations that showed O_3-induced damages likely to the thin-walled and sensitive mesophyll cells around the stomatal cavities. Thus, it was concluded that stomatal closure likely reduced the oxidative pressure but was not able to totally protect *M. officinalis* plants, which therefore were sensitive even to realistic O_3 concentrations. Similar findings have been reported in other studies (Zhang et al., 2012; Shang et al., 2020) that further highlighted the importance of investigating also leaf biochemical processes such as the regulation of ROS and scavenging systems that are crucial in plant defense under stress conditions (Sharma et al., 2012).

The ROS-scavenging of some enzymatic (e.g. SOD) and non-enzymatic antioxidants (e.g. AsA, glutathione and carotenoids) of *M. officinalis* plants has been tested only under relatively high O_3 concentrations (i.e. 200 ppb for 5 h; Pellegrini et al., 2013), pointing out an inability of the antioxidant system to counteract O_3-induced oxidative pressure. Under realistic O_3 concentrations (i.e. 80 ppb for 5 h), investigations of foliar biochemical changes have been focused only on the biosynthesis of rosmarinic acid (RA, i.e. the dominant active phenolic compound in *M. officinalis*; Petersen et al., 2009), highlighting an increase of this compound only in terms of enhanced pharmaceutical properties, and not as a potential component of a complex antioxidant network (Döring et al., 2014b). Thus, interesting but still not answered questions exist about the role of low molecular weight antioxidants in the sensitivity of *M. officinalis* under realistic O_3 concentrations, a gap we addressed in the present study.

Specifically, the objectives of this study were to (i) evaluate how much ROS are induced by realistic O_3 levels, (ii) characterize the antioxidant mechanisms activated under the air pollutant pressure, and (iii) decipher the role of low molecular weight antioxidants in the sensitivity of *M. officinalis* to realistic O_3 concentrations.

2. Materials and methods

2.1. Plant material, culture conditions and ozone exposure

Four-month-old cuttings of *Melissa officinalis* L. were rooted and grown in plastic pots containing a mixture of steam sterilized soil and peat (1:1, in volume) by placing them for two weeks in a controlled environment facility (temperature 20 ± 1 °C, relative humidity 85 ± 5% and photon flux density 500 µmol photons m^{-2} s^{-1} at plant height provided by incandescent lamps with L/D 12:12 photoperiod). Aeration was performed with air filtered through activated charcoal, containing negligible O_3 concentrations (below 5 ppb). Thirty-six uniformly sized plants were transferred in four controlled environment fumigation facilities under the same climatic conditions as reported above, equally divided into two groups (18 plants and two chambers per group) and exposed to O_3-free charcoal filtered air (control plants) or to a target O_3 concentration of 80 ppb for 5 h (in form of a square wave from second to seventh hour of the light period; Pellegrini et al., 2018b). The two youngest mature leaves (fully expanded) per plant were harvested at 0, 3, 5, 12, 24 and 48 h FBE (three plants per treatment were harvested at each time of analysis), instantly frozen in liquid nitrogen and stored at −80 °C until biochemical analyses. The onset and the development of visible symptoms was monitored throughout the whole experiment. The fumigation took place at the field-station of San Piero a Grado of the Phytotoxicology Unit of the Department of Agriculture, Food and Environment of the University of Pisa, Italy (UTMWGS84: E 608510 - N 4837241, 3 m a.s.l.).

2.2. Determination of reactive oxygen species and enzymes involved in H_2O_2 metabolism

Leaf hydrogen peroxide (H_2O_2) production was measured fluorimetrically using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA) at 530 and 590 nm for excitation and emission of fluorescence, respectively, according to Shin et al. (2005). The superoxide radical ($^{\bullet}O_2^-$) production was assessed using a 6505 UV-Vis spectrophotometer (Jenway, Stone, Staffordshire, UK) at 470 nm, according to the method of Able et al. (1998) slightly revised as in Pellegrini et al. (2018b). Superoxide dismutase (EC 1.15.1.1) activity was defined as the amount of SOD required to cause 50 % inhibition of the rate of nitroblue tetrazolium (NBT) reduction, assessed by its absorbance at 560 nm, according to Zhang and Kirkham (1994). Catalase (EC 1.11.1.6) activity was evaluated as the decomposition of H_2O_2 within one minute, assessed as the decrease in absorbance at 240 nm (Zhang and Kirkham, 1994). For enzymatic assays, protein concentrations were assessed according to Bradford (1976). Further details of these analyses are reported as supplementary material.

2.3. Low molecular weight antioxidant compounds

Ascorbate and dehydroascorbate (DHA) content were measured spectrophotometrically at 534 nm, according to Wang et al. (1991). The total ascorbate content (AsA + DHA) was determined through a reduction of DHA to AsA by dithiothreitol and DHA levels were calculated as the difference between total ascorbate and AsA values. The contents of glutathione and oxidized glutathione (GSSG) were assessed spectrophotometrically at 412 nm, according to Pellegrini et al. (2013). The amount of reduced glutathione (GSH) was calculated by subtracting the GSSG amount, as GSH equivalents, from the total glutathione amount. The total content of phenolic compounds was determined spectrophotometrically (at 760 nm), according to Tonelli et al. (2015). Carotenoids were analyzed by High Performance Liquid Chromatography (HPLC; P680 Pump, UVD170U UV-VIS detector, Acclaim 120 column, C18, 5 µm particle size, 4.6 mm internal diameter, 150 mm length; Dionex, Sunnyvale, CA, USA) with photometric detection at 445 nm, according to Cotrozzi et al. (2017). The proline content was determined spectrophotometrically at 520 nm, according to Bates et al. (1973). Further details of these analyses are reported as supplementary material.

2.4. Enzymes involved in RA biosynthesis and RA content

Activity of phenylalanine ammonia-lyase (PAL) was determined by HPLC (Equisil ODS column, 5 µm particle size, 4 mm internal diameter, 250 mm length, 20 mm precolumn; Dr. Maisch HPLC GmbH, Ammerbuch, Germany) with photometric detection at 290 nm, according to Döring et al. (2014b). Activity of rosmarinic acid synthase (RAS), as well as the content of RA, were determined by HPLC with photometric detection at 333 nm, according to Döring et al. (2014b).

Further details of these analyses are reported as supplementary material.

2.5. Antioxidant capacity

The antioxidant capacity was evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method reported by Hanato et al. (1988), and assessed spectrophotometrically at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation: DPPH scavenging effect (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the DPPH, and A_1 is the absorbance of the sample. The antiradical activity was expressed as EC_{50DPPH}, the efficient sample concentration causing a 50 % DPPH inhibition. Further details are reported as supplementary material.

2.6. Statistical analysis

Following the Shapiro-Wilk W test, the effects of O_3 treatment, time and their interaction on leaf traits were analyzed using a two-way analysis of variance (ANOVA) and comparisons among means were performed by the Tukey's HSD post hoc test ($P \leq 0.05$). Statistical analysis was carried out by the NCSS 2000 Statistical Analysis System Software (Kaysville, UT, USA).

