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Abstract

Ozone is considered to be a major phytotoxic pollutant. It is an oxidizing molecule with harmful effects that can affect human health and vegetation. Due to its phytotoxicity, it constitutes a threat to food security in a context of climate change. Proline accumulation is induced in response to numerous stresses and is assumed to be involved in plant antioxidant defense. We therefore addressed the question of the putative involvement of proline in plant ozone responses by analyzing the responses of two Arabidopsis mutants (obtained in the Col-0 genetic background) altered in proline metabolism and different ecotypes with various ozone sensitivity, to controlled ozone treatments. Among the mutants, the *p5cs1* mutant plants accumulated less proline than the double *prodh1xprodh2* (*p1p2*) mutants. Ozone treatments did not induce accumulation of proline in Col-0 nor in the mutant plants. However, the variation of the photosynthetic parameter Fv/Fm in the *p1p2* mutant suggests a positive effect of proline. Proline accumulation induced by ozone was only observed in the most ozone-sensitive ecotypes, Cvi-0 and Ler. Contrary to our expectations, proline accumulation could not be correlated with variations in protein oxidation (carbonylation). On the other hand, flavonols content, measured here, using non-destructive methods, reflected exactly the genotypes ranking according to ozone sensitivity.

Keywords

ozone, proline metabolism, oxidative stress, flavonols, *Arabidopsis thaliana* (L.) Heynh, protein carbonylation

1. Introduction

Plants are subjected to many environmental constraints such as low or high temperatures, drought, ultraviolet (UV) radiation and gaseous pollutants like tropospheric ozone (O_3). Tropospheric ozone is a secondary pollutant formed by photochemical reactions of anthropogenic precursors, mainly nitrogen oxides (NOx) and volatile organic compounds (including the non-anthropogenic plant-emitted volatile organic compounds). Tropospheric ozone concentrations have increased considerably over the past century (Parrish et al., 2012) and predictions show that they will remain high for several decades (Sicard et al., 2017). In addition, ozone is considered as a highly phytotoxic air pollutant (Tiwari and Agrawal, 2018).

The extent of damage to plants induced by ozone is a function of the intensity and duration of the exposure to the pollutant, in other words, of the ozone dose (Mills et al., 2011). Acute exposure, that is, exposure to high concentrations of ozone for short periods of time, can trigger hypersensitivity

responses characterized by the appearance of necrotic lesions on leaf surfaces (Kangasjarvi et al., 2005). In *Arabidopsis thaliana* (L.) Heynh, 93 ecotypes have been ranked according to their sensitivity to ozone, based on such leaf symptoms (Brosché et al., 2010). Plants chronically exposed to current ozone concentrations (Sicard et al., 2017) may not develop necrotic leaf symptoms. Rather, photosynthesis reduction, inhibition of plant growth and, over time, premature senescence may be observed (Gandin et al., 2021; Jolivet et al., 2016). Indeed, current tropospheric ozone concentrations are sufficiently high to reduce yield in many plant species in several regions of the world (Emberson et al., 2018). In poplar, Holm oak, wheat or common bean, as much as 10% in leaf, root, seed and/or total biomass can be lost to ozone (Hayes et al., 2020), even if the impact of ozone varies between species and between cultivars. For example, wheat and bean are considered as sensitive to ozone whereas barley is classified as resistant (Mills et al., 2007).

Ozone is absorbed by plants through stomata, which can lead to a decrease in stomatal conductance. Once in the stomatal cavity, ozone is dissolved in the apoplasm and this results in the formation of reactive oxygen species (ROS) (Kangasjarvi et al., 2005). If the plant's antioxidant systems become overwhelmed by ozone-induced ROS, damage to major cellular biomolecules occurs (proteins, lipids, nucleic acids and chlorophylls) (Foyer and Noctor, 2005). Decrease in leaves chlorophylls has been repeatedly observed, for example in Arabidopsis (Miller et al., 1999), bean (Leitao et al., 2008) or trees (Jolivet et al., 2016) exposed to ozone. Furthermore, changes in chlorophyll fluorescence indicate that the efficiency of the photochemical reaction is negatively affected as a consequence of the inactivation of some proteins associated with photosystem II (Feng et al., 2018). Carbon fixing reactions are also affected since up to 30% of the activity of ribulose-1,5-bisphosphate carboxylase / oxygenase (RuBisCO) was lost in the leaves of plants (bean, wheat, maize or poplar) exposed to ozone (Bagard et al., 2015; Leitao et al., 2008).

ROS formed from O_3 degradation can oxidize the proteins and protein carbonylation is the most common modification induced by ROS. Carbonylation involves the incorporation of an aldehyde or ketone onto an amino acid side chain (Coffey and Gronert, 2016). Protein carbonylation is usually used as a good indicator of stress because, among all cellular oxidative stress markers, it has the advantage of being irreversible. Compared to other markers such as glutathionyled proteins or malondialdehyde, carbonylated proteins are more stable (Dalle-Donne et al., 2003). For all these reasons, level of carbonylated proteins is considered as a good indicator of oxidative stress and is commonly used as a marker of the level of protein oxidation in humans (Mannaa and Hanisch, 2020) as well as in plants (Anjum et al., 2015).

In plants, the amino acid proline is known to participate in the biosynthesis of primary metabolism components, and to play a role during the growth and development (Verbruggen and Hermans, 2008), notably as a component of cell wall proteins (Kavi Kishor et al., 2015). Moreover, proline is known to accumulate in plant tissues in response to many environmental stresses such as drought, high salinity, high temperature, freezing, UV radiation, heavy metals and ozone (Calzone et al., 2019; Szabados and Savouré, 2010; Verbruggen and Hermans, 2008). Under conditions of osmotic stress, proline can act as an osmoprotectant, specifically in halophyte plants, as a molecular chaperone, a stabilizer of cellular structures, a scavenger of free radicals and an energy sink (Szabados and Savouré, 2010). Accumulation of proline confers stress tolerance by maintaining cell turgor or osmotic balance, by stabilizing membranes (thus preventing electrolyte leakage) and by maintaining physiological ROS concentrations (thus preventing/limiting oxidative stress) (Hayat et al., 2012; Smirnoff and Cumbes, 1989). Indeed, proline has been shown to be responsible for the scavenging ROS and other free radicals (Ben Rejeb et al., 2014; Szabados and Savouré, 2010) although its role in the direct scavenging of singlet oxygen could be discussed (Signorelli et al., 2014). Its role in plants response to ozone needs further studying

and directed alteration of proline metabolism could be an appropriate approach. In plants, proline metabolism is compartmentalized and is distributed between cytosol, mitochondria and possibly chloroplasts under stress conditions (Szabados and Savouré, 2010). Proline is mainly synthesized in the cytosol from glutamate that is transformed into Δ'-pyrroline-5-carboxylate (P5C) by Δ'-pyrroline-5-carboxylate synthetase (P5CS) (Funck et al., 2008; Verbruggen and Hermans, 2008). P5C is then reduced to proline by the pyroline-5-carboxylate reductase (P5CR) enzyme. The catabolism of proline occurs in mitochondria and under the command of the proline dehydrogenase (ProDH) that oxidizes proline in P5C, which is then converted to glutamate by the pyroline-5-carboxylate deshydrogenase (P5CDH) enzyme (Hayat et al., 2012). Proline content can be regulated by both biosynthetic and catabolic pathways. P5CS activity was shown to be the rate-limiting step of proline biosynthesis in plants (Kavi Kishor et al., 2015). This enzyme is encoded by two genes in Arabidopsis, *P5CS1* and *P5CS2*. Study of knockout *p5cs1* mutants indicated that this isoform is mainly involved in proline accumulation during osmotic or salt-stress while P5CS2 is required for embryogenesis, during the late stages of seed development (Székely et al., 2008). On the opposite, proline catabolism is mainly induced after stress recovery and senescence, ProDH enzymes being the rate-limiting steps (Cabassa-Hourton et al., 2016; Launay et al., 2019). In Arabidopsis, PRODH is encoded by two genes, *PRODH1* and *PRODH2*, both being involved in abiotic stress responses (Cabassa-Hourton et al., 2016). Both the single *p5cs1* and double *prodh1xprodh2 (p1p2)* mutants were shown to differentially accumulate proline compared to wild type, in response to abiotic stresses like salt stress (for *p5cs1* mutant) and dark induced senescence (for *prodh1xprodh2* mutant) (Cabassa-Hourton et al., 2016; Launay et al., 2019).

In order to test if proline metabolism is linked to ozone stress perception and response, we conducted two experiments, the first one using mutants altered in proline content and the second one using different *Arabidopsis thaliana* ecotypes with variable ozone sensitivity. In line with proline's putative antioxidant properties, we hypothesized that proline accumulation in response to controlled ozone treatments would result in enhanced tolerance to oxidative stress in Arabidopsis plants. As a result, several parameters were analyzed to characterize plant growth and leaf protein oxidation level in relation with the plants ability to accumulate proline.

