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Untangling the role of leaf age specific osmoprotectant and antioxidant responses of two poplar clones under increasing ozone concentrations

Claudia Pisuttu [a], Samuele Risoli [a,b], Lorenzo Cotrozzi [a,c], Cristina Nali [a,c], Elisa Pellegrini [a,c,*], Yasutomo Hoshika [d,e], Barbara Baesso Moura [d,e], Elena Paoletti [d,e]

[a] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124, Pisa, Italy
[b] University of School for Advanced Studies IUSS, Piazza della Vittoria 15, 27100, Pavia, Italy
[c] CIRSEC, Centre for Climate Change Impact, University of Pisa, Italy
[d] Institute of Research on Terrestrial Ecosystems (IRET), National Research Council of Italy (CNR), Via Madonna del Piano 10, I-50019, Sesto Fiorentino, Italy
[e] NBFC, National Biodiversity Future Center, Palermo, 90133, Italy

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ABSTRACT

Plants possess different degrees of tolerance to abiotic stress, which can mitigate the detrimental effect of environmental inputs affecting carbon balance. Less is known about the functions of osmoprotectants in scavenging of reactive oxygen species (ROS), generated at different sites depending on leaf age. This study aimed to clarify the osmotic adjustments adopted by old and young leaves of Oxford and I-214 poplar clones [differing in ozone (O_3) sensitivity] to cope with three levels of O_3 [ambient (AA), and two elevated O_3 levels]. In both clones, the impact of intermediate O_3 concentrations (1.5 × AA) on ROS production appeared to be leaf age-specific, given the accumulation of hydrogen peroxide (H_2O_2) observed only in old leaves of the Oxford plants and in young leaves of the I-214 ones (2- fold higher than AA and +79%, respectively). The induction of an oxidative burst was associated with membrane injury, indicating an inadequate response of the antioxidative systems [decrease of lutein and β-carotene (−37 and −85% in the old leaves of the Oxford plants), accumulation of proline and tocopherols (+60 and +12% in the young leaves of the I-214 ones)]. Intermediate O_3 concentrations reacted with unsaturated lipids of the plasma membrane in old and young leaves of the Oxford plants, leading to an increase of malondialdehyde by-products (more than 2- fold higher than AA), while no effect was recorded for I-214. The impact of the highest O_3 concentrations (2.0 × AA) on ROS production did not appear clone-specific, which may react with cell wall components by leading to oxidative pressure. Outcomes demonstrated the ability of young leaves of I-214 plants in contain O_3 phytotoxic effects.

1. Introduction

In vascular plants, several abiotic stress factors can induce oxidative damage due to the continuous accumulation of reactive oxygen species (ROS; Sachdev et al., 2021). The main target of ROS-provoked damage is the chloroplast (because of its strong photo-oxidative potential) in which various cellular processes may suffer a drastic decline bringing to the hydrolysis of proteins and lipids, leaf senescence and cell death (Ye et al., 2021). Photosynthesis is a fundamental process for plant species and depended by leaf age and developmental stage (Stirbet et al., 2020). It is a well-known fact that the synthesis of components involved in the light- and dark-dependent photosynthetic reactions occurs at various times [e.g., the formation of the core complex of photosystem I (PSI) is ahead of photosystem II (PSII)]. In particular, the activity of Rubisco reaches its maximum value in mature fully expanded leaves and only the oldest ones possess the maximum size of the light-harvesting complex (Nath et al., 2013; Pshybytko et al., 2023). Consequently, the performance of photosystems decreases much faster than the Rubisco activity in aged leaves, and the assimilation of C/N and their redistribution among leaves are different concerning their developmental stage (Sade et al., 2018). For this reason, an initial increase in the photosynthetic rate is observed during leaf expansion, followed by a decrease upon maturation and a sharp drop during senescence: young and fully expanded leaves were found to have the highest photosynthetic rate, older leaves showed a lower rate, and the oldest ones had the lowest (Bielczynski et al., 2017). This continuous reduction of photosynthetic

* Corresponding author. Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124, Pisa, Italy.
E-mail address: elisa.pellegrini@unipi.it (E. Pellegrini).

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capacity in aging leaves can arise from several factors such as reduction in mesophyll conductance, alteration in Rubisco efficiency, accumulation of ROS, photo-inhibition, and photo-oxidation (Pintó-Marijuan and Munné-Bosch, 2014; Pellegrini et al., 2021).

Young leaves, can be considered more valuable than old ones since they are (i) characterized by photosynthetic active tissues where ROS-provoked damage are minimized, and (ii) protected against abiotic stress factors affecting water status and hydric relationships (Sperdouli and Moustakas, 2014). This high plasticity is demonstrated in physiological leaf traits such as stomatal conductance and density. Young leaves have a lower stomatal conductance than old ones where the stomata are typically less dense but well-expanded and developed (Snider et al., 2009; Hoshika et al unpublished). Conversely, young leaves are sensitive to environmental inputs affecting carbon balance (light, putative temperature, and carbon dioxide) because their structural growth requires a net carbon import before the sink-to-source transition (Pantin et al., 2012). The loss of this plasticity can be compensated by the acquisition of structural, physiological and biochemical defenses (Rankenberg et al., 2021). The partially uncoupled changes in water and carbon relations experienced by the growing leaf as it develops make it differentially sensitive to environmental stresses throughout its ontogeny. Moreover, old and young leaves may cross-talk to adjust/adapt their response to various stress factors (Pantin et al., 2012).

Plants possess different degrees of tolerance to abiotic stress, which can mitigate the detrimental effect of environmental inputs affecting carbon and/or water status. According to Hartmann et al. (2020), the investment of carbon and energy reserves, and the consequent osmoregulation, is a crucial process underlying plant acclimation to abiotic stress and influencing the interaction of several metabolites in the conservative carbon-allocation and water-saving strategies. To date, less is known about the adaptive biochemical functions of compatible solutes in the scavenging of ROS that are over-produced under unfavorable environmental conditions. These metabolites (that act as osmoprotectants, antioxidants, low-molecular weight proteins and/or stress signals) can accumulate to high levels without disturbing intracellular biochemistry, being involved in stress tolerance (Wingler and Roistch, 2008). Carbohydrates are a major category of compatible solutes and, being the primary carbon and energy source in plants, could produce reducing power for the biosynthesis of non-enzymatic antioxidants, including phenolic compounds, xanthophylls, carotenoids, ascorbate and glutathione (Keunen et al., 2013). Their production represents a prominent defense trait under stressful conditions, and is mediated by various signals, which are generated at different sites depending on leaf age and developmental stage (Ozden et al., 2009; Boublin et al., 2022).