3. Results

3.1. Visible injury

At the end of O_3-treatment (i.e. 5 h FBE), leaves of all plants appeared symptomless. Small chlorotic spots were recognizable on O_3-treated mature leaves only starting from 24 h FBE, especially on the adaxial leaf surface. These spots coalesced to form necrotic stipples that were visible on both leaf surfaces at 48 h FBE. No visible symptoms were observed on control plants.

3.2. Reactive oxygen species and enzymes involved in H_2O_2 metabolism

The two-way ANOVA of H_2O_2, $^{\bullet}O_2^-$, SOD and CAT data revealed that the effects of O_3, time and their interaction were significant on these traits (Table 1). A time course with two O_3-induced peaks was observed for both H_2O_2 and $^{\bullet}O_2^-$ production (Fig. 1A and B): H_2O_2 transiently peaked at 3 and 5 h FBE (+53 and +56 % in comparison to controls, respectively) and even more at 24 h FBE (+96 %, Fig. 1A); $^{\bullet}O_2^-$ content similarly peaked at 3 h FBE (+90 %) and again at 24 h FBE (+61 %, Fig. 1B). Both H_2O_2 and $^{\bullet}O_2^-$ showed similar values between controls and O_3-treated plants at the other times of analysis. In O_3-treated plants, SOD activity also exhibited a time course with two peaks (Fig. 1 C), being higher than in controls already at 3 h FBE (+18 %), dramatically decreasing below control levels at 5 and 12 h FBE (-33 and -22 %, respectively), increasing again at 24 h FBE (+7%) and finally reaching similar values than plants under filtered air at 48 h FBE. Starting from 3 h FBE, CAT activity was always significantly lower in O_3-treated plants than in controls, except at 24 h FBE when no differences were observed between treatments; minimum values of CAT activity under O_3 were reached at 12 and 48 h FBE (-48 and -65 % in comparison with controls, respectively; Fig. 1 D).

Table 1
F values of two-way ANOVA for the effects of ozone (O_3), time and their interaction on hydrogen peroxide (H_2O_2), superoxide radical ($^{\bullet}O_2^-$), superoxide dismutase (SOD), catalase (CAT), total ascorbate (AsA + DHA), redox state of ascorbate (AsA/(AsA + DHA)), total glutathione (GSH + GSSG), redox state of glutathione (GSH/(GSH + GSSG)), phenols, total carotenoids, proline, phenylalanine ammonia-lyase (PAL), rosmarinic acid synthase (RAS), rosmarinic acid (RA) and antioxidant capacity (DPPH).***: $P \leq 0.001$, **: $P \leq 0.01$; *: $P \leq 0.05$; ns: $P > 0.05$. *d.f.* represents the degrees of freedom.

Effects	O_3	Time	O_3 × time
d.f.	1	5	1
H_2O_2	75.8***	16.6***	19.4***
$^{\bullet}O_2^-$	44.5***	70.1**	13.2***
SOD	16.3***	53.3**	49.2***
CAT	266.0***	24.0***	26.9***
AsA + DHA	194.0***	21.5**	16.6***
AsA/(AsA + DHA)	0.7^{ns}	54.8***	21.1***
GSH + GSSG	120.8***	49.9***	6.9***
GSH/(GSH + GSSG)	1.7^{ns}	5.2**	21.8***
Phenols	1.0^{ns}	9.5^{ns}	9.4***
Total carotenoids	6.5*	41.2***	33.2***
Proline	138.8***	21.2***	20.8***
PAL	96.1***	65.9***	54.8***
RAS	298.5***	57.0***	61.5***
RA	254.9***	78.9***	59.8***
Antioxidant capacity	107.8***	44.1***	37.8***

3.3. Low molecular weight antioxidant compounds

According to the two-way ANOVA, the interactive O_3 × time effect was significant on all the tested traits related to low molecular weight antioxidants, as well as the effect of time alone, except for phenols. The singular O_3 effect was significant on AsA + DHA, GSH + GSSG, total carotenoids and proline (Table 1). The content of AsA + DHA was always significantly higher in O_3-treated plants than in controls, except at 24 h FBE, but especially at 48 h FBE (+43 %, Fig. 2 A). The AsA/(AsA + DHA) ratio showed a variable trend under O_3: it was higher than in controls at 3 and 5 h FBE (+16 and +22 %, respectively; Fig. 2 B), did not show differences at 12 and 24 h FBE, and decreased at the end of the experiment (-30 %). Starting at 5 h FBE, O_3-treatment induced a decrease of GSH + GSSG below control values (-37, -29 and -33 % at 5, 12 and 24 h FBE, respectively; Fig. 2 C), but it finally recovered at 48 h FBE. The GSH/(GSH + GSSG) ratio was higher under O_3 than in controls at 5 h FBE (+20 %), did not show differences at 12 and 24 h FBE, and decreased at the end of the experiment (-21 %; Fig. 2 D). A significant decrease in total phenols was observed under O_3 at 5 and 12 h FBE (-28 and -23 % in comparison with controls, respectively; Fig. 3 A), whereas they increased at 24 h FBE and even more at the last time of analysis (+14 and +54 %, respectively). Total carotenoids were lower in O_3-treated plants than in controls already at 3 and 5 h FBE (-13 and +10 %, respectively; Fig. 3 B), did not show differences between treatments at 12 and 24 h FBE, and increased under O_3 at the end of the experiment (+51 %). Starting at 3 h FBE, the proline content was always significantly lower in O_3-treated plants than in controls, except at 48 h FBE (Fig. 3 C); minimum values of this trait were reached at 5 and 12 h FBE (-35 and -32 % in comparison with controls, respectively).

3.4. Enzymes involved in RA biosynthesis and RA content

The two-way ANOVA of PAL, RAS and RA data revealed that the effects of O_3, time and their interaction were significant on these traits (Table 1). The activity of PAL was lower under O_3 than in controls already at 3 and 5 h FBE (-59 and -89 %; Fig. 4 A), whereas it was higher during the post-fumigation period, reaching its maximum value at 12 h FBE (+64 %). Both, RAS activity and RA content showed a significant decrease in O_3-treated plants at 3 and 5 h FBE (around -25 and 45 % compared to controls, for RAS and RA, respectively; Fig. 4 B and C), recovered to control levels at 12 h FBE and slightly increased at 24 and 48 h FBE (around +10 and 25 %, respectively).

