Science of the Total Environment 822 (2022) 153577

Contents lists available at ScienceDirect

Science of the Total Environment

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

Season-long exposure of bilberry plants to realistic and future ozone pollution improves the nutraceutical quality of fruits

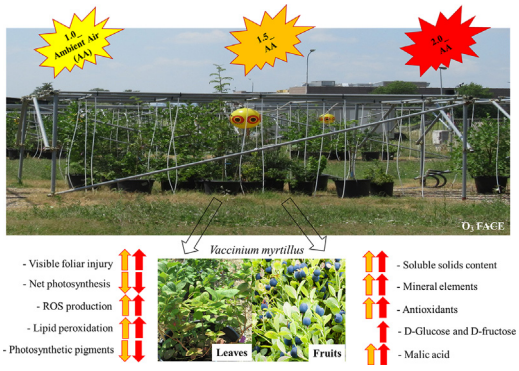
Yasutomo Hoshika [a], Lorenzo Cotrozzi [b,c,d,*], Alessandra Marchica [b], Elisa Carrari [e], Giacomo Lorenzini [b,c,d], Cristina Nali [b,c,d], Elena Paoletti [a], Elisa Pellegrini [b,c,d]

[a] Institute of Research on Terrestrial Ecosystems, IRET-CNR, Via Madonna del Piano 10, 50019, Sesto Fiorentino, Firenze, Italy
[b] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy
[c] CIRSEC, Centre for Climate Change Impact, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy
[d] Nutrafood Research Center, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy
[e] Department of Agriculture, Food, Environment and Forestry, University of Florence, Piazzale delle Cascine 18, 50144, Florence, Italy

HIGHLIGHTS

- Bilberry plants were exposed to increasing ozone using a novel O_3 FACE system.
- Ozone induced oxidative pressure by promoting a shift in carbon partitioning among organs.
- Ozone exposure triggered enzymatic oxidation of phenols to red/purple pigments.
- Ozone exposure resulted in an accumulation of antioxidant compounds in bilberry fruits.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 December 2021
Received in revised form 24 January 2022
Accepted 27 January 2022
Available online 31 January 2022

Editor: Jay Gan

Keywords:
Antioxidants
Carbohydrates
Nutritional value
O_3-FACE
Oxidative stress

ABSTRACT

Ozone (O_3) is a phytotoxic air pollutant capable of limiting plant yield and growth, and altering the quality of edible plant products. This study aimed to investigate the effects of long-term O_3 exposure at realistic and future concentrations (applied during fruit development) not only on morphological, physiological, and biochemical plant/leaf traits of *Vaccinium myrtillus* but also on its fruit yield and quality. Three-year-old saplings were grown from May to July under three levels of O_3 concentration [1.0, 1.5 and 2.0 times the ambient air concentrations, denoted as AA, 1.5_AA and 2.0_AA], using a new-generation O_3 Free Air Controlled Exposure system. Ozone induced oxidative pressure and membrane denaturation as confirmed by the accumulation of anion superoxide, hydrogen peroxide ($^{\bullet}O_2^-$: +39 and + 29%; H_2O_2: +55 and + 59% in 1.5_AA and 2.0_AA, respectively, compared with AA), and malondialdehyde by-product (1.4- and 2.5-fold higher than AA, in 1.5_AA and 2.0_AA, respectively). The observed oxidative burst likely affected several cellular structures interested by photosynthetic processes (e.g., decrease of the maximum rate of carboxylation: −30%). This constraint likely induced a decline in plant vitality and a different partitioning of biomass allocation between above and below organs. An accelerated maturation of bilberries due to O_3 was reported, suggesting that plants grown under harsher environmental conditions suffered from metabolic changes associated with early ripening. Increasing O_3 concentrations might be responsible for an alteration of the ratio between oxidation and reduction processes mechanisms that was followed by a loss of integrity of membranes, so limiting the availability of energy/

* Corresponding author at: Institute of Research on Terrestrial Ecosystems, IRET-CNR, Via Madonna del Piano 10, 50019, Sesto Fiorentino, Firenze, Italy.
E-mail address: lorenzo.cotrozzi@unipi.it (L. Cotrozzi).

http://dx.doi.org/10.1016/j.scitotenv.2022.153577

resources, triggering enzymatic oxidation of phenols to red/purple pigments, and promoting fruit maturation. To the best of our knowledge, this is the first research showing that long-term O_3 exposure during bilberry fruit development influenced not only several plant/leaf traits, but also fruit nutraceutical quality at the time of harvest.

1. Introduction

Ozone (O_3) is no doubt the major phytotoxic air pollutant, challenging wild, agricultural and horticultural plants, as well as inducing considerable quantitative and qualitative impairments to edible plant parts (Wilkinson et al., 2012; Li et al., 2017; Grulke and Heath, 2020). These adverse effects are the result of functional and genetic alterations that finally cause (i) decrease in carbon assimilation, (ii) variation in carbohydrate allocation pattern, (iii) shifts in assimilate distribution, and (iv) reduction of non-photosynthetic organ growth (Puckette et al., 2008; Booker et al., 2009; Gillespie et al., 2015). Ozone-enriched experimental investigations has highlighted species-specific alterations in plant functioning due to different O_3 sensitivity (Mills et al., 2018; Landi et al., 2019; Marchica et al., 2019; Pellegrini et al., 2016, 2019; Hoshika et al., 2020). These divergences mainly concerned senescence mechanisms, photosynthetic processes, and carbohydrate partitioning within the plant (Keutgen and Pawelzik, 2008; Pellegrini et al., 2015; Calzone et al., 2019). Although few investigations have been focused on the effects of O_3 on plant reproductive features such as flowering time and fruit ripening, it has been indicated that O_3 also affect yield and quality of fruits (Leisner and Ainsworth, 2012; Thwe et al., 2014; Gillespie et al., 2015). It is known that an impairment of any steps during reproductive processes may result in reproductive failure, and thus plant yield (Duque et al., 2021). In particular, the timing of O_3 exposure within the plant-life cycle is a key factor determining the net repercussion of O_3 on plant growth and yield, with several species showing higher sensitivity when this detrimental phenomenon happens at the time of or after flowering/anthesis (Cooley and Manning, 1987; Lyons and Barnes, 2008). The demand and supply for assimilates is driven by physiological processes in shoots and roots (e.g., leaf photosynthesis or the development of leaves, roots, stems and fruits), so alterations to these processes due to environmental constraints usually influence the assimilate allocation within a plant (Shipley and Merziane, 2002). As a consequence, changes in growth and/or functioning of some parts may alter further development and growth of the whole plant. The capability to quickly trigger the proper biochemical responses against stressors is strictly linked to carbon metabolism. Thus, the amount of carbon reserves, especially non-structural carbohydrates and starch (Thalmann and Santelia, 2017), and their dynamics within the vegetative and reproductive organs play a pivotal role in acclimation and stress response (Niinemets, 2010).

Ozone is usually approached as a crucial environmental stressor, but some investigations have also highlighted its potential role as 'eustressor' (sensu Marchica et al., 2021). Short-term exposures of plants to adequate concentrations of O_3, under lab conditions, have been proposed as a tool to increase nutraceutical quality, since they commonly trigger antioxidants levels without compromising plant performance (Sachadyn-Król and Agriopoulou, 2020; Marchica et al., 2021). Currently, there is an interest in post-harvest applications of O_3 for decay control and as a potential sanitizer against human pathogens (Conte et al., 2020). Early in 1982 it was "Generally Recognized as Safe" for bottled water disinfection by the U.S. Food and Drug Administration, and in 1999 its application was extended to food treatment, storage, and processing (FDA (Food and Drug Administration), n.d., https://www.accessdata.fda.gov/scripts/cder/daf/). However, despite O_3 can affect fruit features such as sizing, production, biochemistry (e.g., non-structural carbohydrates, ascorbate, and phenol contents) and phenology (e.g., ripening time; Gillespie et al., 2015), little attention has been devoted so far to the direct and indirect impact of O_3 on fruit yield and quality, and the resulting consequences on nutritional value.

Vaccinium myrtillus L. (a member of the Ericaceae plant family) is a spontaneous species native to mountain areas of Northern and Central Europe, largely present in Italian Alps and Apennines. Its fruits ('bilberry') are largely consumed, mainly as high-quality processed products, as well as of ingredients in dietary supplements. For this reason, *V. myrtillus* is now becoming of horticultural interest (Selås et al., 2015) and it is very appreciated for nutraceutical and healthiness values (Jimenez-Garcia et al., 2012). Ripening of the non-climacteric bilberry fruits (Giovannoni, 2001) is associated with high levels of anthocyanins both in peel and flesh, so characterizing peculiar deep blue colour to the ripe fruits. Several studies documented a large annual variation of berry production by indicating the periods of floral initiation and development as particular critical phases because of the pivotal role of the environment as a controlling and synchronizing factor (Heide, 1997). In Central Europe, this species has a vegetative stage in May and then flowering and fruiting phases from June to July, coinciding with high concentrations of O_3 that have the potential to severely affect plant phenology and yields. In a previous study focused on the macroscopic and physiological responses of saplings of *V. myrtillus* to realistic and future O_3 concentrations, we reported a dramatic increase of plant injury index values from June to July, confirming the high susceptibility of this species to O_3 in these months (Hoshika et al., 2020). Despite this, reports of the response of bilberry fruits to long-term O_3 exposure are lacking. Since environmental conditions and source-sink balance within the plant during fruit development determine fruit quality (Thwe et al., 2013), the effects of both realistic and future O_3 concentrations on this fruit feature should be elucidated.

The main objectives of this study were to elucidate the effects of long-term O_3 exposure at realistic and future concentrations (applied during fruit development) not only on morphological, physiological, and biochemical plant/leaf traits of *V. myrtillus* but also on fruit yield and quality, with emphasis on nutraceutical value. In particular, we postulated direct and dose-dependent effects of O_3 on reproductive processes (e.g., shifts in floral initiation and development), resulting in negative effects on vegetative organs (e.g., shifts in resource allocation). To the best of our knowledge, this is the first research highlighting the direct and indirect impact of increasing O_3 concentrations on fruit yield and quality, and the resulting consequences on fruit nutritional value.