2. Materials and Methods

2.1. Plant materials

Four ecotypes of *Arabidopsis thaliana* (L.) Heynh were used: Col-0, Ts-1, L*er*, Cvi-0 kindly provided by Dr Verslues. Two mutants in the Col-0 background, affected in proline metabolism were used: *p5cs1* (salk_063517) and *p1p2* (*prodh1-4* and *prodh2-2* double-mutant (Cabassa-Hourton et al., 2016).

In a first experiment, seeds of Col-0, *p5cs1* and *p1p2* were sown in small pots (7.5 cm in diameter) filled with potting soil/peat (Klashmann-Dailmann, TS3). In a second experiment, seeds of Col-0, Ts-1, L*er* and Cvi-0 were sown on Jiffy-7® peat pellets (Jiffy International, Kristiansand, Norway) placed in smaller pots (4.5 cm in diameter).

In both experiments, after sowing, the pots were placed at 4°C, in darkness for two days (stratification). Plants were thereafter grown for 5 weeks in phytotronic chambers (Adaptis 1000, Conviron) maintained at 20°C/22°C (night/day), 60% of relative humidity, with 8 hours of photoperiod (200 $\mu mol.m^2.s^{-1}$) from 8:30 am to 4:30 pm. Plants were regularly watered by sub-irrigation and fertilized weekly with 5 mL of fertilizer (Algoflash) per pot.

2.2. Ozone exposure and collection of plant material

Ozone generation and regulation in the growth chamber involved an ozone generator (C-Lasky, AirTree Ozone Technology Co. Ltd.), an ozone monitor (106-L, 2B Technologies) coupled to a continuous recorder of ozone concentrations (midi logger GL220, Graphtec). To evaluate plant exposure to ozone, the AOT40 index was calculated. The AOT40 (Accumulated Ozone exposure over a Threshold of 40 ppb; ppb h) was determined as the sum of the differences between hourly ozone concentrations and 40 ppb for each hour when the concentration exceeds 40 ppb over the fumigation period (Fuhrer et al., 1997).

In the first experiment, five-week-old plants (n = 14) were exposed to 240 ppb of ozone during six hours, for 16 consecutive days. Ozone was applied in the middle of the photoperiod, between 9:30 am and 3:30 pm. In parallel, control plants (n = 14) were cultivated under ambient ozone conditions in a similar phytotronic chamber. At the end of the ozone treatment, eight ozonated plants and eight control plants were randomly selected; six mature leaves (leaf ranks 10 to 16; the numbering of leaves was performed from the oldest to the youngest) were collected per plant and per genotype, weighed and immediately frozen in liquid nitrogen for biochemical analyzes. The other six plants (per treatment and per genotype) were used for ecophysiological analyzes.

In the second experiment, five-week-old plants (n = 14) were exposed to 240 ppb of ozone during six hours, for 10 consecutive days. As described previously, ozone was applied between 9:30 am and 3:30 pm. Control plants (n = 14) were cultivated under ambient ozone conditions. After 10 days of ozone treatment, nine ozonated plants and nine control plants were randomly selected; six mature leaves (leaf ranks 10 to 16) were collected and weighed. From these 9 plants, leaves of 3 different plants were pooled and frozen in liquid nitrogen in order to have enough material for biochemical analyzes.

2.3. Estimation of leaf area and biomass

At the end of the experiments, plants were photographed with a Samsung ST73 camera, screwed to a camera stand, at fixed height and angle. The projected areas, determined using the Image J software, were considered as the estimated total leaf areas. The aboveground biomass was determined by weighing each rosette immediately after sampling (fresh biomass) and after drying at 60 °C for 48 hours (dry biomass).

2.4. Determination of the total chlorophyll content and flavonol index

Leaf total chlorophyll and flavonol index were non-destructively measured, using the DUALEX® device (Force-A, Orsay, France; Cerovic et al., 2012). This device is a leafclip optical sensor which estimates chlorophyll and flavonol contents of plant leaves, based on absorbance and transmittance of specific wavelengths of visible and near infrared light. For each plant, five measurements were made on the 12^{th} fully-developed leaf (counting from the oldest one). Thus, the total chlorophyll content and flavonol index values are the means of these five measurements.

2.5. Measurement of chlorophyll fluorescence parameters

In the first experiment, parameters derived from chlorophyll fluorescence were measured, using a Pulse Amplitude Modulation Fluorescence Monitoring System 1 (FMS1, Hansatech Instruments,

Norfolk, UK) (n = 6). After 30 minutes of dark adaptation, F_0 (minimum fluorescence yield) and the Fv/Fm ratio (maximum quantum yield of photosystem II) were quantified on the 12th leaf, by application of a short flash of saturated light (12000 µmol.m^{-2}.s^{-1} for 0.3 s). Following these measurements, the leaves were exposed to a continuous actinic light source (240 µmol.m^{-2}.s^{-1}) for 15 minutes. The parameters Φ PSII (quantum yield of photosystem II), qP (photochemical quenching) and qNP (non-photochemical quenching) were quantified after application of a short flash of saturated light (12000 µmol.m^{-2}.s^{-1} for 0.3 s).

In the second experiment, F_0 (after 30 minutes of dark adaptation) and the Fv/Fm ratio (after application of a short flash of saturated light at 3000 µmol.m^{-2}.s^{-1} for 0.3 s) were measured on the 12th plant leaf, using a HandyPEA (Hansatech Instruments Ltd) (n = 10). Measurements on ozonated Cvi-0 plants were impossible because ozone-induced leaf damage was too important.

2.6. Proline assay

Proline content was spectrophotometrically determined at 520 nm according to Bates' method, using 30 to 40 mg of frozen leaf tissue (Bates et al., 1973).

2.7. Quantification of carbonylated proteins according to the fluorescein-5-thiosemicarbazide (FTC) method

Total soluble leaf protein extraction was carried out in lysis buffer (50 mM Tris, 1 mM EDTA, 1.5% PVPP (w/v), pH 7.5, extemporaneously supplemented with protease inhibitors: 20 µM E-64, 1 mM Pefabloc SC, 1 mM 1,10 phenantroline). After homogenization, samples were centrifuged twice at 21500 *g* for 10 minutes at 4° C. The supernatants were collected and stored on ice.

The Bradford method was used to quantify protein content (Bradford, 1976). Removal of residual nucleic acids and FTC derivatization of proteins were performed according to Havé et al. (2015) with the following modifications: final FTC concentration was 4 mM and the resuspension buffer contained 8 M urea, 1 mM EDTA, 150 mM NaCl, 100 mM Tris, pH 8.0, 20 µM E-64, 1 mM Pefabloc SC and 1 mM 1,10-phenantroline.

Ten µg of FTC-derivatized proteins were separated by SDS-PAGE in 20% acrylamide gels (mini-Criterion, Bio-Rad) using TGS running buffer (Tris/glycine/SDS, pH 8.3 buffer, Bio-Rad). After separation of the proteins, the in-gel capture of the FTC signal was achieved, using an EZ Imager (Bio-Rad) with λ_{em} = 516 nm and λ_{ex}= 492 nm. The gel was then stained with colloidal Coomassie blue according to the protocol of Dyballa and Metzger (2009) and total protein signal acquisition was made with the EZ Imager.

FTC and Coomassie blue signal analyses were performed by densitometry, using the Image Lab™ software (Bio-Rad). FTC signals were normalized to Coomassie blue signals and expressed as arbitrary units.

2.8. Statistical analyses

Statistical analyses were performed using the R software v 3.6.1 (R Core Team, 2020). In order to determine any differences between the ecotypes, treatments and interactions between these two factors, 2-way ANOVA followed by multiple comparison tests were performed if residuals were

normally distributed (Shapiro test) and variances were homogeneous (Bartlett test). When the number of replicates was homogeneous, a type I 2-way ANOVA was performed, followed by a Tukey HSD test whereas when it was not homogeneous, a type II 2-way ANOVA was performed (using the car package) followed by a Pairwise T-test with a Bonferroni correction. If residuals were not normally distributed or variances were not homogeneous, a non-parametric test was performed (Kruskal-Wallis test followed by a Pairwise Wilcoxon test with a Bonferroni correction). Only the significant differences due to ozone treatment were mentioned on the figures and results of p-values are reported in table S2. Results are considered significant at p-value < 0.05 (threshold = 5%).

3. Results and Discussion

3.1. Ozone exposure

The ozone concentrations actually experienced by the plants were recorded throughout the experiments. The means of the daily ozone concentrations were calculated from the hourly ozone averages measured during the fumigation periods (Fig. S1 A and C). It was thus possible to determine the hourly average ozone concentrations, at which the plants were exposed daily during the 6 hours of fumigation. Overall, during the first experiment, the average ozone concentration was 239.4 ppb ± 17.6 ppb and the AOT40 was 21.9 ppm.h after 16 days (Fig. S1 A-B). For the second experiment, the average ozone concentration was 237.9 ppb ± 6.2 ppb and the AOT40 was 11.7 ppm.h after 10 days (Fig. S1 C-D). For this second experiment, ten days into the ozone treatment, the Cvi-0 ecotype showed severe leaf injuries. As a result, the experiment was stopped. Daily variations in hourly ozone concentration are reported in Fig. S1 E and F for experiment 1 and 2 respectively.