Among the abiotic stress, tropospheric ozone (O_3) is an important greenhouse gas and known as the most widespread air pollutant, particularly damaging to forests, grasslands, and crops (Dewan and BamolaLakhani, 2024). Effects and mechanisms of action of O_3 have been extensively described in poplar (Salicaceae), since the species has been proposed as a model tree for scientific studies and it is widely employed (i) as important wood source, (ii) in controlling erosion and (iii) in diversified agricultural systems (e.g., agroforestry; Cotrozzi et al., 2021; Tramacere et al., 2023). Here, we studied two poplar clones differing in shoot growth and O_3 sensitivity. Oxford (*Populus maximowiczii* Henry × *P. berolinensis* Dippel) clone usually concludes shoot development in early August, by exhibiting a "determinate-like" growth (Giovannelli et al., 2019), and is considered extremely sensitive to O_3 in terms of foliar injury (Marzuoli et al., 2009). I-214 (*P. deltoides* W. Bartram ex Marshall × *P. nigra* L.) clone is one of the most used poplars (Bergante et al., 2023), one of the less sensitive to O_3 (Guidi et al., 2001) and has a longer phenological development than Oxford one, by continuing the shoot growth until the end of September (Giovannelli et al., 2007). This phenomenon may have advantages for producing new leaves (as a compensatory response against O_3-induced oxidative damage during the growing season) which may have different structural, physiological and biochemical functional traits.

The present study aimed to clarify the leaf-intrinsic and osmotic adjustments adopted by old and young leaves of Oxford and I-214 clones to cope with various levels of O_3 in an O_3-Free Air Controlled Exposure (O_3-FACE) facility. Specifically, we asked the following questions: (i) Do increasing O_3 concentrations differentially affect the macroscopic responses (in terms of biomass and visible injury) of the examined clones in relation to their sensitivity? (ii) What are the metabolic and cellular mechanisms activated by chronic O_3 exposure in old and young leaves of the two clones? We hypothesized an age-dependent cross-talk among leaf antioxidant and osmotic rearrangements, which regulate the conservative carbon-allocation and water-saving strategies of clones differing in O_3 sensitivity.

2. Materials and methods

2.1. Plant material and experimental design

Experimental activities were carried out in the O_3-FACE facility of Sesto Fiorentino, Florence, Central Italy (43°49′ N, 11°12' E, 55 m a.s.l.; Paoletti et al., 2017). In winter 2019, rooted cuttings of Oxford and I-214 poplar clones were propagated and kept in a refrigerator (4 °C) until planted in nursery pot trays in February. They were moved outside in March and transplanted in plastic pots (volume 10 L) containing a sand:peat:soil mixture (1:1:1 vol), and kept in a nursery until the beginning of O_3 exposure. From 20 May to 31 October 2020, uniform-sized plants (approx. 15 cm in height) were exposed to three concentrations of O_3 (AA, ambient O_3 concentration; 1.5 × AA and 2.0 × AA, 1.5 and 2.0 times AA, respectively). Throughout the whole period of O_3 exposure, the hourly mean O_3 concentrations were 37.5, 52.3 and 73.3 ppb in AA, 1.5 × AA and 2.0 × AA, respectively. Four plants per clone were placed in each of the three replicated plots (5 × 5 × 2 m) per O_3 treatment (in total 36 plants per clone). All plants were watered every day to maintain field capacity.

2.2. Assessment of visible foliar injuries

The O_3-like visible foliar injury was identified on 31st August in 2020 by two well-experienced surveyors, using photoguides (Paoletti et al., 2009) and hand lens (10 × of magnification). The percentage of the number of symptomatic leaves per plant (LA; counted without distinguish among old and young leaves) and the averaged percentage of injured area per each symptomatic leaf (AA: with a 5%-step scale: Calatayud et al., 2007; Hoshika et al., 2020) were visually assessed in each plant. According to these two parameters, the Plant Injury Index (PII) was calculated as PII = (LA × AA)/100.

2.3. Determination of above- and below-ground biomass

Four plants per clone and O_3 level were randomly selected at the end of the experiment and devoted to the biomass assessment in terms of leaves, stems, branches, and fine and coarse roots (⌀ ≤ 2 and >2 mm, respectively). Organs were properly separated and kept in an oven at 80 °C until a constant weight was reached.

2.4. Leaf biochemical analyses

The number of leaves in the main shoot was counted at two-three week intervals throughout the whole period of the experiment in order to estimate the leaf longevity (Kikuzawa and Lechowicz, 2011). Young and old leaves resulted in upper and lower positions, respectively. Young and old leaves were randomly selected and sampled from one to two plants per plot and O_3 treatment on 15th September in 2020, in order to avoid the natural senescence occurring in the fall period. They were immediately frozen in liquid nitrogen and stored at −80 °C until the biochemical analyses reported below.

2.4.1. Hydrogen peroxide content and lipid peroxidation

The content of H_2O_2 was estimated by using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies Corp. Carlsbad, CA, USA) according to the method of Shin et al. (2005). Frozen foliage material (50 mg) was added to 1 mL of 20 mM potassium/phosphate buffer (pH 6.5), centrifuged (12000 *g* for 15 min at 4 °C), and incubated in the dark (for 30 min at 25 °C). The determination was carried out using a Victor3 1420 Multilabel Counter microplate reader (PerkinElmer Inc., Waltham, MA, USA) at 530 and 590 nm for the excitation and emission of resorufin fluorescence.

Lipid peroxidation was evaluated by determining malondialdehyde (MDA) by-products accumulation according to the method of Hodges et al. (1999), with minor modifications as reported by Guidi et al. (2017). Frozen foliage material (100 mg) was added to 1 mL of 90% (v/v in H_2O) ethanol, sonicated 3 times for 10 min, and centrifuged (13000 *g* for 10 min at 4 °C). Then, 100 μL of supernatant were mixed with 400 μL of 20% (w/v) thiobarbituric acid, incubated for 30 min at 95 °C and centrifuged (12000 *g* for 10 min at 4 °C). The determination was carried out by using a spectrophotometer (UV-1900 UV–vis, Shimadzu, Kyoto, Japan) at 440, 532, and 600 nm.

2.4.2. Osmolyte content

The content of abscisic acid (ABA) was estimated by using the Phytodetek® Immunoassay Kit (Agdia, Elkhart, IN, USA; Pisuttu et al., 2023). Foliage material (100 mg) was added to 1 mL of distilled water, and the supernatant was diluted 10 times, according to the manufacturing protocol. The determination was carried out by using the same fluorescence/absorbance microplate reader reported above, at 415 nm.