2. Materials and methods

2.1. Plant material and experimental design

Experimental activities were performed at the O_3-FACE facility of Sesto Fiorentino, Florence, Italy (43°48′59" N, 11°12′01′ E, 55 m a.s.l.; Paoletti et al., 2017). In December 2017, three-year-old saplings of *V. myrtillus* obtained from a local nursery were transplanted into 25-L plastic pots filled with a sand:peat:soil mixture (1:1:1 in vol.) and kept under standard conditions until the beginning of the O_3 exposure. From May 1, 2018 to July 31, 2018, uniform plants were exposed to three levels of O_3 concentration: 1.0 (current O_3 scenario), 1.5 and 2.0 times (future O_3 scenario, Young et al., 2013) the ambient air concentrations (AA, 1.5_AA and 2.0_AA, respectively). Throughout the whole experiment, the hourly mean O_3 concentrations (mean ± S.E.) were 38.4 ± 0.5, 57.6 ± 0.7 and 70.6 ± 0.9 ppb in AA, 1.5_AA and 2.0_AA, respectively; while the Accumulated exposure Over a Threshold of 40 ppb (AOT40, sensu Kärenlampi and Skärby, 1996, Pleijel et al., 2021) values were 13.9, 35.3 and 52.7 ppm h. Three plots (5 × 5 × 2 m) were designated to each O_3 treatment (n = 3) with six plants in each plot (in total 54 plants). All plants were irrigated with water (pH 7.6 and electrical conductivity 5.5 mS cm^{-1}) to maintain field capacity (soil water content = 0.295 m^3 m^{-3}, Paoletti et al., 2017).

2.2. Visible foliar injury

The O_3-like visible foliar injury was identified at the end of exposure using photoguides (Innes et al., 2001; Paoletti et al., 2009). Percentages

of symptomatic leaves per plant and of the injured area in the symptomatic leaves were assessed by two well-experienced surveyors using a 10× magnifying hand lens.

2.3. Plant biometric traits

Six plants per each O_3 level group were randomly selected at the end of the experiment and devoted to the biomass assessments in terms of leaves, stems and roots. After harvesting, organs were properly separated and kept in an oven at 80 °C until constant weights were reached.

2.4. Leaf physiological traits

Photosynthetic parameters of leaves were determined using a portable gas exchange system (CIRAS-2, PP-systems, Hitchin, Hertfordshire, UK) from July 16 to 20. The dependence of net photosynthetic rate (A) to intercellular CO_2 level (C_i), i.e. the A/C_i curve, was measured at 25 °C of leaf temperature, 1500 μmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) and 40 to 50% of relative humidity (RH) with nine CO_2 steps (C_a: 400, 200, 100, 50, 400, 800, 1200, 1600, and 2000 μmol mol^{-1}). Fully expanded and sun-exposed leaves were targeted (one leaf with 5th order from the tip of the plant per one to two plants per each plot per each O_3 treatment). Light-saturated net photosynthetic rate (A_{sat}) was determined at 400 μmol mol^{-1} of CO_2, together with the stomatal conductance for water vapour (G_s). According to the methodology suggested by Farquhar et al. (1980) and Long and Bernacchi (2003), the maximum rate of carboxylation (V_{cmax}), the maximum rate of electron transport (J_{max}) and their ratio (J_{max} /V_{cmax}) were calculated from the A/C_i curve. For this analysis, parameters of the RubisCO Michaelis constants for CO_2 (K_c) and O_2 (K_o), and the CO_2 compensation point in the absence of day respiration (Γ*) were calculated (Bernacchi et al., 2001). Gas exchange measurements were performed between 9:00 and 12:00 a.m. on clear sky days.

2.5. Leaf biochemical traits

At the end of the experiment, fully expanded and sun-exposed leaves (from one to three plants per plot per each O_3 treatment) were harvested from 11:00 a.m. to 1:00 p.m., frozen in liquid nitrogen and kept at −80 °C until biochemical analyses. Anion superoxide ($\bullet O_2^-$) content was measured with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, PerkinElmer, Waltham, MA, USA; Able et al., 1998; Döring et al., 2020). Hydrogen peroxide (H_2O_2) content was measured using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies Corp. Carlsbad, CA, USA; Shin et al., 2005). Oxidative damage was determined in terms of malondialdehyde (MDA) by-product accumulation measured with a spectrophotometer (UV-1900 UV–vis, Shimadzu, Kyoto, Japan; Hodges et al., 1999; Guidi et al., 2017). Leaf pigments [neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, chlorophyll *b* (chl *b*), chlorophyll *a* (chl *a*), and β-carotene; in order of elution time] were determined by ultra-high performance liquid chromatography using a Dionex UltiMate 3000 system equipped with an Acclaim 120 Dionex column and a Dionex UVD 170 U detector (Thermo Scientific, Waltham, MA, USA; Zhang et al., 2018). Total chlorophyll content was calculated as the sum of chl *a* and chl *b*, and then the chl *a* /chl *b* ratio was calculated. Further details about leaf biochemical measurements are reported in the supplementary material.

2.6. Fruit physicochemical and biochemical traits

At the end of the field tests, all fruits produced by each plant were evaluated for their colour based on the Royal Horticultural Society Colour Chart (Royal Horticultural Society, 2007; green: 114D, partly red: 57C, red/blue: 98C). All fruits were harvested at ripening red/blue stage and then sampled for the analysis. Immediately after harvest, fruit characteristics were assessed, and 100 g of fruits were frozen in liquid nitrogen and kept at −80 °C until further analysis. Key fruit quality characteristics were assessed: fresh/dry weights, dry matter content, soluble solids content (SSC, measured by a digital refractometer, Refracto 30PX, Mettler-Toledo, Milan, Italy) and pH of extracts (using a pH-meter; Edge pH/ORP, Hanna Instruments, Padova, Italy). Mineral elements (i.e., K^+, Mg^{2+} and Ca^{2+}) were measured by ion chromatography using a Dionex Aquion system equipped with a CSRS™300, 4 mm Dionex Cation Self-Regenerating Suppressor, a CG12A Dionex IonPac™ pre-column and a CS12A DionexIonPac™ column Dionex (Thermo Scientific, Waltham, MA, USA; Cataldi et al., 2003). Total phenolic, anthocyanin, and ascorbate [ascorbate (AsA) + dehydroascorbate (DHA)] contents were measured with a spectrophotometer (UV-1900 UV–vis, Shimadzu, Kyoto, Japan) according to Waterhouse (2002); Connor et al. (2002), and Kampfenkel et al. (1995), respectively. The antioxidant activity of fruit extracts was estimated by assessing the hydroxyl radical averting capacity (HORAC) and the oxygen radical absorption capacity (ORAC) with the same fluorescence/absorbance microplate reader reported above (Ou et al., 2001, 2002). Soluble sugars (D-glucose, D-fructose and sucrose) were measured using the K-SUFRG commercial kit (Megazyme, Wicklow, Ireland). Organic acids (citric, malic, shikimic and quinic acids) were determined by the same UHPLC reported above equipped with a pre-column Repromer H and a Repromer H column (Eyéghé-Bickong et al., 2012). Further details about fruit biochemical measurements are reported in the supplementary material.

2.7. Statistical analysis

Data were collected from every single plant per each O_3 level condition per each plot in a FACE and plot means were used as statistical unit, i.e., $n = 3$ plots. Normality of collected data (Shapiro-Wilk test) and homogeneity of variance (Levene test) were firstly tested and. Leaf gas exchange data were analysed by a one-way ANOVA to detect the effect of O_3 and then differences among O_3 treatments were evaluated by the Tukey's *post-hoc* test. Plant morphology, leaf biochemical and fruit physicochemical and biochemical data were analysed by a non-parametric Friedman test to determine the effect of O_3. In this case, differences among O_3 treatments were evaluated by the Dunn's *post-hoc* test. Statistical analyses were performed in JMP 11.0 (SAS Institute, Cary, NC, USA) and significant effects were considered for $P \leq 0.05$.

3. Results

3.1. Effects of increasing O_3 concentrations on biometric, gas exchange and biochemical plant/leaf traits

Plants exposed to increasing O_3 concentrations developed visible minute stipples of dark-reddish tissue localized in the interveinal adaxial leaf area at the end of the exposure. The most severe damage occurred under 2.0_AA conditions: 69% of the leaves per plant were affected, and the injured area was on average 35% of their surface per each symptomatic leaf with stippling (*data not shown*).

Root dry weight and root to shoot ratio were slightly decreased by increasing O_3 concentrations, without significant differences between the two higher O_3 concentrations (root dry weight: −19 and −37%; root to shoot ratio: −24 and −33% under 1.5_AA and 2.0_AA, respectively, compared with AA; Fig. 1A and C). No significant O_3 effects were reported on total dry weight (Fig. 1B) and total fruit yield (*data not shown*).

Increasing O_3 concentrations significantly decreased A_{sat} (−26 and −30% in 1.5_AA and 2.0_AA, respectively, compared with AA), although no significant difference in G_s was observed among treatments (Table 1). Leaves exposed to 2.0_AA showed significantly lower V_{cmax} values compared to those under AA. In addition, a significant increase in the J_{max}/V_{cmax} ratio was found under both 1.5_AA and 2.0_AA compared to AA, with no significant changes reported for J_{max}.