Ozone treatments in both experiments were severe. Indeed, the average daily ozone concentrations in Europe does not exceed 35 ppb (Sicard et al., 2020). Moreover, the AOT40 critical level for a reduction of wheat yield is of 3 ppm.h after 3 months (Mills et al., 2007).

3.2. Effects of ozone exposure on Arabidopsis proline-metabolism mutants

3.2.1. Effects of ozone on leaf morphology and biomass

At the end of the ozone treatment (240 ppb over 16 days), no leaf necrosis/chlorosis was visible, regardless of the genotype (Col-0, *p5cs* and *p1p2*). However, all ozonated plants of the three genotypes simultaneously developed leaf curling (Fig. 1A). Average rosette projected areas were between 86 cm^2 and 105 cm^2 (Fig. 1B). Average rosette fresh weights were between 4.08 g and 5.09 g and dry weights between 0.31 g and 0.38 g (Fig. 1C and 1D). No significant difference was observed between genotypes for rosette area, fresh weight and dry weight (Fig. 1B-D), or between control and ozonated plants regardless of the genotype (2-way ANOVA (type I), p-values > 0.05).

3.2.2 Effect of ozone on the fluorescence parameters of chlorophyll

All fluorescence parameters of chlorophyll measured showed similar values for Col-0 and *p5cs* genotypes regardless of the treatment. In the *p1p2* mutant, the ozone treatment had a significant impact (Fig. 1E-F): ozonated *p1p2* plants showed lower F_0 values (≈ -25%) than control plants (Fig. 1E) (Tukey HSD test, p-value = 0.006), and they had a slightly higher maximum quantum yield of photosystem II Fv/Fm ratio (+3%) (Fig. 1F) (Pairwise Wilcoxon test, p-value = 0.032). Regarding Φ PS2, qP and qNP parameters, no significant difference was observed (for Φ PS2, pairwise Wilcoxon test, p-values > 0.05, for qP and qNP, Tukey HSD test, p-values > 0.05; data not shown), regardless of genotypes and treatments.

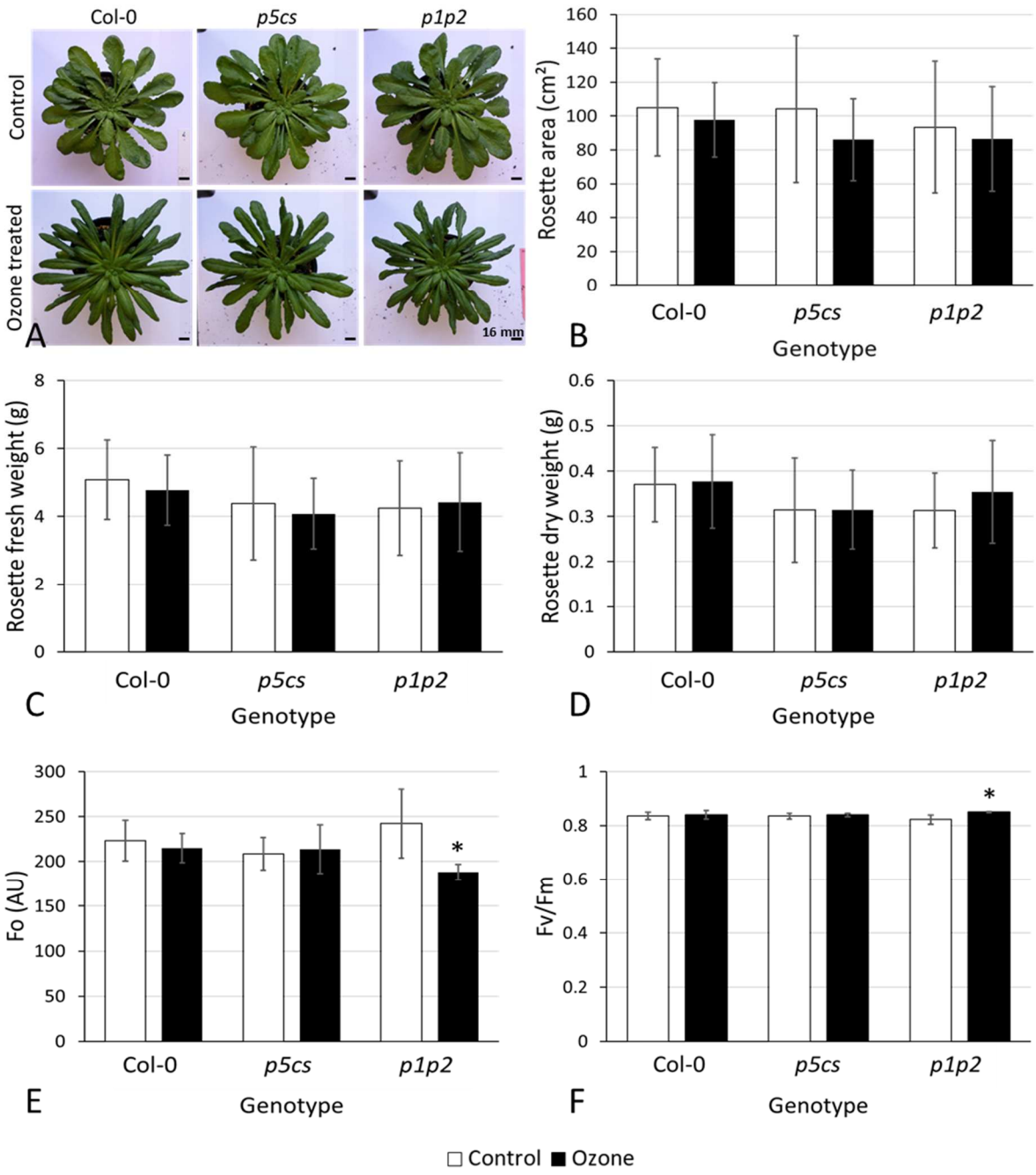

Figure 1: Impacts of ozone (239.4 ppb ± 17.6 ppb over 16 days) on qualitative and quantitative morphological traits (ozonated plants, black bars; control plants, white bars). Col-0: wild-type; *p5cs*: Col-0 mutant affected in the P5CS enzyme (*p5cs1*); *p1p2*: Col-0 mutant affected in the two isoforms of the proline dehydrogenase (*prodh1 prodh2*).

Pictures of representative plants (A); Projected rosette area (cm^2) (B); rosettes fresh (C) and dry (D) weight (g) (n = 14). F_0 (Minimum fluorescence yield, AU (E) and Fv/Fm ratio (maximum quantum yield of photosystem II) (F) (n = 6). Values represented are means ± SD.* represents a significant effect of ozone compared to control (Rosette area, rosette fresh and dry weight, F_o: Tukey HSD test; Fv/Fm: Pairwise Wilcoxon test; threshold = 5%). For ANOVA or Kruskal-Wallis results, see table S2.

3.2.3 Effects of ozone on leaf pigments, soluble protein content, protein carbonylation level and proline content

Total chlorophyll contents and flavonol indexes measured on the 12th leaves were between 24.2 and 27.2 µg/cm² (Fig. 2A) and between 0.16 and 0.19 µg/cm² respectively (Fig. 2B). No significant difference was observed between ozonated and control plants, nor between genotypes for the same treatment, for both parameters (Tukey HSD test, p-values > 0.05). The soluble leaf protein contents were between 9.11 and 10.5 µg/mg of fresh weight (FW) and were statistically equivalent between treatments and genotypes (Fig. 2C) (2-way ANOVA (type I), p-values > 0.05). Protein carbonylation index, quantified using FTC method, was weak for all the genotypes and treatments (Fig. 2D). Furthermore, no significant difference in the carbonylation index was observed between treatments and genotypes (2-way ANOVA (type I), p-values > 0.05).

Wild-type Col-0 plants (both ozonated and controls) had leaf proline contents (2.36 µmol/g FW) intermediate between those of *p5cs* plants (1.52 µmol/g FW) and those of *p1p2* plants (above 3 µmol/g of FW) (Fig. 2E). The proline contents in *p5cs* were significantly lower than those in *p1p2* plants (Tukey HSD test, p-value < 0.003). The ozone treatment did not alter the proline contents in either of the genotypes (2-way Anova, p-values > 0.05; Tukey HSD test, p-values > 0.05).

3.2.4 Discussion of the first experiment with proline-metabolism mutants

These results, obtained after a severe ozone stress (240 ppb), did not show any effects on growth or biochemical parameters in the wild-type Col-0. The lack of ozone effect on aerial biomass agrees with the results of Brosché et al. (2010) where Col-0 was used as a reference genotype for ozone tolerance and did not develop any leaf necrosis. Sharma & Davis (1994), using either 150 or 300 ppb of ozone for durations similar to ours, observed small pinhead-sized dry lesions on leaves, for the 300 ppb treatment only. This can be related to the absence of necrosis observed in our case at 240 ppb (Fig. 1). Nevertheless, in this study, a decrease in the Col-0 ecotype biomass was observed (Sharma and Davis, 1994). High ozone tolerance in the Col-0 ecotype was confirmed by the absence of accumulation of carbonylated proteins, a parameter that is considered as a biomarker of oxidative stress (Dalle-Donne et al., 2003).