The content of proline was determined according to Calzone et al. (2020). Foliage material (20 mg) was added to 200 μL of 70% (v/v in H_2O) ethanol, sonicated three times for 10 min and incubated for 30 min at 90 °C. The determination was carried out by using the same spectrophotometer reported above, at 520 nm.

The content of soluble carbohydrates was evaluated according to Pisuttu et al. (2023), with slight modifications. Leaf material (50 mg) was added to 1 mL of 100% HPLC-demineralized water, incubated for 60 min at 60 °C and centrifuged (16000 *g* for 15 min at 4 °C). The supernatants were filtered through 0.2 μm Minisart® SRT 15 aseptic filters. Soluble carbohydrates were determined by Ultra-High Performance Liquid Chromatography (UHPLC) using a Dionex UltiMate 3000 system (Thermo Scientific, Waltham, MA, USA) equipped with a Repromer H column (9 μm particle size, 8 mm internal diameter × 300 mm length; Dr Maisch, Ammerbuch, Germany), provided with a pre-column (9 μm particle size, 8 mm internal diameter × 20 mm length; Dr Maisch) and maintained at 25 °C. The soluble carbohydrates were eluded using an isocratic mobile phase (9 mM sulphuric acid, flow rate of 1 mL min^{-1}) and detected by their absorbance at 210 nm with a differential refractometer (Shodex, West Berlin, NJ, USA). To quantify soluble carbohydrate contents, known amounts of the pure standard were injected into the UHPLC system. An equation correlating the peak area to each soluble carbohydrate standard concentration was formulated by using Chromeleon Chromatography Management System software, version 7.2.10–2019 (Thermo Scientific). The sum of sucrose, glucose, and fructose was considered as a measure of total soluble carbohydrate content.

2.4.3. Photosynthetic and accessory pigment content

The content of photosynthetic pigments was evaluated according to Pisuttu et al. (2023). Leaf material (100 mg) was added to 1 mL of 100% HPLC-grade methanol, incubated overnight in the dark at 4 °C, and centrifuged (16000 *g* for 15 min at 4 °C). The supernatants were filtered through 0.2 μm Minisart® SRT 15 aseptic filters. Photosynthetic pigments were determined by the same UHPLC (reported above) equipped with a reverse-phase Agilent column (ZORBAX Eclipse plus C18, 5 μm particle size, 4.6 mm internal diameter × 150 mm length; Agilent Technologies, Inc., Santa Clara, CA, USA) maintained at 25 °C. Acetonitrile/methanol (75:25, v/v) was used at 100% for the first 14 min to elute xanthophylls (neoxanthin, violaxanthin, and anteraxanthin), followed by a 1.5 min linear gradient to 100% methanol/ethyl acetate (68:32, v/v), 15 min with 100% methanol/ethyl acetate (68:32, v/v), which was pumped to elute chlorophylls and β-carotene. The flow-rate was 1 mL min^{-1}. Chlorophylls (*a* and *b*), carotenoids (neoxanthin, violaxanthin, anteraxanthin, lutein, and β-carotene), and tocopherols (α, β, γ, and δ) were detected by their absorbance at 445 and 295 nm with a Dionex UVD 170 U UV–Vis detector (Thermo Scientific). To quantify the content of pigments, known amounts of pure authentic standards were injected into the UHPLC system. An equation was formulated correlating the peak area to each chlorophyll/carotenoid/tocopherol standard concentration. Chromatographic data were processed and recorded by the software reported above.

2.5. Statistical analysis

The Shapiro-Wilk's and Levene's tests were used to assess the normal distribution of data and the homogeneity of variance, respectively. The effect of O_3 on visible injury and biometric parameters was investigated by a one-way analysis of variance (ANOVA). The effects of O_3, leaf age and their interaction on biochemical parameters were assessed by a two-way ANOVA for each poplar clone. Comparisons among means were determined by the Tukey's HSD post-hoc test. Effects with $P \leq 0.05$ were considered statistically significant. Statistical analyses were performed with JMP Pro 13.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Visible injury

Both clones exposed to increasing O_3 concentrations developed visible minute (⌀ 1–2 mm) stipples of roundish dark-brown tissue localized in the interveinal areas of the adaxial leaf surface. In the Oxford plants, increased PII values were found as a consequence of O_3 treatments [4-fold higher than AA (throughout the whole text, increased O_3 effects are compared to AA conditions), without significant differences between the two increased O_3 concentrations; Table 1]. The most severe damage occurred in plants grown under 2.0 × AA conditions: 27% of the scored leaves were affected, and the injured leaves had on average 32% of their surface covered by stippling. In I-214 plants, only the highest O_3 concentration significantly increased PII values (90-fold higher than AA).

3.2. Above- and belowground biomass

In the Oxford plants grown under 1.5 × AA conditions, a significant reduction of coarse root biomass was observed (−34%; Table 2).

Table 1
Plant Injury Index (PII) on Oxford and I-214 poplar clones grown under three levels of ozone concentration [ambient air (AA), 1.5 × AA and 2.0 × AA] for five months (May–October). Data are shown as mean ± standard error (n = 3). ANOVA: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$. Since one-way ANOVA revealed a significant ozone effect on PII values according to Tukey's *post-hoc* test, different letters indicate significant differences among means in each clone ($P \leq 0.05$).

Clone	Ozone	PII
Oxford	AA	2.1 ± 0.9 a
	1.5 × AA	8.1 ± 2.9 b
	2.0 × AA	9.8 ± 4.6 b
	P	**
I-214	AA	0.07 ± 0.02 a
	1.5 × AA	2.79 ± 1.53 a
	2.0 × AA	6.30 ± 3.35 b
	P	***

Table 2
Above- and below-ground biomass (expressed as g of dry weight) and shoot/root ratio of Oxford and I-214 poplar clones grown under three levels of ozone concentration [ambient air (AA), 1.5 × AA and 2.0 × AA] for five months (May–October). Data are shown as mean ± standard error (n = 3). ANOVA: $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, ns denotes not significant. Since one-way ANOVA revealed a significant ozone effect on all biometric parameters (except stems and branches in the case of Oxford clone) according to Tukey's *post-hoc* test, different letters indicate significant differences among means in each clone ($P \leq 0.05$).