A significant stimulation of $\bullet O_2^-$ and H_2O_2 production occurred due to increasing O_3, without significant differences between the two high O_3 concentrations ($\bullet O_2^-$: +39 and +29%; H_2O_2: +55 and +59% in 1.5_AA and

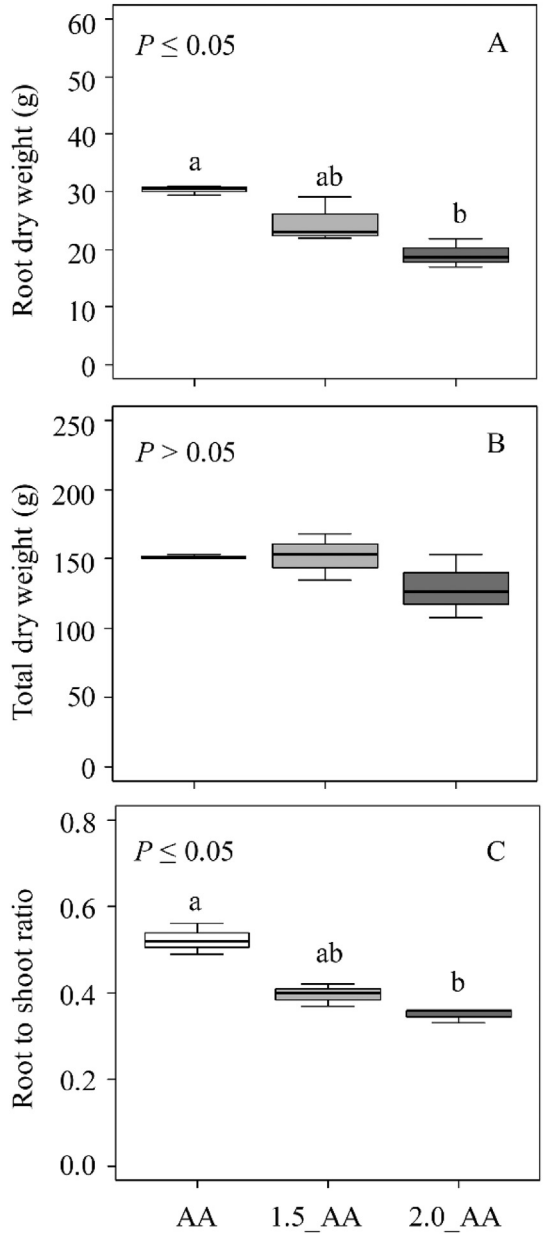


Fig. 1. Root dry weight (A), total dry weight (B) and root to shoot ratio (C) in *Vaccinium myrtillus* plants exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each parameter, the effect of O_3 was tested by the Friedman test. Different letters indicate significant differences among O_3 treatments, according to the Dunn's *post-hoc* test ($P \leq 0.05$, $n = 3$ plots).

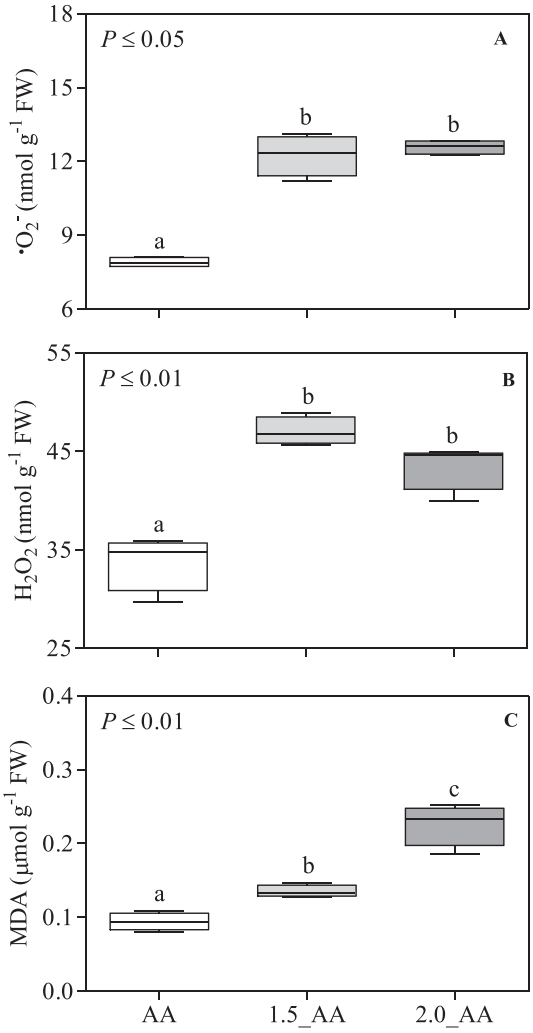


Fig. 2. Anion superoxide ($^{\bullet}O_2^-$; A), hydrogen peroxide (H_2O_2; B) and malondialdehyde by-product (MDA; C) content in *Vaccinium myrtillus* leaves exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). Data are shown are on a fresh weight (FW) basis. For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each parameter, the effect of O_3 was tested by the Friedman test. Different letters indicate significant differences among O_3 treatments, according to the Dunn's *post-hoc* test ($P \leq 0.05$, n = 3 plots).

2.0_AA, respectively, compared with AA; Figs. 2A and B). Accordingly, MDA by-products markedly increased due to increasing O_3 concentrations, but with significant differences between 1.5_AA and 2.0_AA: 1.4- and 2.5-fold higher than AA, respectively (Fig. 2C). Total chlorophylls markedly decreased due to increasing O_3 concentrations, without significant differences

Table 1
Leaf gas exchange traits of *Vaccinium myrtillus* plants exposed to three levels of O_3 concentration (AA, ambient O_3 concentration, 1.5_AA and 2.0_AA) for 3 months (May–July). For each parameter, the effect of O_3 was tested by a one-way ANOVA. Different letters indicate significant differences among O_3 treatments ($P < 0.05$, by Tukey's post-hoc test, n = 3). Each value is the mean (±SE). A_{sat}, light-saturated net photosynthetic rate; G_s, stomatal conductance to water vapour; V_{cmax}, maximum rate of carboxylation; J_{max}, maximum rate of electron transport; J_{max}/V_{cmax}, the ratio of J_{max} to V_{cmax}.

	AA			1.5_AA			2.0_AA			*P* value for ANOVA
A_{sat} (μmol m^{-2} s^{-1})	7.3	(0.4)	b	5.4	(0.3)	a	5.1	(0.3)	a	**0.011**
G_s (mol m^{-2} s^{-1})	0.109	(0.004)	a	0.079	(0.003)	a	0.090	(0.010)	a	0.072
V_{cmax} (μmol m^{-2} s^{-1})	36.7	(2.7)	b	28.8	(1.5)	ab	25.7	(1.5)	a	**0.018**
J_{max} (μmol m^{-2} s^{-1})	91.0	(4.9)	a	85.3	(2.3)	a	79.5	(4.8)	a	0.231
J_{max}/V_{cmax}	2.5	(0.1)	a	3.0	(0.2)	b	3.1	(0.0)	b	**0.011**

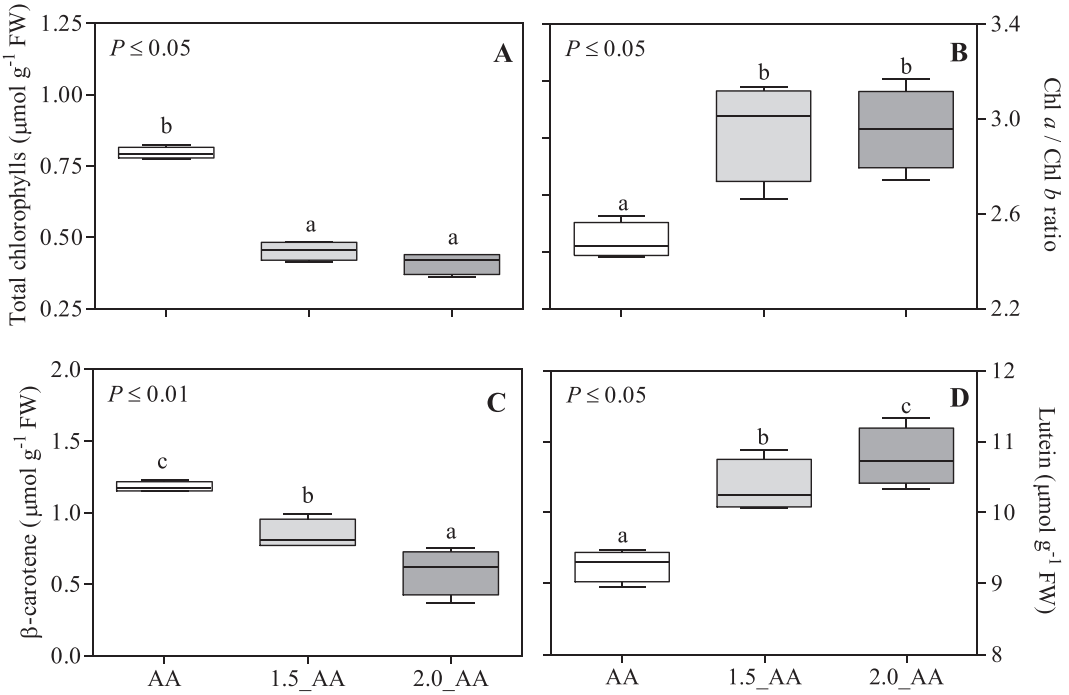


Fig. 3. Total chlorophylls (A), chlorophyll *a* /chlorophyll *b* (Chl *a* / Chl *b*) ratio (B), β-carotene (C) and lutein (D) contents in *Vaccinium myrtillus* leaves exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). Data are shown are on a fresh weight (FW) basis. For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each parameter, the effect of O_3 was tested by the Friedman test. Different letters indicate significant differences among O_3 treatments, according to the Dunn's *post-hoc* test ($P \leq 0.05$, $n = 3$ plots).

between the two higher O_3 concentrations (−43 and −48% in 1.5_AA and 2.0_AA, respectively, compared with AA; Fig. 3A). Conversely, Chl *a* to Chl *b* ratio slightly increased by 16% under both 1.5_AA and 2.0_AA, compared with AA (Fig. 3B). β-carotene content was reduced by 1.5_AA (−28% compared with AA), and even more by 2.0_AA (−50%; Fig. 3C). Conversely, lutein content increased by 1.5_AA (+12% compared with AA) and even more by 2.0_AA (+16%; Fig. 3D).