Our results of rosette areas agree with those of other studies carried out on poplar and maize, where the ozone treatment did not modify leaf surface (Bagard et al., 2008; Leitao et al., 2007a). However, another study, carried out on the L*er* ecotype of *Arabidopsis thaliana*, showed a decrease in the development of the rosette leaves, thus leading to a decrease in the surface of the rosettes (Miller et al., 1999).

Leaf curling has previously been observed in the Col-0 ecotype of Arabidopsis in response to different ozone concentrations. Indeed, Sharma and Davis (1994) observed the apparition of leaf curling after 3 days of treatment at 150 ppb or 300 ppb of ozone. These authors suggested that ethylene could be involved in leaf curling. This is in agreement with the fact that ethylene production corresponds to an early response to ozone in numerous species (reviewed in Langebartels et al., 2002; Vainonen and Kangasjärvi, 2015).

Our results about total chlorophyll contents between ozonated plants and control plants are not in agreement with the results generally observed in the literature for different plant models. Indeed, studies carried out on the L*er* ecotype of Arabidopsis (Miller et al., 1999), poplar (Bagard et al., 2008), bean (Leitao et al., 2008) and maize (Leitao et al., 2007) showed a decrease in total chlorophyll content in the leaves, in response to ozone. However, it was also shown that a mild dose of ozone can lead to

an increase in chlorophyll content in maize leaves (Leitao et al., 2007b), a phenomenon probably linked to hormesis (Agathokleous et al., 2019).

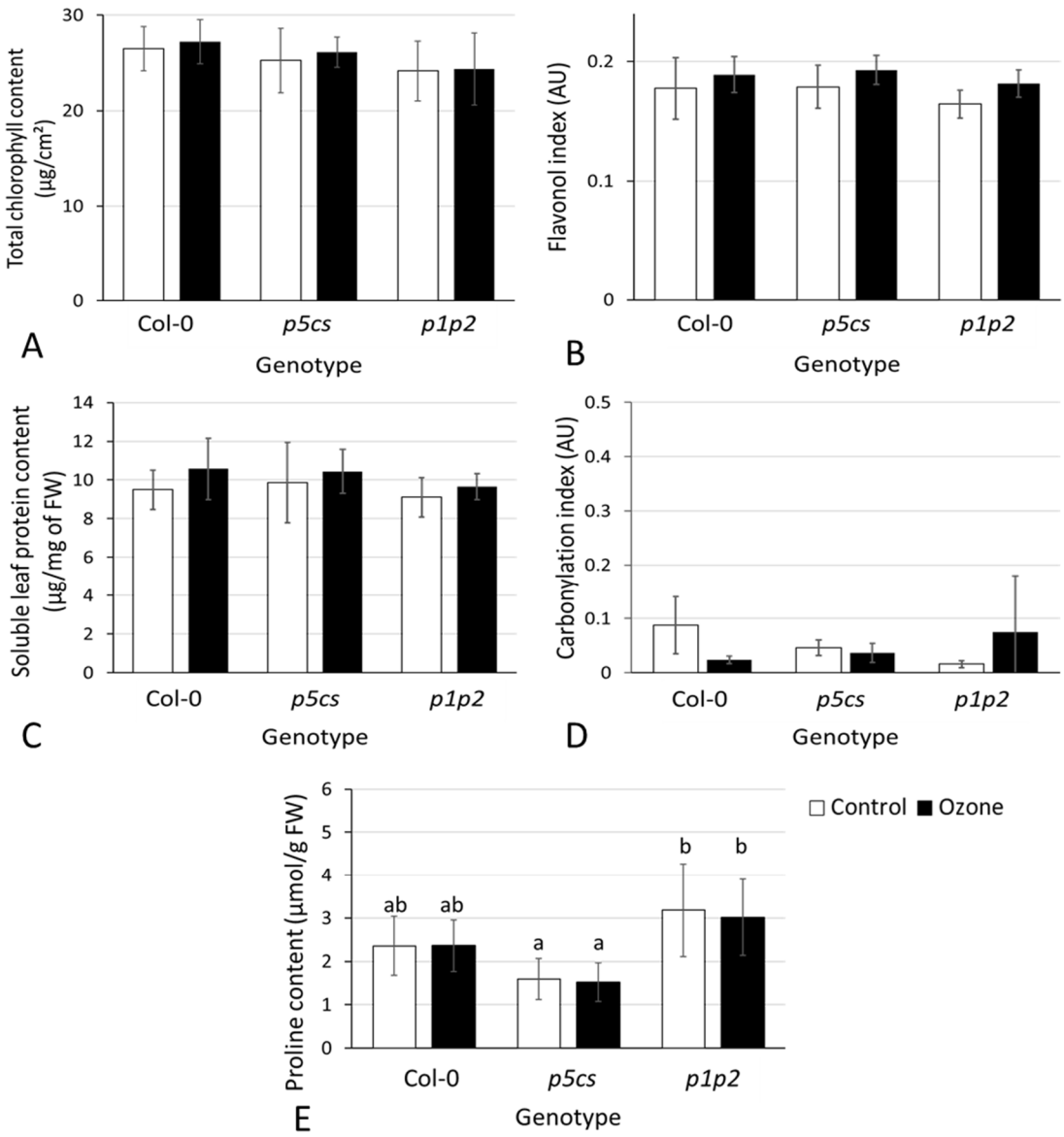

Figure 2: Impacts of ozone (239.4 ppb ± 17.6 ppb over 16 days) on biochemical parameters (ozonated plants, black bars; control plants, white bars). Col-0: wild-type; *p5cs*: Col-0 mutant affected in the P5CS enzyme (*p5cs1*); *p1p2*: Col-0 mutant affected in the two isoforms of the proline dehydrogenase (*prodh1 prodh2*).
Total chlorophyll contents (µg/cm², A) and flavonol index (AU, B) measured on the 12th expanded leaf with Dualex®; total protein content (µg/g of FW, C); carbonylation of proteins revealed by FTC signal (AU, D); and proline content (µmol/g of FW, E). Values represented means ± SD (n = 14 for total chlorophyll content and flavonol index and n = 8 for the other parameters). The letters represent the significant differences between genotypes and treatment (Total chlorophyll content, flavonol index, protein content and proline content: Tukey HSD test; carbonylation index: Pairwise Wilcoxon test; threshold = 5%). For ANOVA or Kruskal-Wallis results, see table S2.

Concerning proline, our results confirmed that the mutations in *P5CS* gene or in the two *ProDH* genes respectively induced lower (-36%) or higher proline (+27%) contents, compared to the wild type, although the difference was only statistically significant between the *p5cs* and *p1p2* genotypes. This is in agreement with previous results obtained by Székely et al. (2008) and Launay et al. (2019) on well-watered plants. Proline is known to accumulate in response to different environmental stresses (Hayat et al., 2012; Zegaoui et al., 2017) and *p5cs* mutants were more sensitive to salt stress (Székely et al., 2008). In our study, no difference in proline content was observed between ozonated and control Arabidopsis plants regardless of genotype.

Since ozone did not impact the proline content of Col-0 and mutant plants, its involvement in tolerance to ozone could be questioned. However, the *p1p2* mutant characterized by the highest proline content also showed the lowest F_0 values and the highest Fv/Fm ratio. Low F_0 was indicative of a physical dissociation between photosystem II reaction centers and light harvesting complexes that is difficult to interpret in the absence of chlorophyll degradation. On the other hand, the high Fv/Fm ratio in this genotype concurrent with high proline contents suggested that proline could nevertheless play a protective role as stresses inducing damage of PSII usually result in reductions in Fv/Fm (Murchie and Lawson, 2013).

Since we concluded to the elevated tolerance to ozone in Col-0, the putative link between ozone and proline had to be further analyzed in other Arabidopsis ecotypes. Thus, a second ozone exposure experiment was conducted, using the natural variability in ozone tolerance amongst Arabidopsis ecotypes, as previously established by Brosché et al., 2010.

3.3. Effects of ozone exposure on different Arabidopsis ecotypes

3.3.1. Effects of ozone on leaf morphology and biomass

In response to the second ozone treatment (237.9 ppb ± 6.2 ppb over 10 days), Ts-1 and Col-0 ecotypes showed no foliar symptoms except leaf curling for Col-0 (Fig. 3A). The L*er* and Cvi-0 ecotypes both showed leaf necrosis and early senescence symptoms, such as chlorosis (Fig. 3A). Average rosette projected areas were between 12 and 42 cm² (Fig. 3B). For all genotypes, rosette fresh weights were between 0.2 g and 0.84 g (Fig. 3C) and the dry weights between 0.03 g and 0.07 g (Fig. 3D). For rosettes areas and fresh weights, no significant difference was observed between control and ozonated plants for Col-0, Ts-1 and L*er* (Pairwise T-test, p-values> 0.05). However, ozonated Cvi-0 plants showed the smallest rosettes areas (-68%) and fresh weight (-71%) (Pairwise T-test, p-values < 0.05). Concerning rosette dry weight, no significant difference was observed between control and ozonated plants regardless of the ecotype (Fig. 3D) (2-way ANOVA (type II), p-value = 0.67).