3.5. Antioxidant capacity

The two-way ANOVA of DPPH data showed that the effects of O_3, time and their interaction were significant also on this trait (Table 1). During the whole O_3-treatment, DPPH content did not show significant

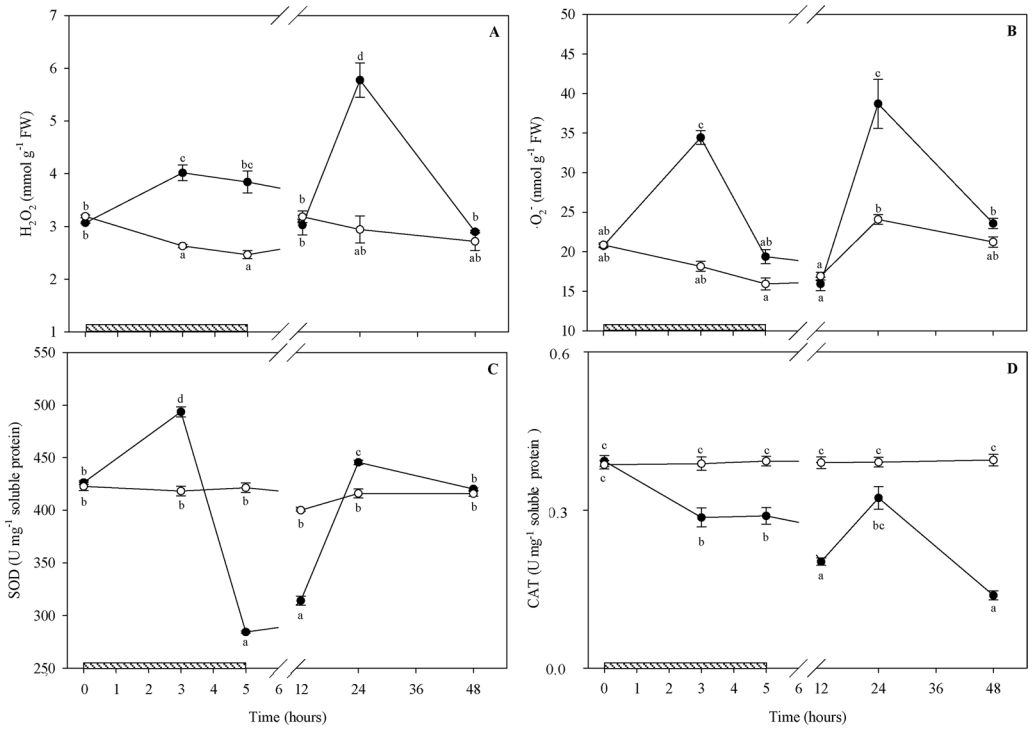

Fig. 1. Time course of hydrogen peroxide (H_2O_2, **A**) content, superoxide radical ($^{\bullet}O_2^-$, **B**) generating rate, and superoxide dismutase (SOD, **C**) and catalase (CAT, **D**) activities in leves of *Melissa officinalis* maintained under filtered air (open circle) or exposed to ozone (80 ppb for 5 h, closed circle). Data are shown as mean ± standard error. Measurements were carried out at 0, 3, 5, 12, 24 and 48 h from the beginning of exposure. Different letters indicate significant differences among means ($P \leq 0.05$). The dashed line indicates the duration of ozone exposure. Abbreviation: FW, fresh weight.

differences between treatments, whereas a marked drop of this trait was observed starting at 12 h FBE until the end of the experiment (-15, -26 and -30 % at 12, 24 and 48 h FBE, respectively; Fig. 5), indicating a prominent increase in the antioxidant capacity.

4. Discussion

According to Levitt's (1972) definition, plants can resist to O_3-induced stress because of two possible mechanisms: avoidance or tolerance. Avoidance may be explained by stomatal closure usually occurring during O_3 exposure. Several studies documented that low stomatal conductance may provide some measures of protection by reducing the flux of O_3 into the leaf intercellular space (e.g. Reich, 1987; Robinson et al., 1998; Cotrozzi et al., 2018b). Tolerance includes cellular repair and O_3-detoxification processes linked to constitutive and induced levels of antioxidant compounds (Dizengremel et al., 2008). As reported in a previous study carried out by our research group (Döring et al., 2014a), *M. officinalis* leaves exposed to 80 ppb of

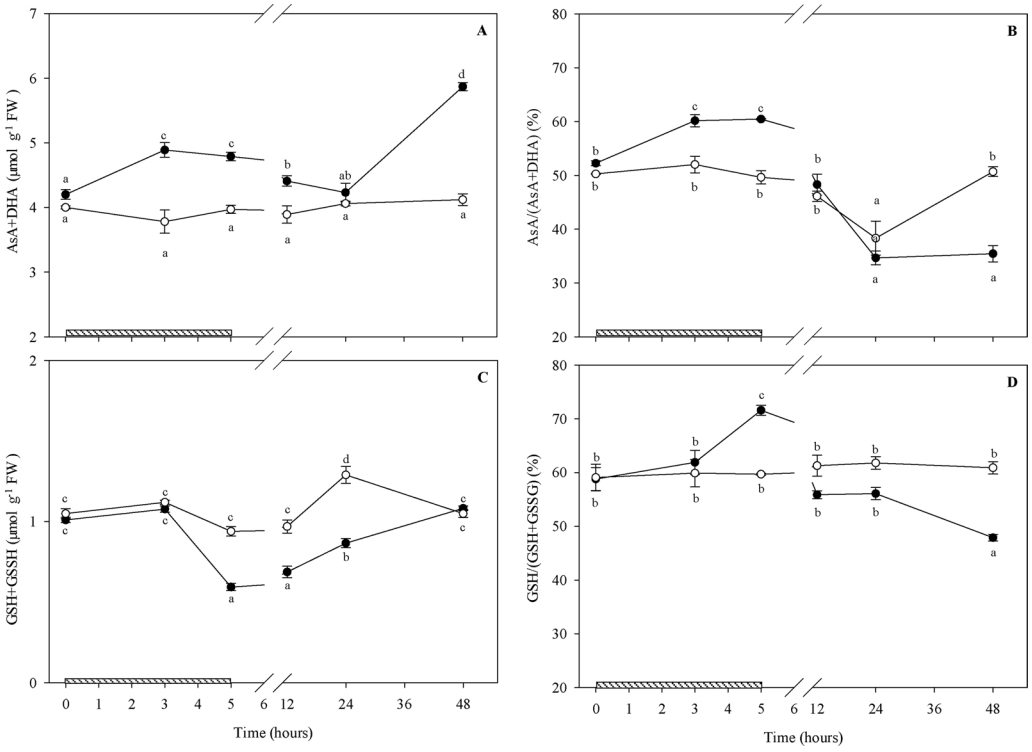

Fig. 2. Time course of total ascorbate content (AsA + DHA, **A**), ascorbate redox state (AsA/(AsA + DHA), **B**), total glutathione content (GSH + GSSG, **C**) and glutathione redox state (GSH/(GSH + GSSG), **D**) activity in leaves of *Melissa officinalis* maintained under filtered air (open circle) or exposed to ozone (80 ppb for 5 h, closed circle). Data are shown as mean ± standard error. Measurements were carried out at 0, 3, 5, 12, 24 and 48 h from the beginning of exposure. Different letters indicate significant differences among means ($P \leq 0.05$). The dashed line indicates the duration of ozone exposure. Abbreviations: AsA, reduced form of ascorbic acid; DHA, oxidized form of ascorbic acid; FW, fresh weight; GSH, reduced form of glutathione; GSSG, oxidized form of glutathione.