3.2. Effects of increasing O_3 concentrations on fruit physico-chemical and quality parameters

At the end of the exposure, most of bilberry fruits collected from plants growing under increasing O_3 concentrations turned red/blue. This phenomenon strongly occurred under 2.0_AA conditions: 44% of the fruits were red/blue at the time of harvest, while 28% was still green in colour (Fig. 4). Conversely, most of berries attached to plants grown under AA conditions, were still green and partly red (54 and 36%, respectively).

No significant O_3 effects was reported on fruit fresh weight (Fig. 5A), while fruit dry matter was slightly decreased by increasing O_3 concentrations, without significant differences between the two higher O_3 concentrations (−23 and −32% in 1.5_AA and 2.0_AA, respectively, compared with AA; Fig. 5B). Differently, SSC values similarly increased because of 1.5_AA and 2.0_AA (+11 and +15% respectively, compared with AA; Fig. 5C). No significant O_3 effects were found on pH values of fruit extracts (Fig. 5D).

Although lower K^+ levels were reported in 2.0_AA fruits than in 1.5_AA ones, no significant differences were found as a consequence of increasing O_3 concentrations compared with AA (Fig. 6A). Fruit Mg^{2+} content slightly increased only under 2.0_AA (+6% compared with AA; Fig. 6B), while

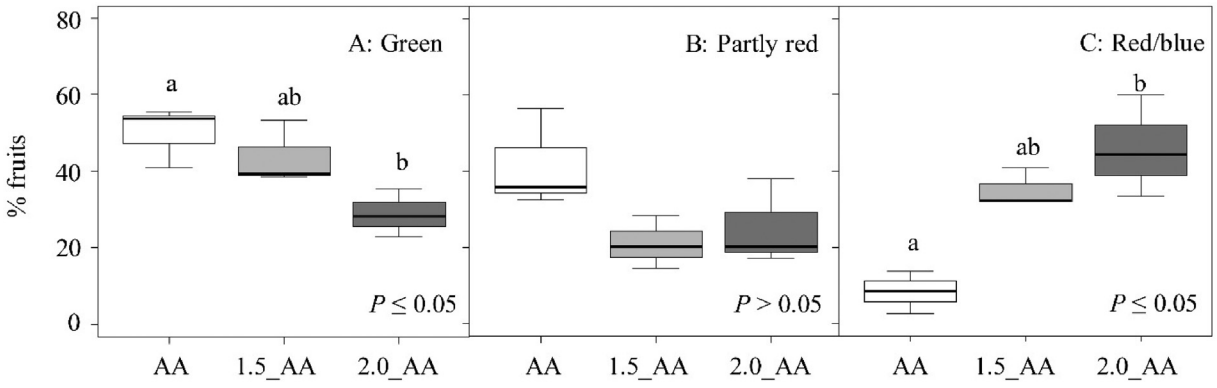


Fig. 4. Percentage of fruits at various stages [green (A), partly red (B) and red/blue (C)] in *Vaccinium myrtillus* leaves exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). Data are shown are on a fresh weight (FW) basis. For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each fruit stage, the effect of O_3 was tested by the Friedman test. For each fruit stage, the effect of O_3 was tested by a Friedman test. Different letters indicate significant differences among O_3 treatments in each fruit stage, according to the Dunn's *post-hoc* test ($P \leq 0.05$, $n = 3$ plots).

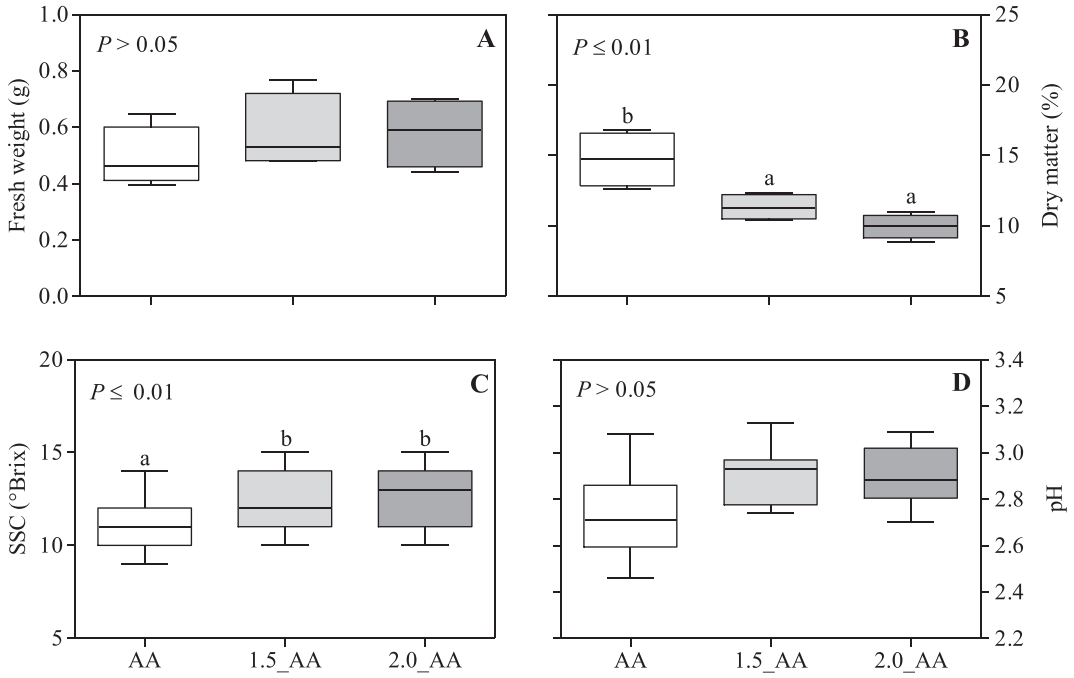


Fig. 5. Fresh weight (A), dry matter (B), soluble solids content (SSC, C) and pH (D) values in ripening fruits of *Vaccinium myrtillus* plants exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each parameter, the effect of O_3 was tested by the Friedman test. Different letters indicate significant differences among O_3 treatments, according to the Dunn's *post-hoc* test ($P \leq 0.05$, $n = 3$ plots).

Ca^{2+} content slightly and similarly increased under both 1.5_AA and 2.0_AA (+5 and +7%, respectively, compared with AA; Fig. 6C).

A significant accumulation of total phenolics and total anthocyanins was found as a consequence of increasing O_3 concentrations, with significant differences between the two higher O_3 concentrations (1.7- and 2.5-fold higher than AA respectively; Fig. 7A-B). Similarly, total ascorbate content strongly increased in 1.5_AA fruits and even more in 2.0_AA ones (1.9- and 2.6-fold higher than AA; Fig. 7C).

A significant increase of HORAC and ORAC values was found as a consequence of increasing O_3 concentrations, with significant differences between the two higher O_3 concentrations: HORAC values increased more under 2.0_AA than under 1.5_AA (1.6- and 3.1-fold higher than AA; Fig. 8A); while ORAC values increased more under 1.5_AA than under 2.0_AA (+36 and +14%, respectively, compared with AA; Fig. 8B).

A slight increase of D-glucose and D-fructose contents was found in fruits grown under 2.0_AA (+29 and +28%, respectively, compared with AA; Fig. 9A-B). Although lower fruit sucrose content was reported under 1.5_AA than under 2.0_AA, no significant differences were found as a consequence of increasing O_3 concentrations compared with AA (Fig. 9C).

A slight increase of citric acid content was only found under 1.5_AA (+23%, compared with AA; Fig. 10A), while malic acid levels significantly increased under both higher O_3 concentrations (about 2-fold higher than AA; Fig. 10B). The content of shikimic acid markedly increased because of 1.5_AA (+34, compared with AA; Fig. 10C), while strongly decreased under 2.0_AA (−39%; Fig. 10C). Although lower quinic acid content was reported under 2.0_AA than under 1.5_AA, no significant differences were found as a consequence of increasing O_3 concentrations compared with AA (Fig. 10D).

4. Discussion

4.1. The impact of increasing O_3 concentrations on biometric, physiological, and biochemical plant/leaf traits

Adverse effects of realistic and future O_3 scenarios on *V. myrtillus* have been highlighted by the present study. It is known that O_3 mainly enters inside the leaves through the stomata and then leads to the production of ROS after reacting with the materials diffused in the apoplast (Schauberger et al., 2019). Here, the accumulation of $^{\bullet}O_2^-$ and H_2O_2 caused by the increased O_3 concentrations was associated with membrane denaturation, confirming the oxidative pressure caused by O_3. Specifically, MDA by-product values followed a similar trend to $^{\bullet}O_2^-$ and H_2O_2, but with significant differences between 1.5_AA and 2.0_AA, suggesting that plants grown under harsher environmental conditions suffered more markedly from peroxidative processes (i.e., dose-dependent effects), and changed the ionic and solute reactions of the membrane cells (Calatayud and Barreno, 2001; Hassan et al., 2018). The observed oxidative burst likely affected several cellular components such as lipids, proteins and membranes involved in photosynthetic processes (e.g., electron transport, photochemistry; Huang et al., 2019), as confirmed by the reduction of photosynthetic capacity found in plants grown under 1.5_AA and 2.0_AA conditions. Increased O_3 exposure did not affect G_s but decreased V_{cmax}, suggesting that O_3-induced decreases in A_{sat} of *V. myrtillus* leaves were principally due to the biochemical limitations associated with a reduced efficiency of Rubisco because of a direct enzyme oxidation. Unchanged values of J_{max} confirmed that there were no limitations to pools of Calvin cycle intermediates and to the energy for RuBP regeneration (Goumenaki et al., 2010). In addition, plants exposed to increased O_3 (regardless of concentration) showed a general reduction in chlorophyll content, suggesting a noticeable alteration of the chlorophyll binding proteins of the Light-Harvesting Complexes (Cotrozzi et al.,2017). The significant decrease of total chlorophylls in leaves commonly occurs under oxidative stress induced by O_3, particularly due to early senescence and nutrient remobilization (Mikkelsen et al., 1995; Köllner and Krause, 2000). This outcome was also confirmed by the larger decrease of Chl *a* than Chl *b* (as documented by the significant increase of Chl *a*/Chl *b* due to the higher O_3 levels), indicating that O_3 not only enhanced the breakdown of chlorophylls, but also impaired de novo chlorophyll synthesis (Saitanis et al., 2001; Döring et al., 2014). The concomitant decrease of β-carotene confirms that increasing O_3 concentrations induced photo-oxidation and a rearrangement of the pigment configuration (Calatayud and Barreno, 2004; Cotrozzi et al., 2017). In particular, the strong reduction of β-carotene found in plants grown under 2.0_AA conditions suggests that this compound can act directly as a thylakoid