3.3.2.Effects of ozone on the fluorescence parameters of chlorophyll

F_0 (Fig. 3E) and Fv/Fm (Fig. 3F) were not affected by ozone treatment in Ts-1 and Col-0 plants (Pairwise Wilcoxon test and Pairwise T-test, p-values > 0.05). However, L*er* showed a significant decrease in F_0 (-22%) (Pairwise T-test, p-value = $1.90*10^{-12}$) and Fv/Fm (-5.5%) (Pairwise Wilcoxon test, p-value = 0.0257) as a response to the ozone treatment. Unfortunately, it was not possible to measure these parameters on ozonated Cvi-0 plants as the leaves selected for analysis were completely dry by the 10^{th} day.

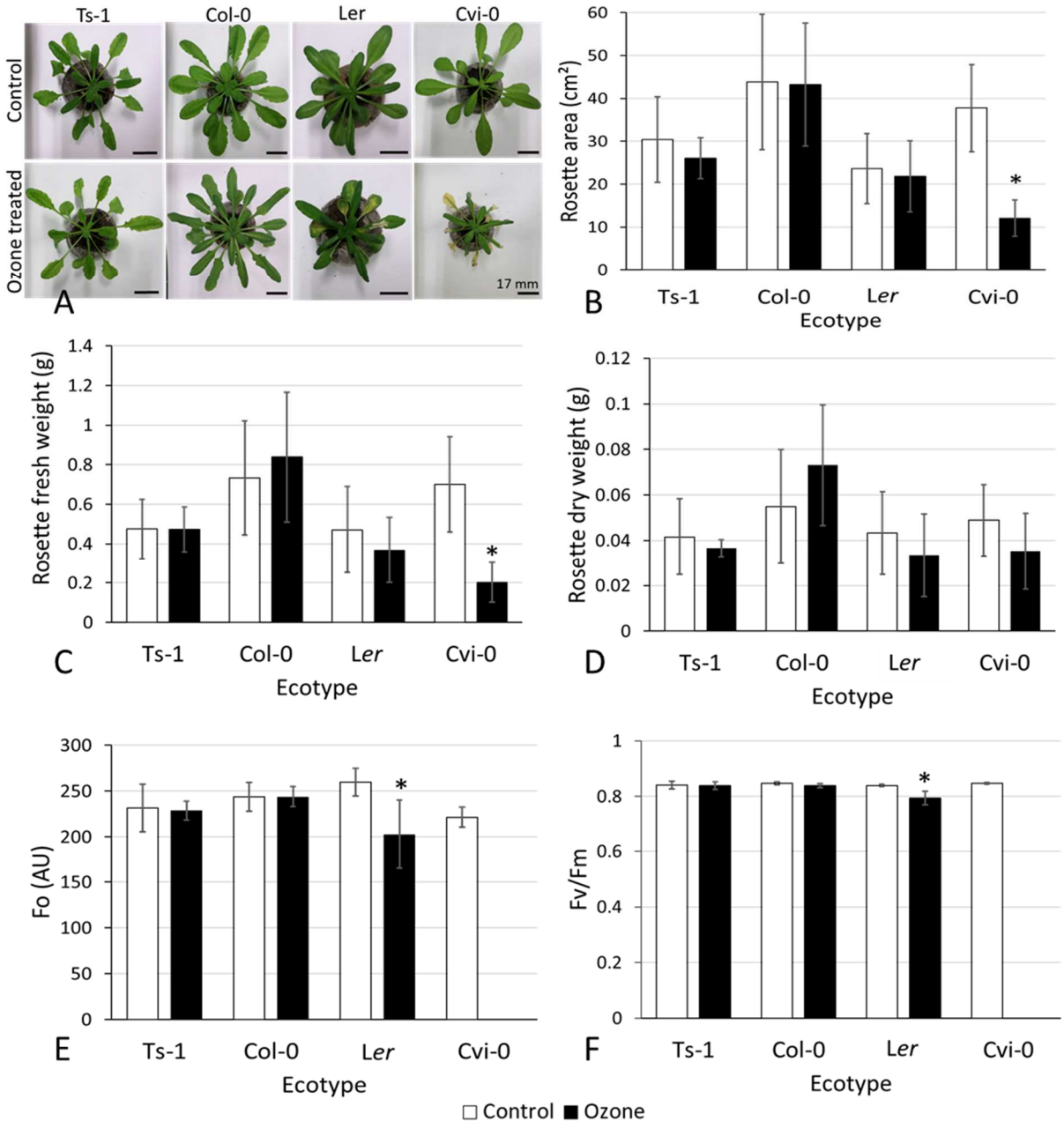

Figure 3: Impacts of ozone (237.9 ppb ± 6.2 ppb over 10 days) on qualitative and quantitative morphological traits (ozonated plants, black bars; control plants, white bars) of different Arabidopsis ecotypes (Ts-1, Col-0, L*er* and Cvi-0).

Pictures of representative plants (A); Projected rosette areas (cm²) (B) (n = 14), rosette fresh (C) (n = 14) and dry (D) (n = 5) weight (g); F_0 (minimum fluorescence yield) (AU) (E) (n = 10) and Fv/Fm ratio (maximum quantum yield of photosystem II) (F) (n = 10). Values represented means ± SD. * represents a significant effect of ozone compared to control (Rosette area, rosette fresh and dry weight and F_0: Pairwise T-test; Fv/Fm: pairwise Wilcoxon test; threshold = 5%). For ANOVA or Kruskal-Wallis results, see table S2

3.3.3. Effects of ozone on leaf pigments, soluble protein content, protein carbonylation level and proline content

Overall, leaf total chlorophyll contents and flavonol indexes were between 4.8 and 19.3 µg/cm² (Fig. 4A) and between 0.10 and 0.41 (Fig. 4B) respectively. In Col-0 and L*er*, ozone had no significant impact on the total chlorophyll contents (Fig. 4A) (Pairwise Wilcoxon test, p-values > 0.05). Ozonated Ts-1 plants accumulated 26% more chlorophylls than the corresponding control plants (Pairwise Wilcoxon

test, p-value = $9.4*10^{-5}$) whereas ozonated Cvi-0 plants had far less chlorophylls than their control (-67%) (Pairwise Wilcoxon test p-value = $6.9*10^{-5}$). Flavonol index increased in response to ozone: Ts-1 (+13%), Col-0 (+30%), L*er* (+60%) and Cvi-0 (+173%) (Fig. 4B, Pairwise Wilcoxon test, p-value < 0.0023). Overall, leaf soluble protein contents were between 7.1 and 11.8 µg/mg of fresh weight and were not significantly influenced by the ozone treatment (Fig. 4C, 2-way ANOVA (type I), p-value = 0.18). It should be noted that under control conditions, L*er* plants presented a significantly higher protein content than Cvi-0 plants (Tukey HSD test, p-value = 0.0132). In ozonated plants, Cvi-0 had a significantly lower protein content than L*er* and Col-0 (Tukey HSD test, p-values < 0.05).