Clone		Parameter				
	Ozone	Leaves	Stems and branches	Coarse roots	Fine roots	Shoot/ root ratio
Oxford	AA	5.6 ± 0.7 b	13.2 ± 3.6	14.1 ± 2.3 b	9.6 ± 2.0 b	0.55 ± 0.12 a
	1.5 × AA	3.9 ± 1.0 ab	11.7 ± 3.0	9.3 ± 0.4 a	6.5 ± 2.0 ab	0.78 ± 0.08 ab
	2.0 × AA	2.0 ± 0.9 a	9.2 ± 3.4	5.3 ± 1.8 a	4.1 ± 1.7 a	1.01 ± 0.14 b
	P	**	ns	**	*	**
I-214	AA	10.0 ± 0.4 b	13.7 ± 1.5 a	17.4 ± 2.2 b	18.5 ± 3.8 b	0.40 ± 0.04 a
	1.5 × AA	8.9 ± 2.5 b	20.0 ± 2.9 b	17.0 ± 3.9 b	13.2 ± 4.2 ab	0.67 ± 0.12 a
	2.0 × AA	3.6 ± 1.4 a	14.1 ± 1.0 a	6.5 ± 1.7 a	5.6 ± 1.8 a	1.39 ± 0.46 b
	P	**	*	**	*	*

Similarly, the highest O_3 concentration decreased leaf, coarse, and fine root biomass (−64, −62 and −57%, respectively) and led to the concomitant increase in shoot/root ratio (+84%). An increase of stem and branch biomass was found in the I-214 plants grown under 1.5 × AA conditions (+46%). Conversely, a significant decrease of leaf, coarse and fine root biomass (−64, −63 and −70%, respectively) and a concomitant increase of shoot/root ratio more than 3-fold higher than AA) was observed as a consequence of the highest O_3 concentration.

3.3. Hydrogen peroxide content and lipid peroxidation

The two-way ANOVA of H_2O_2 content revealed that the interaction between O_3 and leaf age, as well the effects of the singular factors, were significant in both clones (Fig. 1A and B). In the Oxford plants, a significant production of H_2O_2 was found in old leaves as a consequence of O_3 treatment by following the O_3 concentration gradient (about 2- and 3- fold higher than AA under 1.5 × AA and 2.0 × AA, respectively). A similar increase was observed only in young leaves grown under 2.0 × AA conditions (about 3-fold higher; Fig. 1A). In the I-214 plants, only the highest O_3 concentration induced an accumulation of H_2O_2 in old leaves (more than 2-fold higher). A similar increase was found as a consequence of O_3 treatment in young leaves (+79 and + 94%, under 1.5 × AA and 2.0 × AA conditions, respectively; Fig. 1B).

The two-way ANOVA of MDA by-products content revealed that the interaction between O_3 treatment and leaf age, as well the effects of the singular factors, were significant only in Oxford plants (Fig. 1C). In the case of the I-214 plants, only the effect of O_3 *per se* was significant (Fig. 1D). In the Oxford plants, MDA by-products increased due to O_3 treatment in both leaves, following the O_3 concentration gradient (old leaves: 2- and 3-fold higher; young leaves: 3- and 4-fold higher under 1.5 × AA and 2.0 × AA, respectively; Fig. 1C). In both clones grown under AA conditions, the content of H_2O_2 was higher in old leaves than in young ones (about 2-fold). Conversely, no significant differences were observed between old and young leaves regarding MDA by-products content.

3.4. Osmolyte contents

The two-way ANOVA of ABA content revealed that the interaction between O_3 treatment and leaf age, as well as the effects of the singular factors, were significant in both clones (Fig. 2A and 2B). In the Oxford plants, a significant increase of ABA content was found in old leaves as a consequence of O_3 treatment, following the O_3 concentration gradient (about 6- and 12- fold higher under 1.5 × AA and 2.0 × AA,

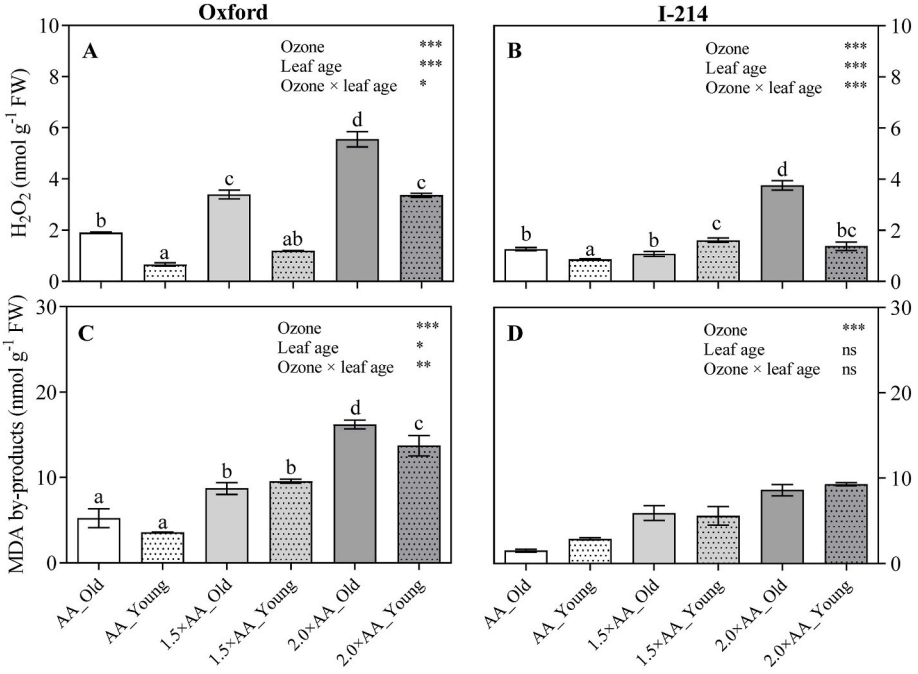

Fig. 1. Hydrogen peroxide (H_2O_2; **A-B**) and malondialdehyde by-products (MDA; **C-D**) content in old (*solid fill*) and young (*pattern fill*) leaves of Oxford and I-214 poplar clones grown under three levels of ozone concentration [ambient air (AA, *white fill*), 1.5 × AA (*light grey fill*) and 2.0 × AA (*dark grey fill*)] for five months (May–October). Data are shown as mean ± standard error (n = 4) on a fresh weight (FW) basis. ANOVA: $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, ns denotes not significant. Since two-way ANOVA revealed a significant ozone and leaf age interaction on H_2O_2 and MDA by-products according to Tukey's *post-hoc* test (except in the case of MDA by-products in the I-214 clone), different letters indicate significant differences among means in each graph ($P \leq 0.05$).