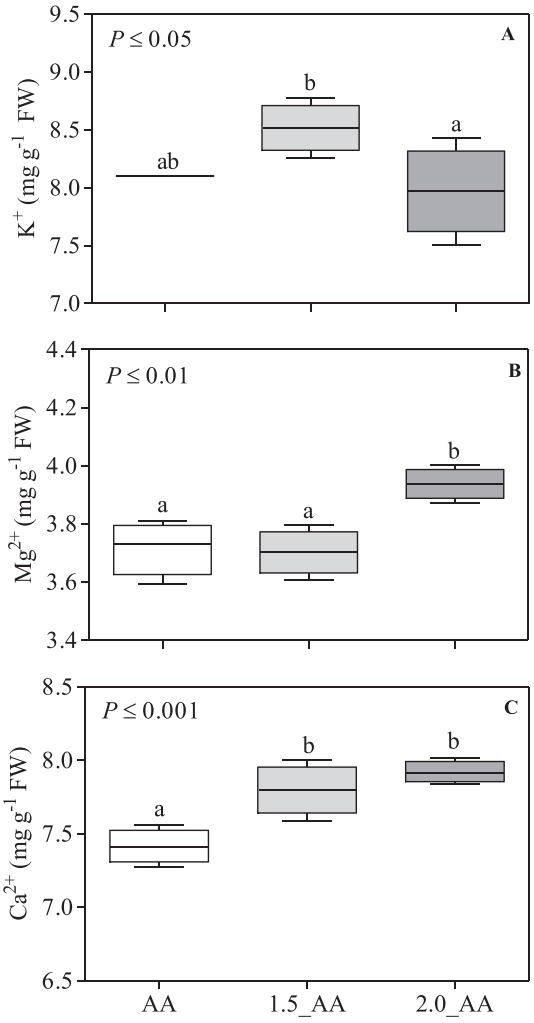


Fig. 6. Potassium (A), magnesium (B) and calcium (C) concentrations in ripening fruits of *Vaccinium myrtillus* plants exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each parameter, the effect of O_3 was tested by the Friedman test. Different letters indicate significant differences among O_3 treatments, according to the Dunn's *post-hoc* test ($P \leq 0.05$, $n = 3$ plots).

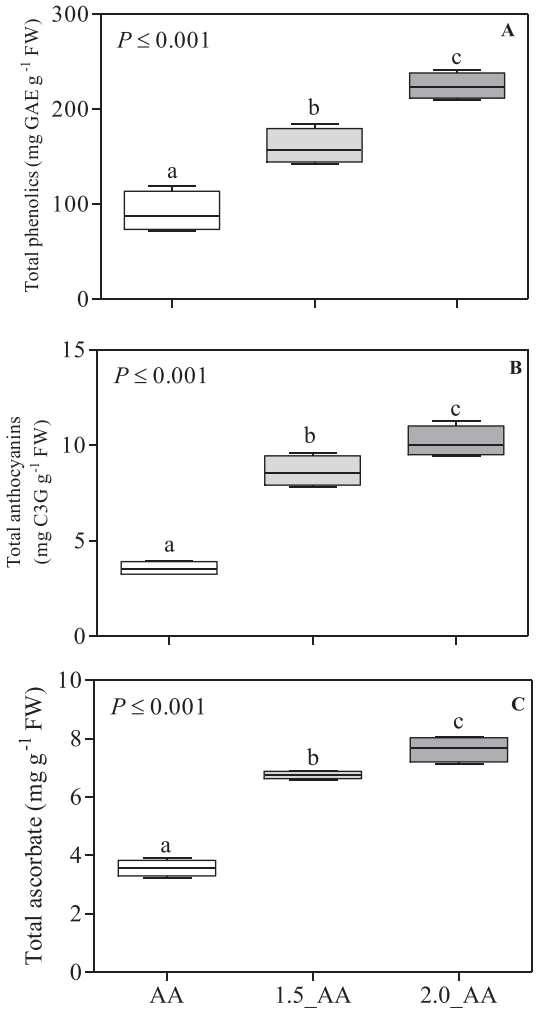


Fig. 7. Total phenolics (A), anthocyanins (B) and ascorbate (C) content in ripening fruits of *Vaccinium myrtillus* plants exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each parameter, the effect of O_3 was tested by the Friedman test. Different letters indicate significant differences among O_3 treatments, according to the Dunn's *post-hoc* test ($P \leq 0.05$, n = 3 plots). Abbreviations: C3G, cyanidin-3-glucoside equivalents; GAE, gallic acid equivalents.

membrane-bound antioxidant against singlet oxygen and/or as a substrate for a de novo biosynthesis of xanthophylls (Havaux et al., 2000). This assumption is confirmed by the significant raise of lutein content which followed the gradient of enhanced O_3 concentrations. A reduced photosynthetic area due to such alteration to leaf pigments may reduce plant vitality. In particular, O_3 can induce a different biomass allocation between above and below organs (as confirmed by the significant reduction in the root/shoot ratio under 2.0_AA conditions; Wittig et al., 2009), even though the duration of O_3 exposure was not long enough to cause significant effects on total biomass (e.g., unchanged values of total dry weight values; Engela et al., 2021). A reduction of root dry weight occurred under elevated O_3, regardless of concentration: this was probably due to a limitation in carbohydrate availability as consequence of leaf injury (Hoshika et al., 2020), alteration of membrane integrity and dysfunction of photosynthetic systems (King et al., 2005; Li et al., 2015; Ghosh et al., 2020). Considered the source-sink competition, the availability of mobile assimilates to a particular sink is defined by the competition among sink tissues (e.g., roots and/or fruits; Ho, 1988) and/or the decreased rate of photosynthesis (Lemoine et al., 2013). When the assimilate supply is limited, the partitioning is driven by the strength of the sink. According to Thwe et al. (2013), carbohydrate distribution is altered by O_3-induced oxidative stress, especially in the presence of reproductive organs. Here, roots gained a limited supply of carbohydrates because of their relative weakness and lesser level of carbon acquisition in competition with developing and ripening fruits, which could be considered the greatest assimilate priority in *V. myrtillus* plants grown under increasing O_3 concentrations. The decreased availability of resources (e.g., water and carbohydrates) and energy likely limited an optimal fruit production and growth, so decreasing the number of well-developed fruits (Thwe et al., 2013; Gillespie et al., 2015). Increasing O_3 concentrations can alter the source-sink relationship within the plant so affecting fruit yield and quality.

4.2. Effects of increasing O_3 concentrations on fruit physico-chemical and quality parameters

In our work, *V. myrtillus* plants exposed to increasing O_3 concentrations were able to activate an adaptation strategy to reduce resource (e.g., carbon and water) spending and divert energy to the fruits, which responded to the O_3-induced oxidative stress by altering development and ripening processes. An accelerated maturation of bilberries occurred under increasing

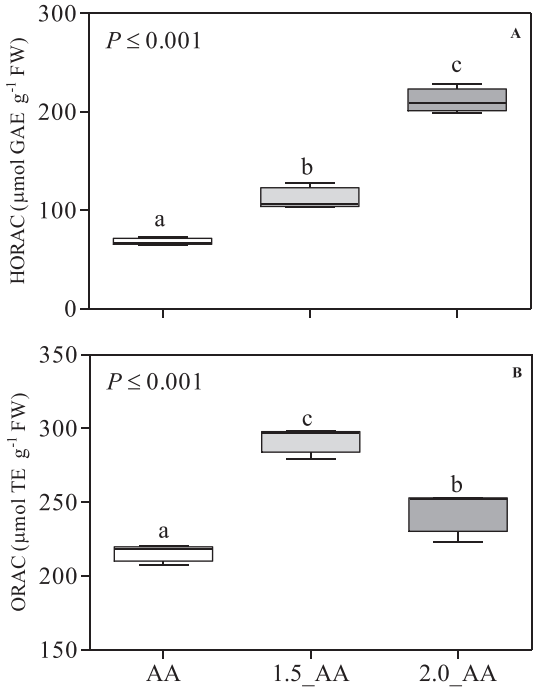


Fig. 8. Antioxidant capacity expressed as hydroxyl radical antioxidant (HORAC, A) and oxygen radical absorbance (ORAC, B) capacity in ripening fruits of *Vaccinium myrtillus* plants exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). Data are shown are on a fresh weight (FW) basis. For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each parameter, the effect of O_3 was tested by the Friedman test. Different letters indicate significant differences among O_3 treatments, according to the Dunn's *post-hoc* test ($P \leq 0.05$, $n = 3$ plots). Abbreviations: GAE, gallic acid equivalents, TE, trolox equivalents.