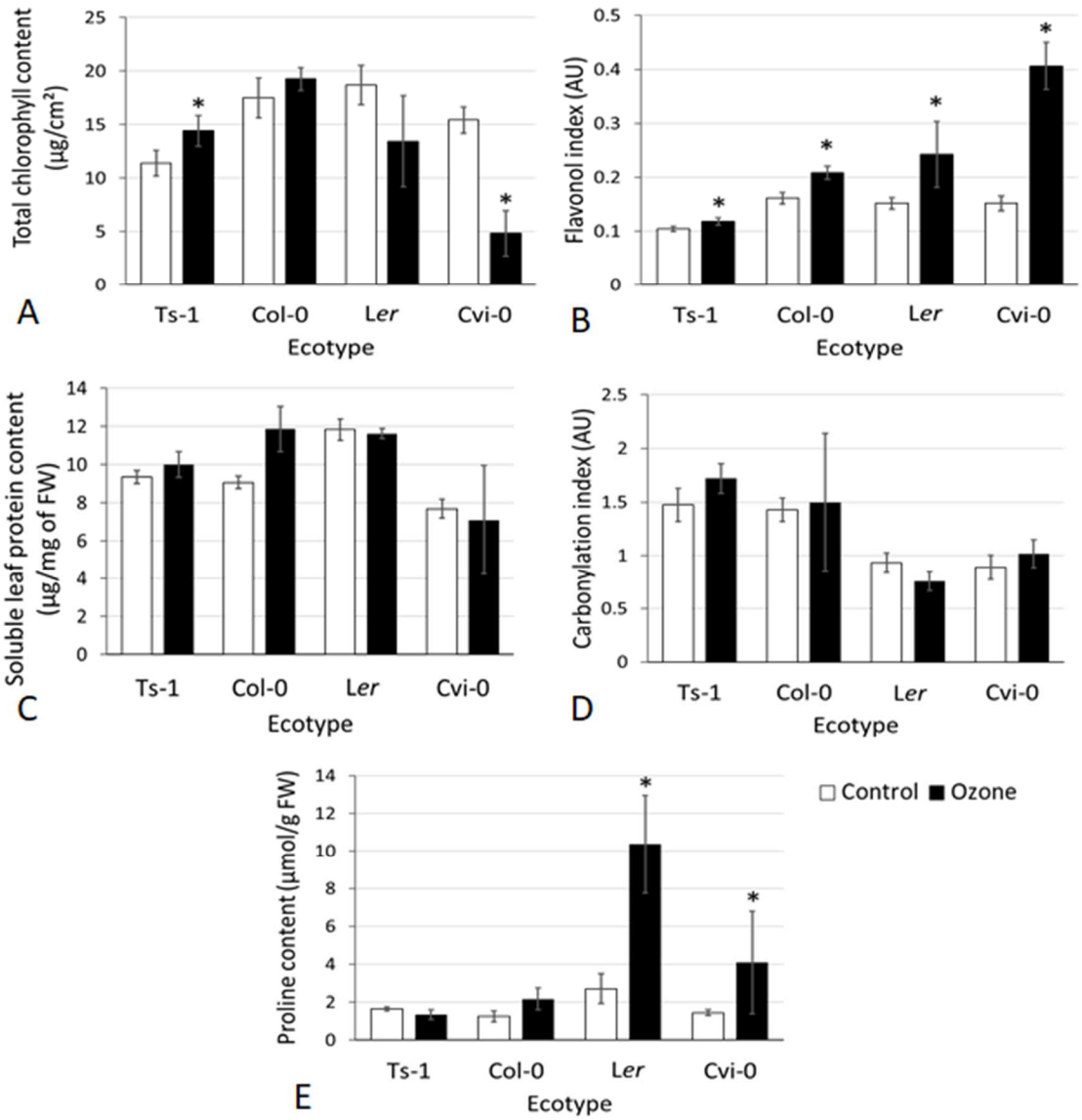

Figure 4: Impacts of ozone (237.9 ± 6.2 ppb over 10 days) on biochemical responses of Ts-1, Col-0, L*er* and Cvi-0 Arabidopsis ecotypes (ozonated plants, black bars; control plants, white bars).
Total chlorophyll contents (µg/cm², A) and flavonol index (AU, B) measured on the 12th expanded leaf with Dualex®; total protein content (µg/g of FW, C); carbonylation of proteins revealed by FTC signal (AU, D); and proline content (µmol/g of FW, E). Values represented means ± SD (n = 14 for total chlorophyll content and flavonol index and n = 3 for the other parameters). * represents a significant effect of ozone compared to control (Total chlorophyll content, flavonol index and proline content: pairwise Wilcoxon test; protein content and carbonylation index: Tukey HSD test threshold = 5%). For ANOVA or Kruskal-Wallis results, see table S2.

In all four ecotypes, there was no significant effect of ozone on leaf protein carbonylation level (Fig. 4D, 2-way ANOVA (type I), p-value = 0.53). Comparison with control plants indicated no significant difference amongst the different ecotypes (Tukey HSD test, p-values > 0.05). However, ozone treated L*er* plants had a significantly lower carbonylation signal than ozonated Ts-1 plants (Tukey HSD test, p-value = 0.0067).

Overall, average proline contents varied from 1.25 to 10.35 µmol/g of fresh weight (Fig. 4E). Col-0 showed proline content similar to those measured the first experiment (Fig. 4E). For control plants, the average proline content was equivalent in all ecotypes (around 2 µmol/g fresh weight). Ozone had a significant impact on leaf proline contents only in L*er* and Cvi-0 (+380% and +280% respectively; Pairwise Wilcoxon test, p-values < 0.05; for Ts-1 and Col-0, Pairwise Wilcoxon test, p-values = 1).

3.3.4. Discussion of the second experiment on Arabidopsis ecotypes

The two ecotypes that presented leaf macroscopic symptoms (necrosis and chlorosis) in response to the second ozone treatment, i.e., L*er* and Cvi-0, accumulated proline in their leaves, while the ozone treatment did not change the proline contents in the Col-0 and Ts-1 ecotypes. Cvi-0, particularly appeared to be the most sensitive ecotype to ozone of the four ecotypes, according to the severe and significant decreases in leaf area, biomass and chlorophyll content. Other experiments confirmed these results (data not shown). Furthermore, they agree with the ozone tolerance classification made by Brosché et al. (2010) who ranked Ts-1, Col-0, L*er* and Cvi-0 in decreasing order of tolerance to ozone, according to macroscopic symptoms. It should be noted that the flavonol index, related to the leaf content in flavonols, also increased with the ozone sensitivity of the four ecotypes. Flavonols accumulation in response to several abiotic stresses, including ozone, has been reported previously (Gandin et al., 2019; Ghosh et al., 2020; Pellegrini et al., 2018; Yamaji et al., 2003) and has been attributed to the antioxidant potential of these compounds (Chapman et al., 2019). However, all plant species and genotypes do not rely on these “low-cost antioxidants” to the same extent, since other systems, such as the ascorbic acid or glutathione antioxidant systems can be favored (Yamaji et al., 2003).

This second experiment clearly showed that proline accumulation occurred in response to ozone only in the two sensitive ecotypes. This explains the lack of differences observed in the mutants derived from the tolerant ecotype Col-0. However, the amounts of accumulated proline did not correlate with the sensitivity of the ecotypes to ozone, suggesting that proline accumulation was a general stress response linked to a its role in oxidative stress detoxification. This response is shared by numerous organisms (Ben Rejeb et al., 2014) but not all, as a reduction in proline content after ozone exposure has recently been reported observed in the biofueling bacteria *Cobetia marina* (Li et al., 2020). Ueno et al. (2021) showed that the pattern of variation in proline concentrations in endophytic-symbiotic plants was inverse to that observed for TBARS (thiobarbituric acid reactive substances, an indicator of lipid peroxidation and oxidative stress). It could be recalled that changes in proline contents in response to ozone varies between species and experiments (Cotrozzi et al., 2017); an increase in proline content was detected in poplar (Podda et al., 2019), wheat (Li et al., 2016; Zheng et al., 2011), *Vigna unguiculata* (Malaiyandi and Natarajan, 2014) or *Lolium multiflorum* (Ueno et al., 2021) whereas it was not observed in pepper (Colunje et al., 2021), oak trees (Cotrozzi et al., 2017) or *Ischaemum rugosum* (Dolker and Agrawal, 2019). El-Khatib (2003) have shown that, in *Medicago sativa*, an ozone sensitive plant species, the proline content strongly correlated with foliar injury, while proline content was not altered in several other species, shown to be tolerant to ozone. Moreover, the response of proline metabolism can vary with the developmental stage, as was observed in winter wheat (Liu et

al., 2015). However, we can assume that the absence of carbonylated proteins in the sensitive ecotypes could be partly related to an increase in protective proline and flavonol content, while the absence of carbonylated proteins in tolerant ecotypes would result from the development of other ROS detoxification mechanisms.

Conclusion

In these experiments, ozone had a negative impact on L*er* and Cvi-0 ecotypes that was not observed in other ecotypes or mutants, results that concur with the classification made by Brosché et al. (2010) from others physiological parameters. Using mutants or natural variability, the results presented here show that proline does not appear to be a major determinant of ozone tolerance but could be a signal induced in sensitive plants when the oxidative stress becomes too high. The slight effect observed for the *p1p2* mutant in response to ozone, characterized by higher proline contents, also suggests a protective role of proline on the photosynthetic apparatus. Literature data show that the effect of ozone on proline accumulation strongly depends on the species and the experiments considered. Even though our results did not allow to correlate the levels of protein carbonylation and proline, an antioxidant role for proline cannot be ruled out. Flavonol index appears particularly interesting in Arabidopsis as it confirmed the ranking of ecotypes according to their sensitivity to ozone, obtained in different experiments. Analysis of the different antioxidant mechanisms (ascorbate, glutathione and enzymatic processes) should help deciphering the roles played by proline and flavonols in ozone responses.