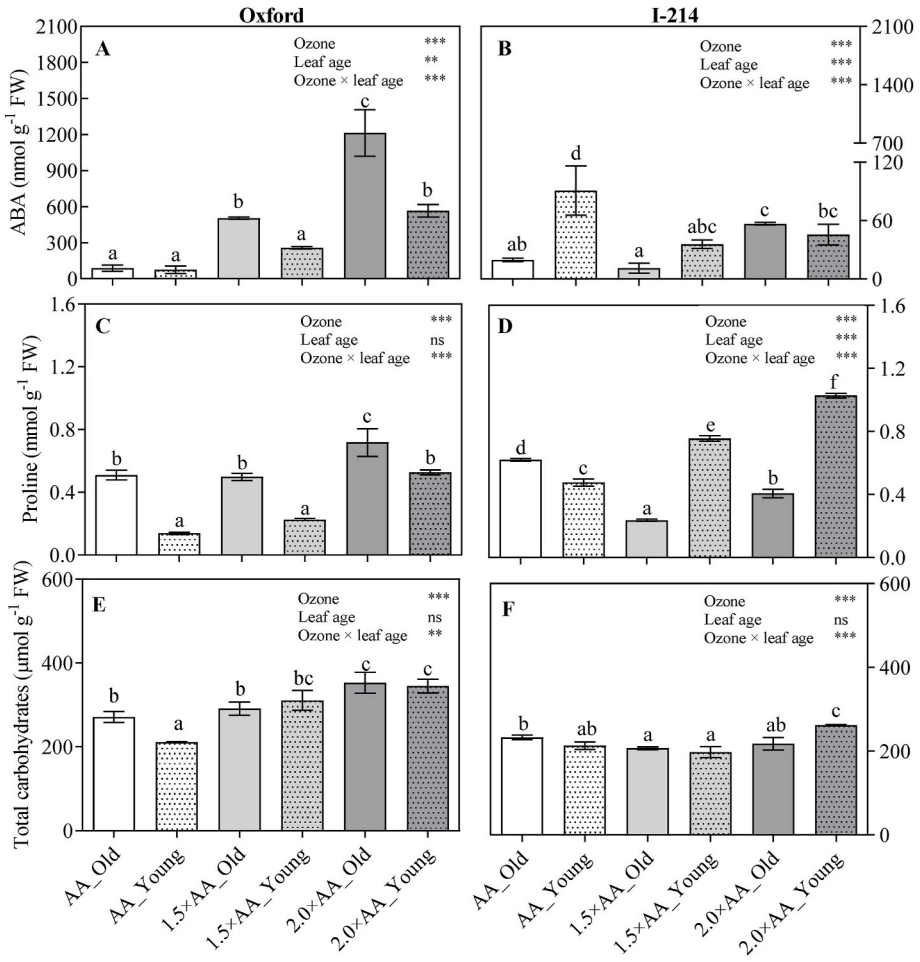

Fig. 2. Abscisic acid (ABA; **A-B**), proline (**C-D**), and total carbohydrates (**E-F**) content in old (*solid fill*) and young (*pattern fill*) leaves of Oxford and I-214 poplar clones grown under three levels of ozone concentration [ambient air (AA, *white fill*), 1.5 × AA (*light grey fill*) and 2.0 × AA (*dark grey fill*)] for five months (May–October). Data are shown as mean ± standard error (n = 4) on a fresh weight (FW) basis. ANOVA: ***$P \leq 0.001$, **$P \leq 0.01$, ns denotes not significant. Since two-way ANOVA revealed a significant ozone and leaf age interaction on ABA, proline, and total carbohydrates according to Tukey's *post-hoc* test, different letters indicate significant differences among means in each graph ($P \leq 0.05$).

respectively). A similar increase was observed only in young leaves grown under 2.0 × AA conditions (about 7-fold higher; Fig. 2A). In the I-214 plants, only the highest O_3 concentration induced an accumulation of ABA in old leaves (more than 3-fold higher). Conversely, a significant decrease was found as a consequence of O_3 treatment in young leaves (about 2-fold lower, regardless of O_3 concentration; Fig. 2B).

The two-way ANOVA of proline content revealed that the interaction between O_3 treatment and leaf age, as well the effects of the singular factors, were significant in both clones (except in the case of "leaf age" in Oxford plants; Fig. 2C and 2D). In the Oxford plants, only the highest O_3 concentration induced an accumulation of proline in old and young leaves (+43 and +80%, respectively; Fig. 2C). In I-214 plants, a significant decrease was found in old leaves as a consequence of O_3 treatment (about 3- and 2-fold lower under 1.5 × AA and 2.0 × AA, respectively). An opposite trend was observed in young leaves: a significant accumulation of proline was found as a consequence of O_3 treatment, following the O_3 concentration gradient (+60 and +118% under 1.5 × AA and 2.0 × AA, respectively; Fig. 2D).

The two-way ANOVA of total carbohydrate content revealed that the interaction between O_3 treatment and leaf age, as well the effects of the "O_3" factor, were significant in both clones (Fig. 2E and 2F). In the Oxford plants, only the highest O_3 concentration induced an increase of total carbohydrates in old leaves (+30%). A similar increase was found as a consequence of O_3 treatment in young leaves (+47 and +64% under 1.5 × AA and 2.0 × AA, respectively; Fig. 2E). In the I-214 plants, a slight decrease of total carbohydrates was found in old leaves grown under 1.5 × AA conditions (−11%). An opposite trend was observed in the young leaves due to the highest O_3 concentration (+23%; Fig. 2F). In the Oxford plants grown under AA conditions, no significant differences were observed between old and young leaves in terms of ABA content. Conversely, the content of ABA was higher in young leaves than old ones in the I-214 plants (about 4-fold). In both clones grown under AA conditions, the content of proline was higher in old leaves than young ones (4- and 2-fold in the Oxford and the I-214 plants, respectively). Concerning the content of total carbohydrates, there was a difference between old and young leaves (−9%) in the Oxford plants grown under AA conditions. Conversely, no significant differences were observed between old and young leaves regarding total carbohydrates in I-214 plants.

3.5. Lipid-soluble antioxidant contents

The two-way ANOVA of lutein content revealed that the interaction between O_3 treatment and leaf age, as well as the effects of the singular factors, were significant only in Oxford plants (Fig. 3A). In the case of the I-214 plants, only the effects of "O_3 treatment" and "leaf age" *per se* were significant (Fig. 3B). In the Oxford plants, a decrease of lutein was found only in the old leaves grown under 1.5 × AA conditions (−37%). No significant differences were observed among O_3 treatments in the young leaves.