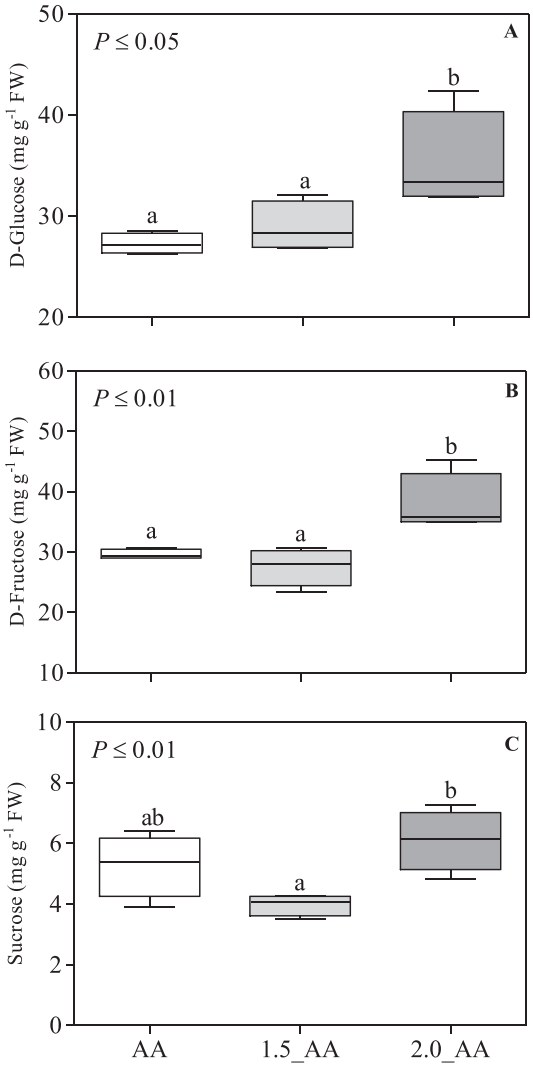


Fig. 9. D-Glucose (A), D-fructose (B) and sucrose (C) content in ripening fruits of *Vaccinium myrtillus* plants exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). Data are shown are on a fresh weight (FW) basis. For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each parameter, the effect of O_3 was tested by the Friedman test. Different letters indicate significant differences among O_3 treatments, according to the Dunn's *post-hoc* test ($P \leq 0.05$, $n = 3$ plots).

O_3, with significant differences between 1.5_AA and 2.0_AA, suggesting that plants grown under harsher environmental conditions markedly suffered from metabolic changes associated with early ripening (Gillespie et al., 2015): dose-dependent effects previously reported on leaf traits were thus observed also on reproductive biology. In fact, increasing O_3 concentrations likely caused an alteration of the oxidative and reductive processes that was followed by membrane damage limiting the availability of energy and resources (as confirmed by the decrease of fruit dry matter), triggering enzymatic oxidation of phenols to red/purple pigments (e.g. accumulation of anthocyanins following the gradient of O_3 concentrations), and promoting maturation of bilberry fruits (as documented by the increase of SSC values; Thwe et al., 2014). Since no O_3 effects were reported on fruit fresh weight and total fruit yield, it seems that increasing O_3 concentrations had less effects on fruit biomass than on fruit quality (Leisner and Ainsworth, 2012). An intricate and coordinated change among metabolites (e.g. soluble carbohydrates, organic acids, and amino acids) likely took place altering organoleptic fruit characteristics and flavour (Osorio et al., 2013). In particular, fruit dry matter originates from the assimilates photosynthesized in the leaves and then transferred to the fruits as carbohydrates which are transformed into organic acids and other compounds. Therefore, the mechanisms regulating the intake in the fruit of water and carbon play a pivotal role in determining not only the fruit size, but also the dry matter concentration of fruit and its overall quality (Batista-Silva et al., 2018). Here, differential responses in chemical composition were observed between red/blue fruits (pH values about 3) harvested from plants exposed to 1.5_AA and 2.0_AA levels. In particular, the organic acids appeared to be more responsive than sugars to carbon limitation due to moderate O_3 concentrations (i.e., 1.5_AA) as confirmed by the accumulation of some intermediates of tricarboxylic acid cycle (TCA; such as citric and malic acids) and the unchanged values of D-glucose, D-fructose and sucrose.

These results suggest that metabolic adjustments on the abundance of different types of carbon compounds occurred under 1.5_AA conditions by providing high amount of energy (probably) required for accelerated ripening (Osorio et al., 2013). . In particular, organic acids served as transient carbon storage molecules (Fahnenstich et al., 2007). Conversely, the soluble sugars appeared to be more responsive to carbon limitation due to higher O_3 levels (i.e., 2.0_AA) than organic acids as confirmed by the increase of fruit's reducing-sugar content (i.e., D-glucose and D-fructose) and the unchanged values of citric and quinic acids. These results suggest that an oxidation of carbohydrates via glycolysis occurred by providing carbon skeleton for the TCA cycle throughout cell respiratory processes, and contributing, not only to the production of intermediates (as confirmed by the rise of malic acid; Batista-Silva et al., 2018), but also to cellular energy supply (Osorio et al., 2013). The fact that there were no significant differences in fruit sucrose content at the end of the experiment suggests that increasing O_3 concentrations differently affected the conversion of sucrose to hexose by activating sucrose biosynthesis or degradation pathways (Dong and Beckels, 2019). Sugars and organic acids are not only crucial sensory

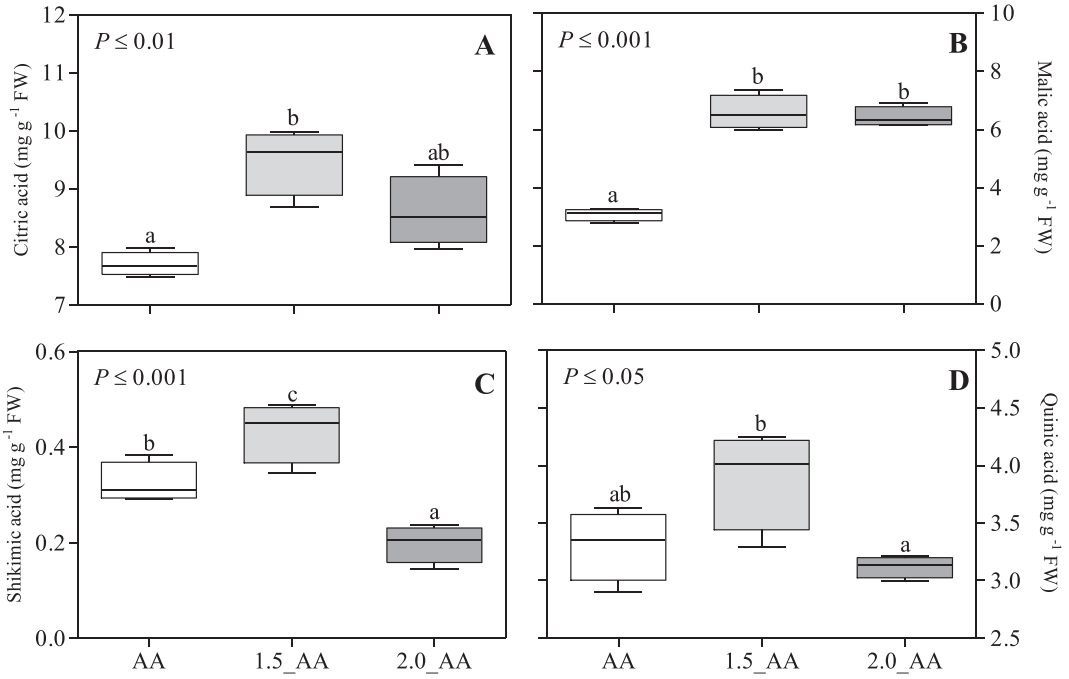


Fig. 10. Citric (A), malic (B), shikimic (C) and quinic acids (D) content in ripening fruits of *Vaccinium myrtillus* plants exposed to three levels of O_3 concentration [ambient air (AA, *white*), 1.5_AA (*grey*) and 2.0_AA (*dark grey*)] for three months (May–July). Data are shown are on a fresh weight (FW) basis. For each boxplot median, 25°-75° percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. For each parameter, the effect of O_3 was tested by the Friedman test. Different letters indicate significant differences among O_3 treatments, according to the Dunn's *post-hoc* test ($P \leq 0.05$, n = 3 plots).

quality parameters in fruits and vegetables (in our study, bilberry fruits from O_3-treated plants may be tasty as documented by the increase of SSC values, regardless of O_3 concentrations), but also the main substrates of primary metabolism. The transformation of these compounds can produce essential precursors by serving as substrates of respiration (as previously reported), and provide efficient energy and small molecular substances for secondary metabolism (e.g., ascorbic and shikimic acids) by contributing to fruit nutritional quality. An accumulation of total phenols, anthocyanins and ascorbate was observed as a consequence of O_3, with significant differences between 1.5_AA and 2.0_AA suggesting that plants grown under harsher environmental conditions produced fruits rich in bioactive compounds and vitamins, which positively contributed to the antioxidant activity of water-soluble fraction against free radicals due to O_3 (as confirmed by the increase of HORAC and ORAC values; Thwe et al., 2015). This response can be also considered an adaptive mechanism to increasing O_3 concentrations that promotes the de novo synthesis of these metabolites with strong antioxidant properties and provides a further fruit resistance to oxidative stress (Bortolin et al., 2016). In particular, the high presence of bioactive compounds in bilberry fruits harvested from plants exposed to 1.5_AA and 2.0_AA levels may have raised sink strength and altered the movement of some nutrient substrates, including minerals, within the plant. These mechanisms could be responsible for the significant increase of Mg^{2+} (only under 2.0_AA conditions) and Ca^{2+} content, which might represent important factors determining fruit nutritional quality. In fact, fruits with high mineral content are less sensitive to many physiological disorders and generally maintain/prolong their shelf-life (Martín-Diana et al., 2007). The absence of significant differences in K^+ content (regardless of O_3 concentrations) at the end of the exposure suggests that increasing O_3 concentrations have a selective effect on macroelements by altering not only the uptake but also the transport of ions to fruits, in relation to their mobility (Ragel et al., 2019). Potassium is generally identified as the major mineral in several fruits (such as bilberry) since it has higher mobility within the xylem than other minerals (e.g., Mg^{2+} and Ca^{2+}), and it is commonly accumulated in the fruit because it is not eliminated during plant transpiration (Moraes et al., 2021).