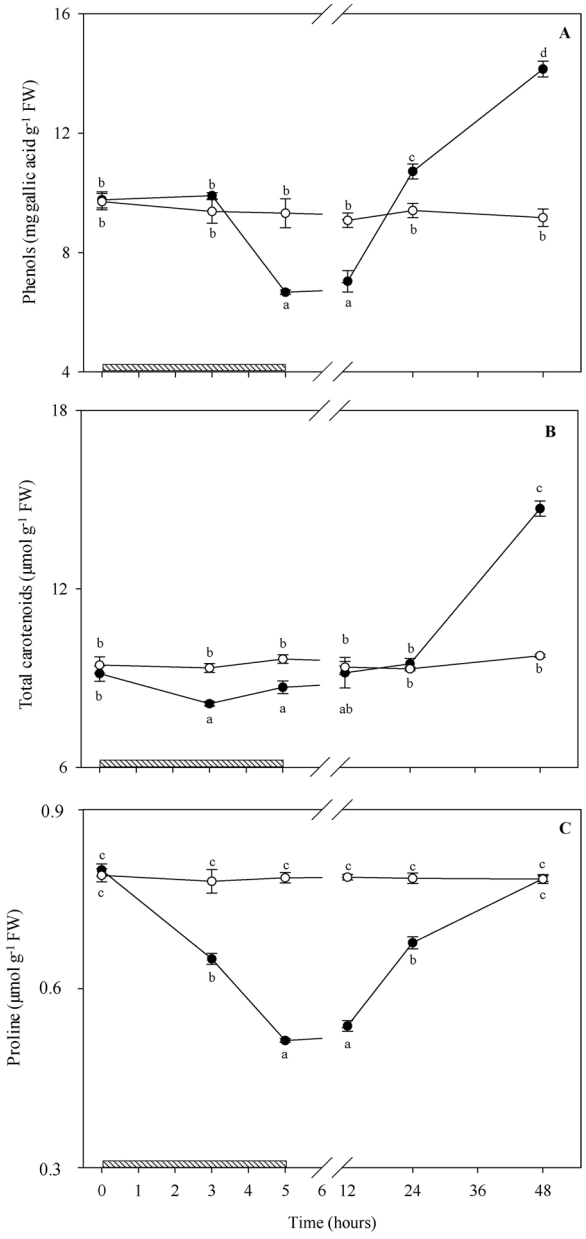

Fig. 3. Time course of total phenols (**A**), total carotenoids (**B**) and proline content (**C**) in leaves of *Melissa officinalis* maintained under filtered air (open circle) and exposed to ozone (80 ppb for 5 h, closed circle). Data are shown as mean ± standard error. Measurements were carried out at 0, 3, 5, 12, 24 and 48 h from the beginning of exposure. Different letters indicate significant differences among means ($P \leq 0.05$). The dashed line indicates the duration of ozone exposure. Abbreviation: FW, fresh weight.

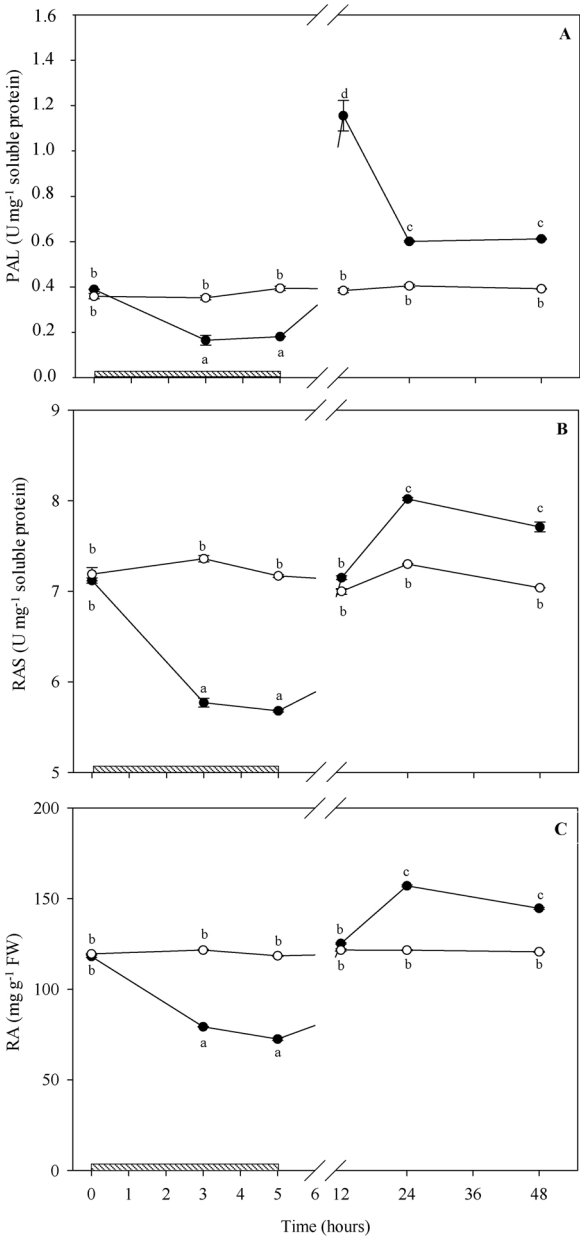

Fig. 4. Time course of phenylalanine ammonia-lyase (PAL, **A**) and rosmarinic acid synthase (RAS, **B**) activities and rosmarinic acid (RA, **C**) content in leaves of *Melissa officinalis* maintained under filtered air (open circle) or exposed to ozone (80 ppb for 5 h, closed circle). Data are shown as mean ± standard error. Measurements were carried out at 0, 3, 5, 12, 24 and 48 h from the beginning of exposure. Different letters indicate significant differences among means ($P \leq 0.05$). The dashed line indicates the duration of ozone exposure. Abbreviation: FW, fresh weight.

O_3 for 5 h (i.e. the same treatment as in the present study) partially closed stomata in order to limit the entry of the gaseous pollutant into the leaf. However, this O_3-avoidance strategy did not completely prevent the oxidative load since O_3 damage was detectable at both macro- and microscopic levels (e.g. dead cells detected by Evans blue staining and H_2O_2 deposits), confirming that an alteration of cell homeostasis and a concomitant metabolic disturbance within the cell occurred (Döring et al., 2014a). In the present study, O_3-treated plants exhibited a production of toxic products peaking twice (i.e. H_2O_2 and $^{\bullet}O_2^-$): the first peak observed during the fumigation was likely due to an O_3-decomposition, whereas the second peak that occurred during recovery, at 24 h FBE, could be entirely due to an O_3-induced overproduction of foliar ROS. Similar findings have been already described in several herbaceous (Mahalingam et al., 2006; Di Baccio et al., 2012; Pellegrini et al., 2013) and tree species (Cotrozzi et al., 2017; Landi et al., 2019). This over-production of ROS likely leaded to an uncontrolled oxidative pressure, exceeding the metabolic capacity of *M. officinalis*. Indeed, the O_3-induced time course of SOD activity, which increased concomitantly with H_2O_2 and $^{\bullet}O_2^-$ peaks at 3 and 24 h FBE, suggests that the activation of this antioxidant enzyme was more tissue damage-related than protective from the ROS (Tuomainen et al., 1996; Pellegrini et al., 2013). This outcome, together with the lacking enhancement of CAT activity throughout the whole experiment, facilitating the persistence of H_2O_2 in the cell (Gill and Tuteja, 2010), allows to conclude that the enzymatic antioxidant system did not perform an optimal ROS