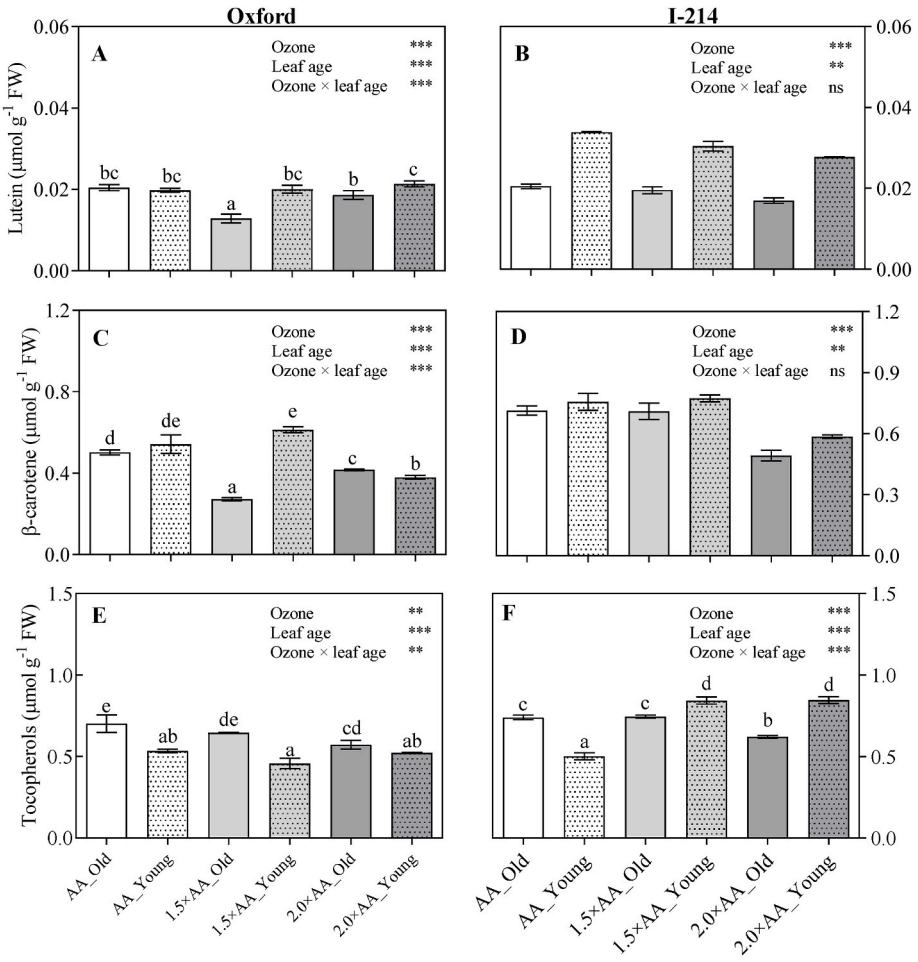

Fig. 3. Lutein (ABA; **A-B**), β-carotene (**C-D**) and tocopherols (**E-F**) content in old (*solid fill*) and young (*pattern fill*) leaves of Oxford and I-214 poplar clones grown under three levels of ozone concentration [ambient air (AA, *white fill*), 1.5 × AA (*light grey fill*) and 2.0 × AA (*dark grey fill*)] for five months (May–October). Data are shown as mean ± standard error (n = 4) on a fresh weight (FW) basis. ANOVA: ***$P \leq 0.001$, **$P \leq 0.01$, ns denotes not significant. Since two-way ANOVA revealed a significant ozone and leaf age interaction on lutein and β-carotene in the case of the Oxford clone, and tocopherols according to Tukey's *post-hoc* test, different letters indicate significant differences among means in each graph ($P \leq 0.05$).

The two-way ANOVA of β-carotene content revealed that the interaction between O_3 treatment and leaf age, as well as the effects of the singular factors, were significant only in the Oxford plants (Fig. 3C). In the case of the I-214 plants, only the effects of "O_3 treatment" and "leaf age" *per se* were significant (Fig. 3D). In the Oxford plants, a significant decrease was found in the old leaves as consequence of O_3 treatment (−85 and −32% under 1.5 × AA and 2.0 × AA, respectively; Fig. 3C). A similar trend was observed in the young leaves due to the highest O_3 concentration (−21%; Fig. 3D).

The two-way ANOVA of tocopherols content revealed that the interaction between O_3 treatment and leaf age, as well as the effects of the "O_3" factor, were significant in both clones (Fig. 3E and 3F). In the Oxford plants, only the highest O_3 concentration induced a decrease of tocopherols in the old leaves (−23%). No significant differences were observed among O_3 treatments in the young leaves. In the I-214 plants, a slight decrease of tocopherols was found in the old leaves grown under 2.0 × AA conditions (−19%). An opposite trend was observed in the young leaves due to O_3 treatments (+12 and +13% under 1.5 × AA and 2.0 × AA, respectively; Fig. 3F). In the Oxford plants grown under AA conditions, no significant differences were observed between old and young leaves in terms of lutein and β-carotene. In both clones grown under AA conditions, the content of tocopherols was higher in old leaves than young ones (+31 and + 48% in the Oxford and I-214 plants, respectively).

3.6. Photosynthetic pigment contents

The two-way ANOVA of total chlorophylls content revealed that the interaction between O_3 treatment and leaf age, as well as the effects of the "O_3" factor, were significant in both clones (Fig. 4A and 4B). In the Oxford plants, a significant decrease of total chlorophylls was found in old leaves grown under 1.5 × AA and 2.0 × AA conditions (−53% as average). In the young leaves, only the highest O_3 concentration induced a decrease of total chlorophylls (−25%). In the I-214 plants, a decrease of total chlorophylls was found in the old and young leaves grown under 2.0 × AA conditions (−26% as average).