In conclusion, the present study shows that long-term O_3 exposure during bilberry fruit development induced plant/leaf biometric, physiological, and biochemical disorders, which occurred simultaneously, by altering the fruit nutraceutical quality at the time of harvest. In particular, O_3 induced oxidative pressure and membrane denaturation by affecting several cellular structures involved in photosynthetic reactions. This constraint likely induced (i) changes in C carbon, (ii) different partitioning of biomass allocation between above and below organs, (iii) decline in plant vitality, and (iv) alteration of fruit ripening (and other important visual quality parameters). However, no significant differences in fruit fresh weight were reported suggesting that a compensatory mechanism occurred in the reproductive organs. In addition, the accumulation of antioxidant compounds (anthocyanins, phenols, and ascorbate) and the concomitant increase of soluble carbohydrates (e.g. glucose and fructose) in bilberry fruits harvested from plants exposed to 1.5_AA and 2.0_AA improved their nutritional value and sensory quality. The characterization of vegetative and reproductive organs showed two major outcomes: first, O_3 exposure resulted in a direct (dose-dependent) effect on reproductive biology; second, the long-term exposure induced a shift in resource partitioning because of indirect effects of O_3 via leaf damage. Overall, our study highlights the need to better understand the impact of realistic and future O_3 concentrations on plants also during their reproductive phases, especially focusing on fruit yield, quality and nutraceutical value. Plant treatments with increasing O_3 may lead to tastier (due to higher contents of both sugars and organic acids, which determine the sweetness and sour taste), healthier (because of the enhanced bioactive compounds content), and safer fruits.

CRediT authorship contribution statement

Conceptualization, Y.H., L.C., C.N., E.Pa., E.Pe.; Analysis, Y.H., A.M., E.C.; data curation, Y.H., L.C., E.Pe.; writing—original draft preparation, Y.H., E.Pe.; writing—review and editing, L.C., A.M., E.C., G.L., C.N., E.Pa.; supervision, C.N., E.Pa., E.Pe. All authors have read and agreed to the published version of the manuscript.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153577.

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.

Batista-Silva, W., Nascimento, V.L., Medeiros, D.B., Nunes-Nesi, A., Ribeiro, D.M., Zsögön, et al., 2018. Modifications in organic acids profiles during fruit development and ripening: correlation or causation? Front. Plant Sci. 9, 1689.

Bernacchi, C.J., Singsaas, E.L., Pimentel, C., Portis Jr., A.R., Long, S.P., 2001. Improved temperature response functions for models of rubisco-limited photosynthesis. Plant Cell Environ. 24, 253–259.

Booker, F., Muntifering, R., Mcgrath, M., Burkey, K., Decoteau, D., Fiscus, E., 2009. The ozone component of global change: potential effects on agricultural and horticultural plant yield, product quality and interactions with invasive species. J. Integr. Plant Biol. 51, 337–351.

Bortolin, R.C., Caregnato, F.F., Divan Junior, A.M., Zanotto-Filho, A., Moresco, K.S., de Oliveira Rios, A., et al., 2016. Chronic ozone exposure alters the secondary metabolite profile, antioxidant potential, anti-inflammatory property, and quality of red pepper fruit from *Capsicum baccatum*. Ecotoxicol. Environ. Safe. 129, 16–24.

Calatayud, V., Barreno, E., 2001. Chlorophyll fluorescence, antioxidant enzymes and lipid peroxidation in tomato in response to ozone and benomyl. Environ. Pollut. 155, 283–289.

Calatayud, A., Barreno, E., 2004. Response to ozone in two lettuce varieties on chlorophyll a fluorescence, photosynthetic pigments and lipid peroxidation. Plant Physiol. Biochem. 42, 549–555.

Calzone, A., Podda, A., Lorenzini, G., Maserti, B.E., Carrari, E., Deleanu, E., et al., 2019. Cross-talk between physiological and biochemical adjustments by *Punica granatum* cv. Dente di cavallo mitigates the effects of salinity and ozone stress. Sci. Total Environ. 656, 589–597.

Cataldi, T.R., Margiotta, G., Del Fiore, A., Bufo, S.A., 2003. Ionic content in plant extracts determined by ion chromatography with conductivity detection. Phytochem. Anal. 14, 176–183.

Connor, A.M., Luby, J.J., Tong, C.B.S., Finn, C.E., Hancock, F.J., 2002. Genotypic and environmental variation in antioxidant activity, total phenolic content, and anthocyanin content among blueberry cultivars. J. Am. Soc. Hortic. Sci. 127, 89–97.

Conte, G., Fontanelli, M., Galli, F., Cotrozzi, L., Pagni, L., Pellegrini, E., 2020. Mycotoxins in feed and food and the role of ozone in their detoxification and degradation: an update. Toxins 12, 486.

Cooley, D.R., Manning, W.J., 1987. The impact of ozone on assimilate partitioning in plants: a review. Environ. Pollut. 47, 5–113.

Cotrozzi, L., Remorini, D., Pellegrini, E., Guidi, L., Nali, C., Lorenzini, G., et al., 2017. Living in a Mediterranean city in 2050: broadleaf or evergreen "citizens"? Environ. Sci. Pollut. Res. 25, 8161–8173.

Dong, S., Beckels, D.M., 2019. Dynamic changes in the starch-sugar interconversion within plant source and sink tissues promote a better abiotic stress response. J. Plant Physiol. 234–235, 80–93.

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

Döring, A.S., Cotrozzi, L., Lorenzini, G., Nali, C., Petersen, M., Pellegrini, E., 2020. Deciphering the role of low molecular weight antioxidants in the sensitivity of *Melissa officinalis* L. to realistic ozone concentrations. Ind. Crop. Prod. 150, 112369.

Duque, L., Poelman, E.H., Steffan-Dewenter, I., 2021. Effects of ozone stress on flowering phenology, plant-pollinator interactions and plant reproductive success. Environ. Pollut. 272, 115953.

Engela, M.R.G.S., Furlan, C.M., Esposito, M.P., Fernandes, F.F., Carrari, E., Domingos, M., et al., 2021. Metabolic and physiological alterations indicate that tropical broadleaf tree *Eugenia uniflora* L. is sensitive to ozone. Sci. Total Environ. 769, 145080.

Eyéghé-Bickong, H.A., Alexandersson, E.O., Gouws, L.M., Young, P.R., Vivier, M.A., 2012. Optimisation of an HPLC method for the simultaneous quantification of the major sugars and organic acids in grapevine berries. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci . 15, 885–886. https://doi.org/10.1016/j.jchromb.2011.12.011.

Fahnenstich, H., Saigo, M., Niessen, M., Zanor, M.I., Andreo, C.S., Fernie, A.R., et al., 2007. Alteration of organic acid metabolism in arabidopsis overexpressing the maize C4 NADP-malic enzyme causes accelerated senescence during extended darkness. Plant Physiol. 145, 640–652.

Farquhar, G.D., von Caemmerer, S., Berry, J.A., 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C3 species. Planta 149, 78–90.

FDA (Food and Drug Administration), n.d.FDA (Food and Drug Administration) Direct Food Substances Affirmed as Generally Recognized as Safe. [(accessed on 13 September 2021)]; Available online: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.Cfm?fr=184.1563.

Ghosh, A., Agrawal, M., Agrawal, S.B., 2020. Effect of water deficit stress on an indian wheat cultivar (*Triticum aestivum* L. HD 2967) under ambient and elevated level of ozone. Sci. Total Environ. 714, 136837.

Gillespie, C., Stabler, D., Tallentire, E., Goumenaki, E., Barnes, J., 2015. Exposure to environmentally-relevant levels of ozone negatively influence pollen and fruit development. Environ. Pollut. 206, 494–501.

Giovannoni, J., 2001. Molecular biology of fruit maturation and ripening. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 725–749.

Goumenaki, E., Taybi, T., Borland, A., Barnes, J., 2010. Mechanism underlying the impacts of ozone on photosynthetic performance. Environ. Exp. Bot. 69, 259–266.

Guidi, L., Remorini, D., Cotrozzi, L., Giordani, T., Lorenzini, G., Massai, R., et al., 2017. The harsh life of an urban tree: the effect of a single pulse of ozone in salt-stressed *Quercus ilex* saplings. Tree Physiol. 37, 246–260.

Grulke, N.E., Heath, R.L., 2020. Ozone effects on plants in natural ecosystems. Plant Biol. 22, 12–37.

Hassan, I.A., Bell, N.B., Ashmore, M.R., Cotrozzi, L., Haiba, N.S., Basahi, J.M.N., 2018. Radish (*Raphanus sativus* L.) cultivar-specific response to O_3: patterns of biochemical and plant growth characteristics. Clean-Soil Air Water 46, 1800124.

Havaux, M., Bonfils, J., Lütz, C., Niyogi, K., 2000. Photodamage of the photosynthetic apparatus and its dependence on the leaf development stage in the npq1 Arabidopsis mutant deficient in the xanthophyll cycle enzyme violaxanthin de-epoxidase. Plant Physiol. 124, 273–284.

Heide, O.M., 1997. Environmental control of flowering in some northern *Carex* species. Ann. Bot. 79, 319–327.

Ho, L.C., 1988. Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. Annu. Rev. Plant Physiol. 39, 355–378.

Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric acid reactive substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207, 604–611.

Hoshika, Y., Carrari, E., Mariotti, B., Martini, S., De Marco, A., Sicard, P., et al., 2020. Flux-based ozone risk assessment for a plant injury index (PII) in three european cool-temperate deciduous tree species. Forests 11, 82.

Huang, H., Ullak, F., Zhou, D.X., Yi, M., Zhao, Y., 2019. Regulation of plant development and stress responses. Front. Plant Sci. 10, 800.

Innes, J.L., Skelly, J.M., Schaub, M., 2001. Ozone and broadleaved species. A Guide to the Identification of Ozone-induced Foliar Injury. Paul Haupt Verlag Bern, Switzerland.

Jimenez-Garcia, S.N., Guevara-Gonzalez, R.G., Miranda-Lopez, R., Feregrino-Perez, A.A., Torres-Pacheco, I., Vazquez-Cruz, M.A., 2012. Functional properties and quality characteristics of bioactive compounds in berries: biochemistry, biotechnology, and genomics. Food Res. Int. 54, 1195–1207.