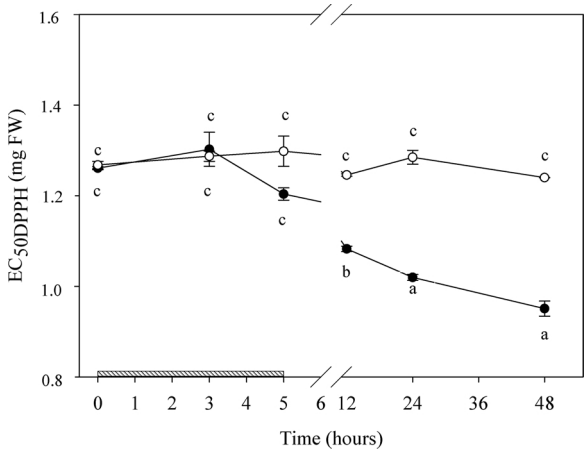

Fig. 5. Time course of antioxidant capacity (EC_{50DDPH}, the concentration required to cause a 50 % DPPH inhibition) in leaves of *Melissa officinalis* maintained under filtered air (open circle) or exposed to ozone (80 ppb for 5 h, closed circle). Data are shown as mean ± standard error. Measurements were carried out at 0, 3, 5, 12, 24 and 48 h from the beginning of exposure. Different letters indicate significant differences among means ($P \leq 0.05$). The dashed line indicates the duration of ozone exposure. Abbreviation: FW, fresh weight.

scavenging in relation to the O_3-triggered ROS production. This interpretation would explain the marked increase in the levels of MDA byproducts and the decrease in the photosynthetic activity previously reported in *M. officinalis* under O_3 (Döring et al., 2014a), as well as the development of O_3-induced visible symptoms observed in the present study (similar to those reported by Dias et al., 2013).

Plants have also evolved efficient non-enzymatic antioxidant systems to protect themselves against toxic O_2 intermediates (Foyer and Noctor, 2011). The O_3-induced increase of the total ascorbate content observed throughout the whole experiment (except at 24 h FBE), together with the enhanced AsA reduced rate [i.e. increased AsA/(AsA + DHA) ratio] that occurred during the fumigation, confirmed the role of AsA in maintaining an optimal reduced state within cells, as well as in limiting the ROS-induced oxidative pressure under O_3. This is supported by the unchanged $^{\bullet}O_2^-$ values observed at 5 h FBE in conjunction with still enhanced levels of H_2O_2 content (Meyer, 2008). Conversely, the inability of maintaining a favourable AsA reduced rate [AsA/(AsA + DHA) ratio even decreased] during the recovery period was likely the reason of the strong increase in ROS production observed at 24 h FBE, confirming the role of this biochemical mechanism in counterbalancing the oxidative alteration. These impairments in the AsA regeneration system were further confirmed by the O_3-induced alterations of the total GSH content and its redox state: the decrease of the GSH pool observed at the end of O_3-treatment and during the recovery period (except at 48 h FBE) demonstrated a decreased synthesis or a degradation of this system (Foyer and Noctor, 2005); and a predominant GSH oxidation took place only at 48 FBE [i.e. marked decrease of GSH/(GSH + GSSG) ratio]. It is thus possible to conclude that the whole Halliwell-Asada cycle was likely not sufficient to mitigate the negative effects of O_3 in terms of ROS production and membrane denaturation. These results suggest that AsA was more important as non-enzymatic antioxidant directly scavenging ROS than as a member of the Halliwell-Asada cycle involved in redox homeostasis (Foyer and Noctor, 2011). As largely reported, AsA is a cofactor of several plant-specific enzymes that are involved in important pathways leading to the biosynthesis of (i) cell wall hydroxyproline-rich proteins; (ii) plant hormones (Gest et al., 2013); and (iii) redox-active metabolites (such as carotenoids, tocopherols and phenols; Potters et al., 2010).

Phenols are well suited to constitute a "secondary" antioxidant system with a central role in plant defense against severe constraints by avoiding the generation of ROS and by quenching ROS once they are formed (Brunetti et al., 2015). In our study, the significant decrease of total phenols observed at 5 and 12 h FBE may suggest that these antioxidants were consumed by the cell to counteract the ROS generation/accumulation due to increased oxidative metabolism and the resulting cellular damage (Pellegrini et al., 2019). The significant induction of total phenols observed later at 24 h FBE and even more at 48 h FBE further suggests a great capacity of these compounds to regulate the levels of ROS and the cellular redox state, as confirmed by the unchanged levels of ROS at the end of the experiment. However, also this antioxidative mechanism was not able to preserve *M. officinalis* from the negative effects of O_3, given the already mentioned impairments at the photosynthetic performance and cell structure (Döring et al., 2014a). Oxidative stress affects not only total phenols, but also the amount of single metabolites. Thus, the significant decrease of RA levels which occurred at 3 and 5 h FBE indicates that RA was likely decomposed by O_3 due to its strong oxidizing potential (Döring et al., 2014b), whereas the slight accumulation of RA observed at 24 and 48 h FBE confirmed the activation of secondary metabolites as a detoxifying system. This outcome was further proved by the positive relation between the O_3-induced regulations in RA content and those in the activities of enzymes involved in RA biosynthesis (i.e. PAL and RAS). Phenylalanine ammonia-lyase catalyzes the initial step in the biosynthesis of many plant phenolics and operates as key regulatory and branch point enzyme in secondary (phenylpropanoid) metabolism forming a multitude of phenolic compounds (Dixon and Paiva, 1995). Rosmarinic acid synthase links the two different pathways to form RA as end product (Petersen and Alfermann, 1988) and it can be considered a specific enzyme leading to this secondary product. The decrease of these enzyme activities during the O_3 treatment confirmed the known detrimental effect of O_3 on proteins (Rao et al., 1995). On the other hand, their increase during the recovery period, with the PAL activity peaking at 12 h FBE in concomitance with unchanged levels of RAS activity, confirmed the role of the specialized metabolism in plant defense under O_3 pressure, as well as the possible fine regulation of this mechanism through time (Eckey-Kaltenbach et al., 1994; Cotrozzi et al., 2018a). Some phenolic compounds could act as antioxidants by retarding protein oxidation or by binding to the proteins. The antiradical mechanisms in phenol-protein aggregates may be due to the ability of phenolic compounds to transfer oxidative pressure from one phenolic site to another, protecting proteins from oxidation (Štajner et al., 2007). This hypothesis is supported by the significant increase of the free radical scavenging observed during the recovery period by the DPPH assay, where the antioxidant activity mainly depends on the dissociation of hydrogen radicals from phenolic substances to form a stable compound with DPPH radicals (Tonelli et al., 2015).