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References

Agathokleous, E., Araminiene, V., Belz, R.G., Calatayud, V., De Marco, A., Domingos, M., Feng, Z., Hoshika, Y., Kitao, M., Koike, T., Paoletti, E., Saitanis, C.J., Sicard, P., Calabrese, E.J., 2019b. A quantitative assessment of hormetic responses of plants to ozone. Environ. Res. 176, 108527. https://doi.org/10.1016/j.envres.2019.108527

Anjum, N.A., Sofo, A., Scopa, A., Roychoudhury, A., Gill, S.S., Iqbal, M., Lukatkin, A.S., Pereira, E., Duarte, A.C., Ahmad, I., 2015. Lipids and proteins—major targets of oxidative modifications in abiotic stressed plants. Environ. Sci. Pollut. Res. 22, 4099–4121. https://doi.org/10.1007/s11356-014-3917-1

Bagard, M., Jolivet, Y., Hasenfratz-Sauder, M.-P., Gérard, J., Dizengremel, P., Le Thiec, D., 2015. Ozone exposure and flux-based response functions for photosynthetic traits in wheat, maize and poplar. Environ. Pollut. 206, 411–420. https://doi.org/10.1016/j.envpol.2015.07.046

Bagard, M., Le Thiec, D., Delacote, E., Hasenfratz-Sauder, M.-P., Banvoy, J., Gérard, J., Dizengremel, P., Jolivet, Y., 2008. Ozone-induced changes in photosynthesis and

photorespiration of hybrid poplar in relation to the developmental stage of the leaves. Physiol. Plant. 134, 559–574. https://doi.org/10.1111/j.1399-3054.2008.01160.x
Bates, L.S., Waldren, R.P., Teare, I.D., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205–207. https://doi.org/10.1007/BF00018060
Ben Rejeb, K., Abdelly, C., Savouré, A., 2014. How reactive oxygen species and proline face stress together. Plant Physiol. Biochem. 80, 278–284. https://doi.org/10.1016/j.plaphy.2014.04.007
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1006/abio.1976.9999
Brosché, M., Merilo, E., Mayer, F., Pechter, P., Puzõrjova, I., Brader, G., Kangasjärvi, J., Kollist, H., 2010. Natural variation in ozone sensitivity among Arabidopsis thaliana accessions and its relation to stomatal conductance: Natural variation of Arabidopsis ozone sensitivity. Plant Cell Environ. 33, 914–925. https://doi.org/10.1111/j.1365-3040.2010.02116.x
Cabassa-Hourton, C., Schertl, P., Bordenave-Jacquemin, M., Saadallah, K., Guivarc'h, A., Lebreton, S., Planchais, S., Klodmann, J., Eubel, H., Crilat, E., Lefebvre-De Vos, D., Ghelis, T., Richard, L., Abdelly, C., Carol, P., Braun, H.-P., Savouré, A., 2016. Proteomic and functional analysis of proline dehydrogenase 1 link proline catabolism to mitochondrial electron transport in Arabidopsis thaliana. Biochem. J. 473, 2623–2634. https://doi.org/10.1042/BCJ20160314
Calzone, A., Podda, A., Lorenzini, G., Maserti, B.E., Carrari, E., Deleanu, E., Hoshika, Y., Haworth, M., Nali, C., Badea, O., Pellegrini, E., Fares, S., Paoletti, E., 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. https://doi.org/10.1016/j.scitotenv.2018.11.402
Cerovic, Z.G., Masdoumier, G., Ghozlen, N.B., Latouche, G., 2012. A new optical leaf-clip meter for simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids. Physiol. Plant. 146, 251–260. https://doi.org/10.1111/j.1399-3054.2012.01639.x
Chapman, J.M., Muhlemann, J.K., Gayomba, S.R., Muday, G.K., 2019. RBOH-Dependent ROS Synthesis and ROS Scavenging by Plant Specialized Metabolites To Modulate Plant Development and Stress Responses. Chem. Res. Toxicol. 32, 370–396. https://doi.org/10.1021/acs.chemrestox.9b00028
Coffey, C.M., Gronert, S., 2016. A cleavable biotin tagging reagent that enables the enrichment and identification of carbonylation sites in proteins. Anal. Bioanal. Chem. 408, 865–874. https://doi.org/10.1007/s00216-015-9176-2
Colunje, J., Garcia-Caparros, P., Moreira, J.F., Lao, M.T., 2021. Effect of Ozonated Fertigation in Pepper Cultivation under Greenhouse Conditions. Agronomy 11, 544. https://doi.org/10.3390/agronomy11030544
Cotrozzi, L., Remorini, D., Pellegrini, E., Guidi, L., Lorenzini, G., Massai, R., Nali, C., Landi, M., 2017. Cross-Talk between Physiological and Metabolic Adjustments Adopted by Quercus cerris to Mitigate the Effects of Severe Drought and Realistic Future Ozone Concentrations. Forests 8, 148. https://doi.org/10.3390/f8050148

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38. https://doi.org/10.1016/S0009-8981(03)00003-2

Dolker, T., Agrawal, M., 2019. Negative impacts of elevated ozone on dominant species of semi-natural grassland vegetation in Indo-Gangetic plain. Ecotoxicol. Environ. Saf. 182, 109404. https://doi.org/10.1016/j.ecoenv.2019.109404

Dyballa, N., Metzger, S., 2009. Fast and Sensitive Colloidal Coomassie G-250 Staining for Proteins in Polyacrylamide Gels. J. Vis. Exp. 1431. https://doi.org/10.3791/1431

El-Khatib, A.A., 2003. The response of some common Egyptian plants to ozone and their use as biomonitors. Environ. Pollut. 124, 419–428. https://doi.org/10.1016/S0269-7491(03)00045-9

Emberson, L.D., Pleijel, H., Ainsworth, E.A., van den Berg, M., Ren, W., Osborne, S., Mills, G., Pandey, D., Dentener, F., Büker, P., Ewert, F., Koeble, R., Van Dingenen, R., 2018. Ozone effects on crops and consideration in crop models. Eur. J. Agron. 100, 19–34. https://doi.org/10.1016/j.eja.2018.06.002

Feng, Z., Calatayud, V., Zhu, J., Kobayashi, K., 2018. Ozone exposure- and flux-based response relationships with photosynthesis of winter wheat under fully open air condition. Sci. Total Environ. 619–620, 1538–1544. https://doi.org/10.1016/j.scitotenv.2017.10.089

Foyer, C.H., Noctor, G., 2005. Redox Homeostasis and Antioxidant Signaling: A Metabolic Interface between Stress Perception and Physiological Responses. Plant Cell 17, 1866–1875. https://doi.org/10.1105/tpc.105.033589

Fuhrer, J., Skärby, L., Ashmore, M.R., 1997. Critical levels for ozone effects on vegetation in Europe. Environ. Pollut. 97, 91–106. https://doi.org/10.1016/S0269-7491(97)00067-5

Funck, D., Stadelhofer, B., Koch, W., 2008. Ornithine-δ-aminotransferase is essential for Arginine Catabolism but not for Proline Biosynthesis. BMC Plant Biol. 8, 40. https://doi.org/10.1186/1471-2229-8-40

Gandin, A., Davrinche, A., Jolivet, Y., 2019. Deciphering the main determinants of O3 tolerance in Euramerican poplar genotypes. Sci. Total Environ. 656, 681–690. https://doi.org/10.1016/j.scitotenv.2018.11.307

Gandin, A., Dizengremel, P., Jolivet, Y., 2021. Integrative role of plant mitochondria facing oxidative stress: The case of ozone. Plant Physiol. Biochem. 159, 202–210. https://doi.org/10.1016/j.plaphy.2020.12.019

Ghosh, A., Pandey, B., Agrawal, M., Agrawal, S.B., 2020. Interactive effects and competitive shift between Triticum aestivum L. (wheat) and Chenopodium album L. (fat-hen) under ambient and elevated ozone. Environ. Pollut. 265, 114764. https://doi.org/10.1016/j.envpol.2020.114764

Havé, M., Leitao, L., Bagard, M., Castell, J.-F., Repellin, A., 2015. Protein carbonylation during natural leaf senescence in winter wheat, as probed by fluorescein-5-thiosemicarbazide. Plant Biol. 17, 973–979. https://doi.org/10.1111/plb.12315

Hayat, S., Hayat, Q., Alyemeni, M.N., Wani, A.S., Pichtel, J., Ahmad, A., 2012. Role of proline under changing environments: A review. Plant Signal. Behav. 7, 1456–1466. https://doi.org/10.4161/psb.21949

Hayes, F., Sharps, K., Harmens, H., Roberts, I., Mills, G., 2020. Tropospheric ozone pollution reduces the yield of African crops. J. Agron. Crop Sci. 206, 214–228. https://doi.org/10.1111/jac.12376

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

Kangasjarvi, J., Jaspers, P., Kollist, H., 2005. Signalling and cell death in ozone-exposed plants. Plant Cell Environ. 28, 1021–1036. https://doi.org/10.1111/j.1365-3040.2005.01325.x

Kavi Kishor, P.B., Hima Kumari, P., Sunita, M.S.L., Sreenivasulu, N., 2015. Role of proline in cell wall synthesis and plant development and its implications in plant ontogeny. Front. Plant Sci. 6, 544. https://doi.org/10.3389/fpls.2015.00544

Langebartels, C., Wohlgemuth, H., Kschieschan, S., Grün, S., Sandermann, H., 2002. Oxidative burst and cell death in ozone-exposed plants. Plant Physiol. Biochem. 40, 567–575. https://doi.org/10.1016/S0981-9428(02)01416-X