Kampfenkel, K., Van Montagu, M., Inzé, D., 1995. Extraction and determination of ascorbate and dehydroascorbate from plant tissue. Anal. Biochem. 225, 165–167.

Critical levels for ozone in Europe: testing and finalizing the concepts. In: Kärenlampi, L., Skärby, L. (Eds.), UN/ECE Workshop Report. Department of Ecology and Environmental Science. University of Kuopio, Finland, p. 366.

King, J.S., Kubiske, M.E., Pregitzer, K.S., Hendrey, G.R., McDonald, E.P., Giardina, C.P., Quinn, V.S., Karnosky, D.F., 2005. Tropospheric O_3 compromises net primary production in young stands of trembling aspen, paper birch and sugar maple in response to elevated atmospheric CO_2. New Phytol. 168, 623–636.

Keutgen, A., Pawelzik, E., 2008. Influence of pre-harvest ozone exposure on quality of strawberry fruit under simulated retail conditions. Postharvest Biol. Technol. 49, 10–18.

Köllner, B., Krause, G.H.M., 2000. Changes in carbohydrates, leaf pigments and yield in potatoes induced by different ozone exposure regimes. Agric. Ecosyst. Environ. 78, 149–158.

Leisner, C.P., Ainsworth, E.A., 2012. Quantifying the effects of ozone on plant reproductive growth and development. Glob. Change Biol. 18, 606–616.

Lemoine, R., La Camera, S., Atanassova, R., Dédaldéchamp, F., Allario, T., Pourtau, N., et al., 2013. Source-to-sink transport of sugar and regulation by environmental factors. Front. Plant Sci. 4, 272.

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. Total Environ. 657, 379–390.

Li, L., Manning, W.J., Tong, L., Wang, X., 2015. Chronic drought stress reduced but not protected shantung maple (*Acer truncatum* Bunge) from adverse effects of ozone (O3) on growth and physiology in the suburb of BeijingChina. Environ. Pollut. 201, 31–41.

Li, P., Feng, Z., Calatayud, V., Yuan, X., Xu, Y., Paoletti, E., 2017. A meta-analysis on growth, physiological, and biochemical responses of woody species to ground-level ozone highlights the role of plant functional types. Plant Cell Environ. 40, 2369–2380.

Long, S.P., Bernacchi, C.J., 2003. Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. J. Exp. Bot. 54, 2393–2401.

Lyons, T.M., Barnes, J.D., 2008. Influence of plant age on ozone resistance in *Plantago major*. New Phytol. 138, 83–89.

Marchica, A., Ascrizzi, R., Flamini, G., Cotrozzi, L., Tonelli, M., Lorenzini, G., et al., 2021. Ozone as eustress for enhancing secondary metabolites and bioactive properties in *Salvia officinalis*. Ind. Crop. Prod. 170, 113730.

Marchica, A, Loré, S, Cotrozzi, L, Lorenzini, G, Nali, C, Pellegrini, E, Remorini, D, 2019. Early detection of sage (*Salvia officinalis* L.) responses to ozone using reflectance spectroscopy. Plants 8 (9), 346. https://doi.org/10.3390/plants8090346.

Martín-Diana, A.B., Rico, D., Frías, J.M., Barat, J.M., Henehan, G.T.M., Barry-Ryan, C., 2007. Calcium for extending the shelf life of fresh whole and minimally processed fruits and vegetables: a review. Trends Food Sci. Technol. 18, 210–218.

Mikkelsen, T.N., Dodell, B., Lütz, C., 1995. Changes in pigment concentration and composition in Norway spruce induced by long-term exposure to low levels of ozone. Environ. Pollut. 87, 197–205.

Mills, G., Sharps, K., Simpson, Pleijel, H., Frei, M., Burkey, K., et al., 2018. Closing the global ozone yield gap: quantification and cobenefits for multistress tolerance. Glob. Change Biol. 24, 4869–4893.

Moraes, D.P., Chim, J.F., Barin, J.S., Vizzotto, M., Farias, C.A.A., Ballus, C.A., et al., 2021. Influence of the cultivar on the composition of blackberry (*Rubus* spp.) minerals. J. Food Compos. Anal. 100, 103913.

Niinemets, Ü., 2010. Responses of forest trees to single and multiple environmental stresses from seedlings to mature plants: past stress history, stress interactions, tolerance and acclimation. Forest Ecol. Manag. 260, 1623–1639.

Osorio, S., Scossa, F., Fernie, A., 2013. Molecular regulation of fruit ripening. Front. Plant Sci. 4, 198.

Ou, B., Hampsch-Woodill, M., Prior, R.L., 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J. Agric. Food Chem. 49, 4619–4626.

Ou, B., Hampsch-Woodill, M., Flanagan, J., Deemer, E.K., Prior, R.L., Huang, D., 2002. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. J. Agric. Food Chem. 50, 2772–2777.

Paoletti, E., Ferrara, A.M., Calatayud, V., Cerveró, J., Giannetti, F., Sanz, M.J., Manning, W.J., 2009. Deciduous shrubs for ozone bioindication: *Hibiscus syriacus* as an example. Environ. Pollut. 157, 865–870.

Paoletti, E., Materassi, A., Fasano, G., Hoshika, Y., Carriero, G., Silaghi, D., 2017. A new generation 3D ozone FACE (free air controlled exposure). Sci. Total Environ. 575, 1407–1414.

Pellegrini, E., Campanella, A., Paolocci, M., Trivellini, A., Gennai, C., Muganu, M., et al., 2015. Functional leaf traits and diurnal dynamics of photosynthetic parameters predict the behaviour of grapevine varieties towards ozone. PlosOne 10, e0135056.

Pellegrini, E., Hoshika, Y., Dusart, N., Cotrozzi, L., Gérard, J., Nali, C., et al., 2019. Antioxidative responses of three oak species under ozone and water stress conditions. Sci. Total Environ. 647, 390–399.

Pellegrini, E, Trivellini, A, Cotrozzi, L, Vernieri, P, Nali, C, 2016. Involvement of phytohormones in plant responses to ozone. Plant Hormones under Challenging Environmental Factors. Springer Netherlands, pp. 215–245.

Pleijel, H., Broberg, M.C., Uddling, J., Kobayashi, K., 2021. Letter to the editor regarding Pleijel et al. 2019: ozone sensitivity of wheat in different continents - an addendum. Sci. Total Environ. 773, 146335.

Puckette, M.C., Tang, Y., Mahalingam, R., 2008. Transcriptomic changes induced by acute ozone in resistant and sensitive *Medicago truncatula* accessions. BMC Plant Biol. 8, 46.

Ragel, P., Raddatz, N., Leidi, E.O., Quintero, F.J., Pardo, J.M., 2019. Regulation of K^+ nutrition in plants. Front. Plant Sci. 10, 281.

Royal Horticultural Society, 2007. RHS Colour Chart. 5th ed. RHS, London, UK.

Sachadyn-Król, M., Agriopoulou, S., 2020. Ozonation as a method of abiotic elicitation improving the health-promoting properties of plant products - a review. Molecules 25 (10), 2416.

Saitanis, C.J., Riga-Karandinos, A.N., Karandinos, M.G., 2001. Effects of ozone on chlorophyll and quantum yield of tobacco (*Nicotiana tabacum* L.) varieties. Chemosphere 42, 945–953.

Schauberger, B., Rolinski, S., Shaphoff, S., Müller, C., 2019. Global historical soybean and wheat yield loss estimates from ozone pollution considering water and temperature as modifying effects. Agric. For. Meteorol. 265, 1–15.

Selås, V., Sønateby, A., Heide, O.M., Opstad, N., 2015. Climatic and seasonal control of annual growth rhythm and flower formation in *Vaccinium myrtillus* (Ericaceae), and the impact on annual variation in berry production. Plant Ecol. Evol. 148, 350–360.

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.

Shipley, B., Merziane, D., 2002. The balanced-growth hypothesis and the allometry of leaf and root biomass allocation. Funct. Ecol. 16, 326–331.

Thalmann, M., Santelia, D., 2017. Starch as a determinant of plant fitness under abiotic stress. New Phytol. 214, 943–951.

Thwe, A.A., Vercambre, G., Gautier, H., Pagès, L., Jourdan, C., Gay, F., et al., 2013. Dynamic shoot and root growth at different developmental stages of tomato (*Solanum lycopersicum* mill.) under acute ozone stress. Sci. Hortic. 150, 317–325.

Thwe, A.A., Vercambre, G., Gautier, H., Gay, F., Phattaralerphong, J., Kasemsap, P., 2014. Effects of acute ozone stress on reproductive traits of tomato, fruit yield and fruit composition. J. Sci. Food Agric. 95, 614–620.

Waterhouse, A., 2002. Determination of total phenolics. Food Current Protocols in Food Analytical Chemistry. 6, pp. E1.2.1–1.5.8.

Wilkinson, S., Mills, G., Illdge, R., Davies, W.J., 2012. How is ozone pollution reducing our food supply? J. Exp. Bot. 63, 527–536.

Wittig, V.E., Ainsworth, E.A., Naidu, S.L., Karnosky, D.F., Long, S.P., 2009. Quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: a quantitative meta-analysis. Glob. Change Biol. 15, 396–424.

Young, P.J., Archibald, A.T., Bowman, K.W., Lamarque, J.-F., Naik, V., Stevenson, D.S., et al., 2013. Pre-industrial to end 21st century projections of tropospheric ozone from the atmospheric chemistry and climate model intercomparison project (ACCMIP). Atmos. Chem. Phys. 13, 2063–2090.

Zhang, L., Hoshika, Y., Carrari, E., Cotrozzi, L., Pellegrini, E., Paoletti, E., 2018. Effects of nitrogen and phosphorous imbalance on photosynthetic traits of poplar Oxford clone under ozone pollution. J. Plant Res. 131, 915–924.