It is known that products of the phenylpropanoid pathway are directly involved in photoprotection in response to stress as well as in ROS scavenging (Dixon and Paiva, 1995; Gill and Tuteja, 2010). Carotenoids are lipophilic antioxidants that play an important photoprotective role by (i) dissipating excess excitation energy as heat, (ii) scavenging ROS, and (iii) suppressing lipid peroxidation (Havaux et al., 2005). Similarly to the regulation of the phenol contents, the slight decrease of total carotenoids at 3 and 5 h FBE was likely due to their consumption by the cell to improve its tolerance to the photo-oxidative stress induced by O_3 (Pellegrini et al., 2019), whereas the significant induction of total carotenoids observed at the end of the experiment was likely aimed to limit ROS formation. However, also this defense mechanism was not sufficient to stabilize the lipid phase of the thylakoid membranes and to offer photoprotection to the leaves (Sharma et al., 2012), even if it was concurrent with the regulation of the phenol contents.

In addition to the main antioxidants such as AsA, GSH and secondary metabolites (Queval and Noctor, 2007; Potters et al., 2010), plants contain many other organic compounds of low molecular weight involved in osmotic adjustments (Hare et al., 1998). Proline, for example, not only facilitates water uptake, but also protects the cell

against ROS accumulation under stress conditions by recycling of NADPH via its synthesis from glutamate and by acting as a free radical scavenger (Soares et al., 2018). In our study, the significant decrease of proline that occurred throughout the whole experiment (except at 48 h FBE) indicates that this amino acid was not involved in a direct antioxidative protection or served as AsA regeneration to buffer the cellular redox potential. On the contrary, it is possible to speculate that proline likely triggered a premature cell death instead of preventing the oxidative damage of membrane lipids (Zhang and Becker, 2015).

Even though the relationship between the activation of low molecular weight antioxidant compounds and stress tolerance has not so far been fully elucidated, it is known that the biosynthesis of these compounds increases more in stress-sensitive than in tolerant species (Shang et al., 2020). In addition, changes in their oxidation/reduction state is associated with the exposure level and the duration of O_3 treatment, as well as the leaf age and the growth stage (Jolivet et al., 2016). In our study, *M. officinalis* plants tried to cope with O_3 by improving the synthesis of low molecular weight antioxidant compounds (except proline). These cellular processes induced a partial and transient control of ROS production, but they were not able to protect the cell structure, as demonstrated by the observed macroscopic damages. It is worth to note that the concept of a cellular redox state, which regulates these biochemical responses (e.g. increase of AsA + DHA content during O_3-treatment; induction of total phenols, RA and total carotenoids during the recovery period), is inherently associated with the kinetics of O_3 stress and especially with the balance between damage and acclimation responses. Although *M. officinalis* was shown to be sensitive to the gaseous air pollutant, the present study highlights the potential of using O_3 as a technological application for increasing the production of biologically active compounds such as phenols, with meaningful antioxidant properties.

CRediT authorship contribution statement

Anne S. Döring: Investigation, Formal analysis. **Lorenzo Cotrozzi:** Methodology, Writing - original draft. **Giacomo Lorenzini:** Supervision, Writing - review & editing. **Cristina Nali:** Supervision, Project administration, Writing - review & editing. **Maike Petersen:** Methodology, Supervision, Project administration, Writing - review & editing. **Elisa Pellegrini:** Supervision, Project administration, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2020.112369.

References

Able, A.J., Guest, D.I., Sutherland, M.W., 1998. Use of a new tetrazolium-based assay to study the production of superoxide radicals by tobacco cell cultures challenged with avirulent zoospores of *Phytophthora parasitica* var *nicotianae*. Plant Physiol. 117, 491–499. https://doi.org/10.1104/pp.117.2.491.

Bates, L.S., Waldren, R.P., Teare, J.D., 1973. Rapid determination of free proline for water stress studies. Plant Soil 39, 205–207. https://doi.org/10.1007/BF00018060.

Bellini, E., De Tullio, M.C., 2019. Ascorbic acid and ozone: novel perspectives to explain an elusive relationship. Plants 8, 122. https://doi.org/10.3390/plants8050122.

Bhuiyan, M.A., Khan, H.U.R., Zaman, K., Hishan, S.S., 2018. Measuring the impact of global tropospheric ozone, carbon dioxide and sulfur dioxide concentrations on biodiversity loss. Environ. Res. 160, 398–411. https://doi.org/10.1016/j.envres.2017.10.013.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brunetti, C., Guidi, L., Sebastiani, F., Tattini, M., 2015. Isoprenoids and phenylpropanoids are key components of the antioxidant defense system of plants facing severe excess light stress. Environ. Exp. Bot. 119, 54–62. https://doi.org/10.1016/j.envexpbot.2015.04.007.

Cotrozzi, L., Pellegrini, E., Guidi, L., Landi, M., Lorenzini, G., Massai, R., Remorini, D., Tonelli, M., Trivellini, A., Vernieri, P., Nali, C., 2017. Losing the warning signal: drought compromises the cross-talk of signalling molecules in *Quercus ilex* exposed to ozone. Front. Plant Sci. 8, 1020. https://doi.org/10.3389/fpls.2017.01020.

Cotrozzi, L., Campanella, A., Pellegrini, E., Lorenzini, G., Nali, C., Paoletti, E., 2018a. Phenylpropanoids are key players in the antioxidant defense to ozone of European ash, *Fraxinus excelsior*. Environ. Sci. Pollut. Res. 25, 8137–8147. https://doi.org/10.1007/s11356-016-8194-8.

Cotrozzi, L., Remorini, D., Pellegrini, E., Guidi, L., Nali, C., Lorenzini, G., Massai, R., Landi, M., 2018b. Living in a Mediterranean city in 2050: broadleaf or evergreen 'citizens'? Environ. Sci. Pollut. Res. 9https://doi.org/10.1007/s11356-017-9316-7. 9161-8173.

Cotrozzi, L., Townsend, P.A., Pellegrini, E., Nali, C., Couture, J.J., 2018c. Reflectance spectroscopy: a novel approach to better understand and monitor the impact of air pollution on Mediterranean plants. Environ. Sci. Pollut. Res. 9, 8249–8267. https://doi.org/10.1007/s11356-017-9568-2.

Di Baccio, D., Ederli, L., Marabottini, R., Badiani, M., Francini, A., Nali, C., Antonelli, M., Santangelo, E., Sebastiani, L., Pasqualini, S., 2012. Similar foliar lesions but opposite hormonal patterns in a tomato mutant impaired in ethylene perception and its near isogenic wild type challenged with ozone. Environ. Exp. Bot. 75, 286–297. https://doi.org/10.1016/j.envexpbot.2011.08.001.