Launay, A., Cabassa-Hourton, C., Eubel, H., Maldiney, R., Guivarc'h, A., Crilat, E., Planchais, S., Lacoste, J., Bordenave-Jacquemin, M., Clément, G., Richard, L., Carol, P., Braun, H.-P., Lebreton, S., Savouré, A., 2019. Proline oxidation fuels mitochondrial respiration during dark-induced leaf senescence in Arabidopsis thaliana. J. Exp. Bot. 70, 6203–6214. https://doi.org/10.1093/jxb/erz351

Leitao, L., Bethenod, O., Biolley, J.-P., 2007a. The Impact of Ozone on Juvenile Maize (Zea mays L.) Plant Photosynthesis: Effects on Vegetative Biomass, Pigmentation, and Carboxylases (PEPc and Rubisco). Plant Biol. 9, 478–488. https://doi.org/10.1055/s-2007-964942

Leitao, L., Delacôte, E., Dizengremel, P., Le Thiec, D., Biolley, J.-P., 2007b. Assessment of the impact of increasing concentrations of ozone on photosynthetic components of maize (Zea mays L.), a C4 plant. Environ. Pollut. 146, 5–8. https://doi.org/10.1016/j.envpol.2006.05.019

Leitao, L., Dizengremel, P., Biolley, J.-P., 2008. Foliar CO2 fixation in bean (Phaseolus vulgaris L.) submitted to elevated ozone: Distinct changes in Rubisco and PEPc activities in relation to pigment content. Ecotoxicol. Environ. Saf. 69, 531–540. https://doi.org/10.1016/j.ecoenv.2006.10.010

Li, C., Meng, J., Guo, L., Jiang, G., 2016. Effects of ozone pollution on yield and quality of winter wheat under flixweed competition. Environ. Exp. Bot. 129, 77–84. https://doi.org/10.1016/j.envexpbot.2015.11.011

Li, J., Rumancev, C., Lutze, H.V., Schmidt, T.C., Rosenhahn, A., Schmitz, O.J., 2020. Effect of ozone stress on the intracellular metabolites from Cobetia marina. Anal. Bioanal. Chem. 412, 5853–5861. https://doi.org/10.1007/s00216-020-02810-6

Liu, X., Sui, L., Huang, Y., Geng, C., Yin, B., 2015. Physiological and visible injury responses in different growth stages of winter wheat to ozone stress and the protection of spermidine. Atmospheric Pollut. Res. 6, 596–604. https://doi.org/10.5094/APR.2015.067

Malaiyandi, M., Natarajan, M., 2014. Impact of Ozone on Morphological, Physiological, and Biochemical Changes in Cow Pea (*Vigna unguiculata* [L.] Walp.). Ozone Sci. Eng. 36, 36–42. https://doi.org/10.1080/01919512.2013.824817

Mannaa, A., Hanisch, F.-G., 2020. Redox Proteomes in Human Physiology and Disease Mechanisms. J. Proteome Res. 19, 1–17. https://doi.org/10.1021/acs.jproteome.9b00586

Miller, J.D., Arteca, R.N., Pell, E.J., 1999. Senescence-Associated Gene Expression during Ozone-Induced Leaf Senescence in Arabidopsis. Plant Physiol. 120, 1015–1024. https://doi.org/10.1104/pp.120.4.1015

Mills, G., Buse, A., Gimeno, B., Bermejo, V., Holland, M., Emberson, L., Pleijel, H., 2007. A synthesis of AOT40-based response functions and critical levels of ozone for agricultural and horticultural crops. Atmos. Environ. 41, 2630–2643. https://doi.org/10.1016/j.atmosenv.2006.11.016

Mills, G., Hayes, F., Simpson, D., Emberson, L., Norris, D., Harmens, H., Büker, P., 2011. Evidence of widespread effects of ozone on crops and (semi-)natural vegetation in Europe (1990-2006) in relation to AOT40- and flux-based risk maps. Glob. Change Biol. 17, 592–613. https://doi.org/10.1111/j.1365-2486.2010.02217.x

Murchie, E.H., Lawson, T., 2013. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. J. Exp. Bot. 64, 3983–3998. https://doi.org/10.1093/jxb/ert208

Parrish, D.D., Law, K.S., Staehelin, J., Derwent, R., Cooper, O.R., Tanimoto, H., Volz-Thomas, A., Gilge, S., 2012. Long-term changes in lower tropospheric baseline ozone concentrations at northern mid-latitudes. Atmos Chem Phys 21.

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

Podda, A., Pisuttu, C., Hoshika, Y., Pellegrini, E., Carrari, E., Lorenzini, G., Nali, C., Cotrozzi, L., Zhang, L., Baraldi, R., Neri, L., Paoletti, E., 2019. Can nutrient fertilization mitigate the effects of ozone exposure on an ozone-sensitive poplar clone? Sci. Total Environ. 657, 340–350. https://doi.org/10.1016/j.scitotenv.2018.11.459

R Core Team. 2020. R: A Language and Environment for Statistical Computing. Vienna, Austria: https://www.r-project.org/., n.d.

Sharma, Y.K., Davis, K.R., 1994. Ozone-Induced Expression of Stress-Related Genes in Arabidopsis thaliana. Plant Physiol. 105, 1089–1096.

Sicard, P., Anav, A., De Marco, A., Paoletti, E., 2017. Projected global ground-level ozone impacts on vegetation under different emission and climate scenarios. Atmospheric Chem. Phys. 17, 12177–12196. https://doi.org/10.5194/acp-17-12177-2017

Sicard, P., Paoletti, E., Agathokleous, E., Araminienė, V., Proietti, C., Coulibaly, F., De Marco, A., 2020. Ozone weekend effect in cities: Deep insights for urban air pollution control. Environ. Res. 191, 110193. https://doi.org/10.1016/j.envres.2020.110193

Signorelli, S., Coitiño, E.L., Borsani, O., Monza, J., 2014. Molecular Mechanisms for the Reaction Between $^{\bullet}$OH Radicals and Proline: Insights on the Role as Reactive Oxygen Species Scavenger in Plant Stress. J. Phys. Chem. B 118, 37–47. https://doi.org/10.1021/jp407773u

Smirnoff, N., Cumbes, Q.J., 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28, 1057–1060. https://doi.org/10.1016/0031-9422(89)80182-7

Szabados, L., Savouré, A., 2010. Proline: a multifunctional amino acid. Trends Plant Sci. 15, 89–97. https://doi.org/10.1016/j.tplants.2009.11.009

Székely, G., Abraham, E., Cseplo, A., Rigo, G., Zsigmond, L., Csiszar, J., Ayaydin, F., Strizhov, N., Jasik, J., Schmelzer, E., Koncz, C., Szabados, L., 2008. Duplicated *P5CS* genes of Arabidopsis play distinct roles in stress regulation and developmental control of

proline biosynthesis. Plant J. 53, 11–28. https://doi.org/10.1111/j.1365-313X.2007.03318.x
Tiwari, S., Agrawal, M., 2018. Tropospheric Ozone and its Impacts on Crop Plants. Springer International Publishing, Cham. https://doi.org/10.1007/978-3-319-71873-6
Ueno, A.C., Gundel, P.E., Molina-Montenegro, M.A., Ramos, P., Ghersa, C.M., Martínez-Ghersa, M.A., 2021. Getting ready for the ozone battle: Vertically transmitted fungal endophytes have transgenerational positive effects in plants. Plant Cell Environ. pce.14047. https://doi.org/10.1111/pce.14047
Vainonen, J.P., Kangasjärvi, J., 2015. Plant signalling in acute ozone exposure: Ozone action on plants. Plant Cell Environ. 38, 240–252. https://doi.org/10.1111/pce.12273
Verbruggen, N., Hermans, C., 2008. Proline accumulation in plants: a review. Amino Acids 35, 753–759. https://doi.org/10.1007/s00726-008-0061-6
Yamaji, K., Julkunen-Tiitto, R., Rousi, M., Freiwald, V., Oksanen, E., 2003. Ozone exposure over two growing seasons alters root-to-shoot ratio and chemical composition of birch (*Betula pendula* Roth): Ozone alters chemical composition of birch. Glob. Change Biol. 9, 1363–1377. https://doi.org/10.1046/j.1365-2486.2003.00669.x
Zegaoui, Z., Planchais, S., Cabassa, C., Djebbar, R., Belbachir, O.A., Carol, P., 2017. Variation in relative water content, proline accumulation and stress gene expression in two cowpea landraces under drought. J. Plant Physiol. 218, 26–34. https://doi.org/10.1016/j.jplph.2017.07.009
Zheng, Y.H., Li, Y.G., Xia, W.R., Xu, H., Su, B.Y., Jiang, G.M., Ning, T.Y., 2011. Responses of gas exchange, cellular membrane integrity, and antioxidant enzymes activities of salinity-stressed winter wheat to ozone pollution. Photosynthetica 49. https://doi.org/10.1007/s11099-011-0047-6