The two-way ANOVA of chlorophylls *a/b* ratio revealed that the interaction between O_3 treatment and leaf age, as well as the effects of the "O_3" factor, were significant in both clones (Fig. 4C and 4D). In the Oxford plants, a slight increase of chlorophylls *a/b* ratio was found in the old leaves grown under 2.0 × AA conditions (+11%). No significant differences were observed among O_3 treatments in young leaves. An opposite trend was observed in the I-214 plants regardless of the leaf age, except in the case of young leaves grown under 1.5 × AA conditions (−21 and −35% in old leaves grown under 1.5 × AA and 2.0 × AA conditions, −39% in young leaves grown under 2.0 × AA conditions). In the Oxford plants grown under AA conditions, the total content of chlorophylls was higher in young leaves than old ones (+22%). In both clones grown under AA conditions, no significant differences were observed between old and young leaves in terms of chlorophylls *a/b*

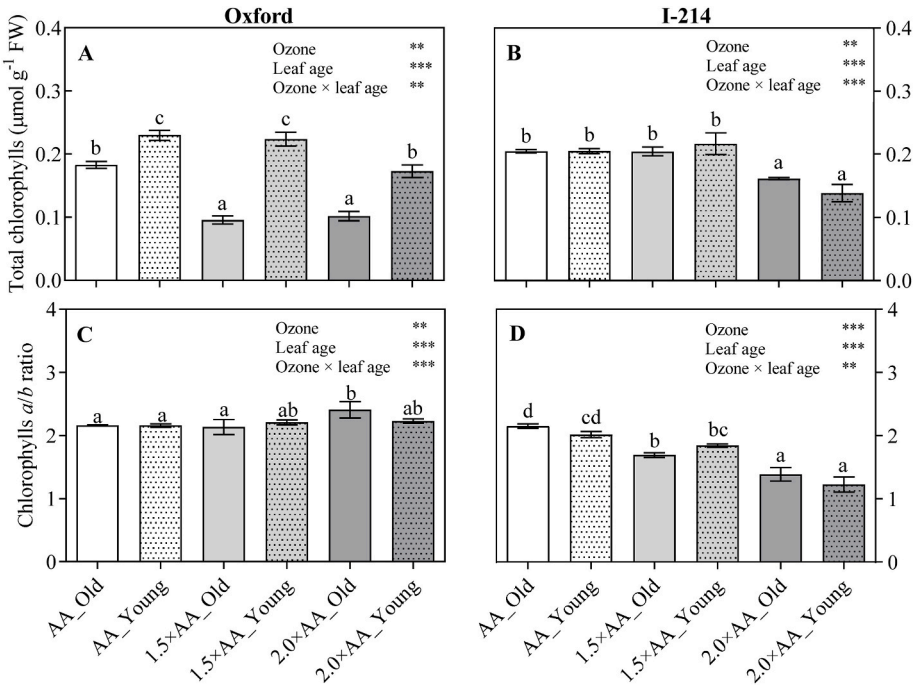

Fig. 4. Total chlorophylls content (**A-B**) and chlorophylls *a/b* ratio (**C-D**) in old (*solid fill*) and young (*pattern fill*) leaves of Oxford and I-214 poplar clones grown under three levels of ozone concentration [ambient air (AA, *white fill*), 1.5 × AA (*light grey fill*) and 2.0 × AA (*dark grey fill*)] for five months (May–October). Data are shown as mean ± standard error (n = 4) on a fresh weight (FW) basis. ANOVA: ***$P \leq 0.001$, **$P \leq 0.01$. Since two-way ANOVA revealed a significant ozone and leaf age interaction on total chlorophylls and chlorophylls *a/b* ratio according to Tukey's *post-hoc* test, different letters indicate significant differences among means in each graph ($P \leq 0.05$).

ratio.

4. Discussion

4.1. Do increasing O_3 concentrations differentially affect the macroscopic responses (in terms of biomass and visible injury) of examined clones in relation to their sensitivity?

Differential macroscopic responses were observed between O_3-sensitive and -resistant clones grown under 1.5 × AA conditions. In the Oxford plants, a significant reduction of coarse root biomass - which supports them mechanically - was documented, while fine roots, - which provide nutrients and water,- and aboveground biomass were not significantly affected. The impact of intermediate O_3 concentration seemed unequal between root classes, and the imbalance in carbon allocation/partitioning towards roots (i.e., biomass reduction) could be attributed to the appearance of visible foliar injury, and a limited production and translocation of new photo assimilates, although leaf biomass remained unchanged (Paoletti et al., 2021). In the I-214 plants exposed to 1.5 × AA, leaf and belowground biomass were not significantly affected, while a significant increase in stem and branch biomass was observed. Because new leaf development was enhanced considerably in O_3-exposed I-214 but not in the Oxford clones (Hoshika et al unpublished), it is possible that the peculiar indeterminate growth pattern of I-214 poplar shoots may have an ability to shift allocation resources to stems and branches. Considering the unchanged PII values, this ability could help the I-214 plants to cope the O_3-induced foliage damage as similarly reported in *Alnus glutinosa* with a continuous shoot growth (Hoshika et al., 2020). In both clones, the highest O_3 concentration (2.0 × AA) induced a decrease of belowground biomass regardless of the root class, which was attributed to the fact that leaves were injured, their growth were significantly suppressed and their capability to produce photosynthates was altered (Agathokleous et al., 2016). The observed increase of shoot/root ratio suggests that roots were more stressed than shoots in both clones grown under 2.0 × AA conditions, independently to their sensitivity/resistance to O_3). This could be probably due to (i) limited photosynthate production in the foliage with less carbon flux in the other organs, (ii) accumulation of photosynthates in above-ground biomass rather than belowground one, (iii) maintenance of leaves to replace O_3-injured tissues, and/or (iv) production of antioxidant and osmoprotectant compounds (Riikonen et al., 2020). The sensitivity of biomass allocation and carbon partitioning to highest O_3 concentrations did not differ between the examined clones, but may depend on leaf ontology, morphology and anatomy (Rankenberg et al., 2021). In particular, responses between old and young leaves could vary according to the increasing O_3 concentrations.

4.2. What are the metabolic and cellular mechanisms activated by chronic O_3 exposure in old and young leaves of the examined clones?

In both clones, the impact of intermediate O_3 concentrations on ROS production appeared to be leaf age-specific in view of the accumulation of H_2O_2 observed only in old leaves of the Oxford plants (where the basal levels of this ROS were significant high) and in young leaves of the I-214 ones. The induction of an oxidative burst was associated with membrane injury, indicating an inadequate response of the antioxidative systems. In old leaves of Oxford plants grown under 1.5 × AA conditions, the concomitant reduction of lutein and β-carotene indicates that these lipid-soluble antioxidants could be consumed by cells in order to counteract the ROS generation. However, these additional antioxidative mechanisms were not able to prevent the (i) rearrangement of the photosynthetic apparatus, (ii) partial breakdown of chlorophylls, and (iii) oxidative cleavage of carotenoids, that led to the production of ABA (Havaux, 2013; Pellegrini et al., 2015). In young leaves of the I-214 plants grown under 1.5 × AA conditions, on the other hand, the marked accumulation of proline and tocopherols suggests that these metabolites could provide antioxidative protection to chloroplasts, as confirmed by the unchanged content of total chlorophylls (Mesa and Munné-Bosch, 2023). Indeed, proline is anamino acid which is known to play a highly beneficial role in plants exposed to abiotic stress being an osmolyte, a