Dias, M.C., Moura, B.B., da Silva Pedros, G., Ribeiro de Souza, S., Cardoso-Gustavson, P., 2013. The role of non-glandular emergences in *Croton floribundus* (Euphorbiaceae) upon elevated ozone exposures. Nord. J. Bot. e02338. https://doi.org/10.1111/njb.02338.

Dixon, R.A., Paiva, N.L., 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085–1097. https://doi.org/10.1105/tpc.7.7.1085.

Dizengremel, P., Le Thiec, D., Bagard, M., Jolivet, Y., 2008. Ozone risk assessment for plants: central role of metabolism-dependent changes in reducing power. Environ. Pollut. 156, 11–15. https://doi.org/10.1016/j.envpol.2007.12.024.

Döring, A.S., Pellegrini, E., Campanella, A., Trivellini, A., Gennai, C., Petersen, M., et al., 2014a. How sensitive is *Melissa officinalis* to realistic ozone concentrations? Plant Physiol. Biochem. 74, 156–164.

Döring, A.S., Pellegrini, E., Della Batola, M., Nali, C., Lorenzini, G., Petersen, M., Nali, C., Lorenzini, G., 2014b. How do background ozone concentrations affect the biosynthesis of rosmarinic acid in *Melissa officinalis*? J. Plant Physiol. 171, 35–41. https://doi.org/10.1016/j.plaphy.2013.11.006.

Eckey-Kaltenbach, H., Ernst, D., Heller, W., Sandermann, H.Jr., 1994. Biochemical plant responses to ozone IV. Cross-induction of defensive pathways in parsley plants. Plant Physiol. 104, 67–74. https://doi.org/10.1104/pp.104.1.67.

EEA (European Envionment Agency), 2019. Air Quality in Europe. Technical Report n. 10/2019. . https://www.eea.europa.eu/publications/air-quality-in-europe-2019.

Fernandes, F.F., Esposito, M.P., da Silva Engela, M.R.G., Cardoso-Gustavson, P., Furlan, C.M., Hoshika, Y., Carrari, E., Magni, G., Domingos, M., Paoletti, E., 2019. The passion fruit liana (*Passiflora edulis* Sims, Passifloraceae) is tolerant to ozone. Sci. Tot. Environ. 656, 1091–1101. https://doi.org/10.1016/j.scitotenv.2018.11.425.

Foyer, C.H., Noctor, G., 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell 17, 1866–1875. https://doi.org/10.1105/tpc.105.033589.

Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 155, 2–18. https://doi.org/10.1104/pp.110.167569.

Gest, N., Gautier, H., Stevens, R., 2013. Ascorbate as seen through plant evolution: the rise of a successful molecule? J. Exp. Bot. 64, 33–53. https://doi.org/10.1093/jxb/ers297.

Gill, S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930. https://doi.org/10.1016/j.plaphy.2010.08.016.

Hanato, T., Kagawa, H., Yasuhara, T., Okuda, T., 1988. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effect. Chem. Pharm. Bul. 36, 1090–1097. https://doi.org/10.1248/cpb.36.2090.

Hare, P.D., Cress, W.A., Staden, J.V., 1998. Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ. 21, 535–553. https://doi.org/10.1046/j.1365-3040.1998.00309.x.

Havaux, M., Eymery, F., Porfirova, S., Rey, P., Dörmann, P., 2005. Vitamin E protects against photoinhibition and photooxidative stress in *Arabidopsis thaliana*. Plant Cell 17, 3451–3469. https://doi.org/10.1105/tpc.105.037036.

Jolivet, Y., Bagard, M., Cabané, M., Vaultier, M.N., Gandin, A., Afif, D., Dizengremel, P., Le Thiec, D., 2016. Deciphering the ozone-induced changes in cellular processes: a prerequisite for ozone risk assessment at the tree and forest levels. Ann. For. Sci. 73, 923–943. https://doi.org/10.1007/s13595-016-0580-3.

Krupa, S., McGrath, M.T., Andersen, C.P., Booker, F.L., Burkey, K.O., Chappelka, A.H., et al., 2001. Ambient ozone and plant health. Plant Dis. 85, 4–17. https://doi.org/10.1094/PDIS.2001.85.1.4.

Landi, M., Cotrozzi, L., Pellegrini, E., Remorini, D., Tonelli, M., Trivellini, A., Nali, C.,

Guidi, L., Massai, R., Vernieri, P., Lorenzini, G., 2019. 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. Sci. Tot. Environ. 657, 379–390. https://doi.org/10.1016/j.scitotenv.2018.12.012.

Lefohn, A.S., Malley, C.S., Smith, L., Wells, B., Hazucha, M., Simon, H., et al., 2018. Tropospheric ozone assessment report: global ozone metrics for climate change, human health, and crop/ecosystem research. Elementa 6, 28. https://doi.org/10.1525/elementa.279.

Levitt, J., 1980. Responses of Plants to Environmental Stresses. Academic Press, New York.

Mahalingam, R., Jambunathan, N., Gunjan, S.K., Faustin, E., Weng, H., Ayoubi, P., 2006. Analysis of oxidative signalling induced by ozone in *Arabidopsis thaliana*. Plant Cell Environ. 29, 1357–1371. https://doi.org/10.1111/j.1365-3040.2006.01516.x.

Meyer, A.J., 2008. The integration of glutathione homeostasis and redox signaling. J. Plant Physiol. 165, 1390–1403. https://doi.org/10.1016/j.jplph.2007.10.015.

Moradkhani, H., Sargsyan, E., Bibak, H., Naseri, B., Sadat-Hosseini, M., Fayazi-Barjin, A., Meftahizade, H., 2010. *Melissa officinalis* L., a valuable medicinal plant: a review. J. Med. Plant Res. 4, 2753–2759.

Nali, C., Paoletti, E., Marabottini, R., Della Rocca, G., Lorenzini, G., Paolacci, A.R., Ciaffi, M., Badiani, M., 2004. Ecophysiological and biochemical strategies of response to ozone in Mediterranean evergreen broadleaf species. Atmos. Environ. 38, 2247–2257. https://doi.org/10.1016/j.atmosenv.2003.11.043.

Nuvolone, D., Petri, D., Voller, F., 2018. The effects of ozone on human health. Environ. Sci. Pollut. Res. 25, 8074–8088. https://doi.org/10.1007/s11356-017-9239-3.

Pasqualini, S., Antonielli, M., Ederli, L., Piccioni, C., Loreto, F., 2002. Ozone uptake and its effect on photosynthetic parameters of two tobacco cultivars with contrasting ozone sensitivity. Plant Physiol. Biochem. 40, 599–603. https://doi.org/10.1016/S0981-9428(02)01426-2.

Pellegrini, E., Carucci, M.G., Campanella, A., Lorenzini, G., Nali, C., 2011. Ozone stress in *Melissa officinalis* plants assessed by photosynthetic function. Environ. Exp. Bot. 73, 94–101. https://doi.org/10.1016/j.envexpbot.2010.10.006.

Pellegrini, E., Trivellini, A., Campanella, A., Francini, A., Lorenzini, G., Nali, C., Vernieri, P., 2013. Signaling molecules and cell death in *Melissa officinalis* plants exposed to ozone. Plant Cell Rep. 32, 1965–1980. https://doi.org/10.1007/s00299-013-1508-0.

Pellegrini, E., Campanella, A., Cotrozzi, L., Tonelli, M., Nali, C., Lorenzini, G., 2018a. What about the detoxification mechanisms underlying ozone sensitivity in *Liriodendron tulipifera*? Environ. Sci. Pollut. Res. 25, 8148–8160. https://doi.org/10.1007/s11356-017-8818-7.

Pellegrini, E., Campanella, A., Cotrozzi, L., Tonelli, M., Nali, C., Lorenzini, G., 2018b. Ozone primes changes in phytochemical parameters in the medicinal herb *Hypericum perforatum* (St. John’s wort). Ind. Crop. Prod. 126, 119–128. https://doi.org/10.1016/j.indcrop.2018.10.002.

Pellegrini, E., Hoshika, Y., Dusart, N., Cotrozzi, L., Gérard, J., Nali, C., Vaultier, M.-N., Jolivet, Y., Lorenzini, G., Paoletti, E., 2019. Antioxidative responses of three oak species under ozone and water stress conditions. Sci. Tot. Environ. 647, 390–399. https://doi.org/10.1016/j.scitotenv.2018.07.413.

Petersen, M., Alfermann, A.W., 1988. Two new enzymes of rosmarinic acid biosynthesis from cell cultures of *Coleus blumei*: hydrophenylpyruvate reductase and rosmarinic acid synthase. Zeitschrift für Naturforschung 43c, 501–504. https://doi.org/10.1515/znc-1988-7-804.

Petersen, M., Abdullah, Y., Benner, J., Eberle, D., Gehlen, K., Hücherig, S., Janiak, V., Kim, K.H., Sander, M., Weitzel, C., Wolters, S., 2009. Evolution of rosmarinic acid biosynthesis. Phytochemistry 70, 1663–1679. https://doi.org/10.1016/j.phytochem.2009.05.010.

Potters, G., Horemans, N., Jansen, M.A.L., 2010. The cellular redox state in plant stress biology - a charging concept. Plant Physiol. Biochem. 48, 292–300. https://doi.org/10.1016/j.plaphy.2009.12.007.

Queval, G., Noctor, G., 2007. A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: application to redox profiling during *Arabidopsis* rosette development. Anal. Biochem. 363, 58–69. https://doi.org/10.1016/j.ab.2007.01.005.

Rao, M.V., Hale, B.A., Ormrod, D.P., 1995. Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. Plant Physiol. 109, 421–432. https://doi.org/10.1104/pp.109.2.421.

Reich, P.B., 1987. Quantifying plant response to ozone: a unifying theory. Tree Physiol. 3, 63–91.

Robinson, M.F., Heat, J., Mansfield, T.A., 1998. Disturbance in stomatal behavior caused by air pollutants. J. Exp. Bot. 49, 461–469. https://doi.org/10.1093/treephys/3.1.63.

Shang, B., Feng, Z.Z., Gao, F., Calatayud, V., 2020. The ozone sensitivity of five poplar clones is not related to stomatal conductance, constitutive antioxidant levels and morphology of leaves. Sci. Tot. Environ. 699, 134402. https://doi.org/10.1016/j.scitotenv.2019.134402.

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanisms in plants under stressful conditions. J. Bot., 217037. https://doi.org/10.1155/2012/217037.

Shin, R., Berg, R.H., Schachtman, D.P., 2005. Reactive oxygen species and root hairs in *Arabidopsis* root response to nitrogen, phosphorus and potassium deficiency. Plant Cell Physiol. 46, 1350–1357. https://doi.org/10.1093/pcp/pci145.

Soares, C., Carvalho, M.E.A., Azevedo, R.A., Fidalgo, F., 2018. Plants facing oxidative challenges - a little help from the antioxidant networks. Environ. Exp. Bot. 161, 4–25. https://doi.org/10.1016/j.envexpbot.2018.12.009.

Štajner, D., Milošević, M., Popović, B.M., 2007. Irradiation effects on phenolic content, lipid and protein oxidation and scavenger ability of soybean seeds. Int. J. Mol. Sci. 8, 618–627.

The Royal Society, 2008. Ground-level Ozone in the 21st Century: Future Trends, Impacts and Policy Implications. Science Policy Report 15/08. The Royal Society, London.

Tonelli, M., Pellegrini, E., D’Angiolillo, F., Petersen, M., Nali, C., Pistelli, L., Lorenzini, G., 2015. Ozone-elicited secondary metabolites in shoot cultures of *Melissa officinalis* L. Plant Cell Tissue Organ Cult. 120, 617–629. https://doi.org/10.1007/s11240-014-0628-8.

Treutter, D., 2005. Significance of flavonoids in plant resistance and enhancement of their biosynthesis. Plant Biol. 7, 581–591. https://doi.org/10.1055/s-2005-873009.

Tuomainen, J., Pellinen, R., Roy, S., Kiiskinen, M., Eloranta, T., Karjalainen, R., Kangasjärvi, J., 1996. Ozone affects birch (*Betula pendula* Roth) phenylpropanoid, polyamine and active oxygen detoxifying pathways at biochemical and gene expression level. J. Plant Physiol. 148, 179–188. https://doi.org/10.1016/S0176-1617(96)80312-9.

Wang, S., Jiao, H.J., Faust, M., 1991. Changes in ascorbate, glutathione and related enzyme activities during thidiazuron-induced bud break apple. Physiol. Plant. 82, 231–236. https://doi.org/10.1111/j.1399-3054.1991.tb00086.x.

Yang, N., Cotrozzi, L., Zhang, Y., Wang, X., Ouyang, Z., Zheng, F., 2018. First defense mechanisms of ornamental species suitable for urban green spaces in China exposed to ozone. Urban For. Urban Green. 36, 68–75. https://doi.org/10.1016/j.ufug.2018.10.007.

Zhang, L., Becker, D.F., 2015. Connecting proline metabolism and signaling pathways in plant senescence. Front. Plant Sci. 6, 552. https://doi.org/10.3389/fpls.2015.00552.

Zhang, J., Kirkham, M.B., 1994. Drought-stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. Plant Cell Physiol. 35, 783–791. https://doi.org/10.1093/oxfordjournals.pcp.a078658.

Zhang, W., Feng, Z., Wang, Z., Niu, J., 2012. Responses of native broadleaved woody species to elevated ozone in subtropical China. Environ. Pollut. 163, 149–157. https://doi.org/10.1016/j.envpol.2011.12.035.

Zhang, L., Hoshika, Y., Carrari, E., Cotrozzi, L., Pellegrini, E., Paoletti, E., 2018. Effects of nitrogen and phosphorus imbalance on photosynthetic traits of poplar Oxford clone under ozone pollution. J. Plant Res. 131, 915–924. https://doi.org/10.1007/s10665-018-1071-4.