free radical scavenger, a carbon and nitrogen reserve, and a signaling molecule (Szabados and Savouré, 2010; Hayat et al., 2012). Tocopherols are the most abundant antioxidants in the chloroplast membranes and are known to protect plants against photo-oxidative damage due to an unique ability to quench singlet oxygen by a charge transfer mechanism (Neelam et al., 2023). However, the abovementioned cellular processes were not able to inhibit the peroxidation action of free radicals and protect the cell structure,as demonstrated by the observed macroscopic responses. Abscisic acid is a phytohormone that regulates many aspects of plant physiology, development and growth, and abiotic stress responses (Cutler et al., 2010). Several works documented the activation of its signalling pathway during O_3 exposure, with a significant increase in terms of content and abundance of ABA-related genes (Cotrozzi et al., 2017; McAdam et al., 2017; Landi et al., 2019). In this work, it is worth noting that ABA level decreased and seemed not to be involved in osmoregulation, but may have a pivotal role in O_3-eliciting adaptive mechanisms as the stimulation of phenylpropanoid biosynthesis; (González-Villagra et al., 2018). Conversely, intermediate O_3 concentrations did not elicit cellular H_2O_2 production in young leaves of Oxford plants and in old leaves of I-214 ones, where the basal levels of this ROS were significant high. Consequently, O_3 reacting spontaneously with unsaturated lipids of the plasma membrane could lead to the production of peroxidative processes, as confirmed by the significant increase of MDA by-products levels (Kangasjärvi et al., 2005). In young leaves of the Oxford plants grown under 1.5 × AA conditions, an accumulation of total carbohydrates was observed, but the other examined osmoprotectants did not change. This leads to exclude the involvement of carbohydrates in the osmoregulation of this sensitive clone. In particular, the occurrence of other mechanisms may justify the carbohydrate accumulation in plants as (i) remobilization of stored starch into hexoses, (ii) excess C availability due to decreased amino acid synthesis, and (iii) reduced sugars export and translocation (Liu et al., 2013; Sade et al., 2018). In old leaves of I-214 plants grown under 1.5 × AA conditions, the concomitant decrease of proline and total carbohydrates indicates that these compounds may serve as non-enzymatic antioxidants regeneration to buffer the cellular redox potential and accelerate defensive mechanisms, as confirmed by the reduced chlorophylls *a/b* ratio (Rathore and Chadhary, 2021).

The impact of the highest O_3 concentrations on ROS production did not appear to be clone-specific in view of the increased H_2O_2 levels, which may react with some plasma membrane and cell wall components by leading to uncontrolled oxidative pressure and lipid peroxidation (as confirmed by the increased MDA by-products values; Czarnocka and Karpińsky, 2018). It is worth noting that old leaves of Oxford plants showed a more severe oxidative damage as compared to young ones, probably due to a less efficacious and inadequate antioxidative system. In both young and old leaves, regardless of their age, a degradation of chlorophyll content was observed, and it did not compensate for the degradation of lipid-soluble antioxidants in the chloroplast [i.e., β-carotene and tocopherols (only in old leaves)]. The rearrangement of the pigment composition of the photosynthetic apparatus was not sufficient to stabilize the lipid phase of the thylakoid membranes and preserve the PSII photochemistry, even if it was concurrent with an accumulation of ABA and proline (this was most evident in old leaves; Wei et al., 2015). Consequently, the decline of total chlorophylls may be considered an O_3-induced secondary effect related to accelerated aging and/or nutrient remobilization,as confirmed by the significant increase in total carbohydrates (Pellegrini et al., 2015). In I-214 plants grown under 2.0 × AA conditions, differential metabolic responses were observed in relation to leaf age. In old leaves, tocopherols degradation exceeds their synthesis by inducing chlorophylls loss, lipid peroxidation and oxidative cleavage of lipid-soluble antioxidants (e.g., lutein and β-carotene), which led the production of ABA. The significant decrease of proline indicates the role of this molecule as non-enzymatic antioxidant involved in the defensive mechanisms against O_3 (Rathore and Chadhary, 2021). In young leaves, a marked accumulation of tocopherols and proline was observed suggesting that these metabolites could provide antioxidative protection to chloroplasts, as confirmed by the reduced chlorophylls *a/b* ratio (Mesa and Munné-Bosch, 2023). However, these cellular processes were not able to inhibit the peroxidation action of free radicals and protect the cell structure, as demonstrated by the observed macroscopic responses. Consequently, the decline in chlorophyll and the concomitant increase of total carbohydrates indicates that the highest O_3 concentrations could trigger early senescence or damage. It is worth noting that ABA seemed not to be involved in premature leaf death, as confirmed by its significant decrease, but may have a pivotal role in O_3-eliciting adaptive mechanisms (e.g., stimulation of phenylpropanoid biosynthesis; González-Villagra et al., 2018).

5. Conclusions

Old leaves are known to be more sensitive to O_3 in terms of visible injury than those still in expansion (Turc et al., 2021). However, the mechanisms underlying the higher O_3 tolerance in young leaves remain unclear and the differential responses as a function of the leaf development stage require further investigations. Here, we untangled the age-specific antioxidant and osmotic adjustments (and their cross-talk) that may reduce/prevent the impact of increasing O_3 concentrations on two poplar clones differing in O_3 sensitivity. Different metabolic and cellular mechanisms were activated in examined poplar clones concerning leaf age. In I-214 poplar plants grown under 1.5 × AA conditions, young leaves were less sensitive to oxidative stress due to the biochemical traits that regulate the degree of tolerance (in terms of visible injury), carbon allocation strategy, detoxification and/or repair processes, leading to high tolerance against the negative impacts of O_3 on plant biomass development.

CRediT authorship contribution statement

Claudia Pisuttu: Writing – original draft, Investigation, Formal analysis. **Samuele Risoli:** Writing – review & editing, Data curation. **Lorenzo Cotrozzi:** Writing – review & editing, Validation. **Cristina Nali:** Writing – review & editing, Supervision, Conceptualization. **Elisa Pellegrini:** Writing – original draft, Supervision, Resources, Conceptualization. **Yasutomo Hoshika:** Writing – original draft, Validation, Investigation. **Barbara Baesso Moura:** Writing – review & editing, Investigation, Formal analysis. **Elena Paoletti:** Writing – review & editing, Supervision, Resources.

Declaration of competing interest

The authors declare no competing financial interest.

Data availability

The authors do not have permission to share data.

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