



## Cross-talk between physiological and biochemical adjustments by *Punica granatum* cv. Dente di cavallo mitigates the effects of salinity and ozone stress

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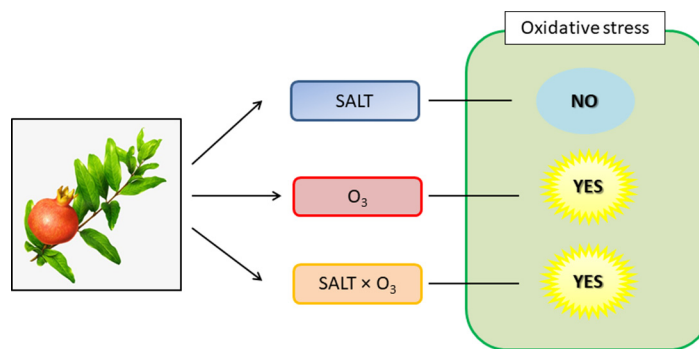
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### HIGHLIGHTS

- No evidence of visible injury due to salt (e.g. tip yellow-brown necrosis) was found.
- Physiological and biochemical adjustments were induced by salt and/or O<sub>3</sub> exposure.
- In the combined treatment, salt did not ameliorate the negative effects of O<sub>3</sub>.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Plants are exposed to a broad range of environmental stresses, such as salinity and ozone (O<sub>3</sub>), and survive due to their ability to adjust their metabolism. The aim of this study was to evaluate the physiological and biochemical adjustments adopted by pomegranate (*Punica granatum* L. cv. Dente di cavallo) under realistic field conditions. One-year-old saplings were exposed to O<sub>3</sub> [two levels denoted as ambient (AO) and elevated (EO) O<sub>3</sub> concentrations] and salinity [S (salt, 50 mM NaCl)] for three consecutive months. No salt (NS) plants received distilled water. Despite the accumulation of Na<sup>+</sup> and Cl<sup>-</sup> in the aboveground biomass, no evidence of visible injury due to salt (e.g. tip yellow-brown lesions) was found. The maintenance of leaf water status (i.e. unchanged values of electrolytic leakage and relative water content), the significant increase of abscisic acid, proline and starch content (+98, +65 and +59% compared to AO\_NS) and stomatal closure (−24%) are suggested to act as adaptive mechanisms against salt stress in AO\_S plants. By contrast, EO\_NS plants were unable to protect cells against the negative impact of O<sub>3</sub>, as confirmed by the reduction of the CO<sub>2</sub> assimilation rate (−21%), accumulation of reactive oxygen species (+10 and +225% of superoxide anion and hydrogen peroxide) and malondialdehyde

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Antioxidants  
Multiple stress

by-product (about 2-fold higher than AO\_NS). Plants tried to preserve themselves from further oxidative damage by adopting some biochemical adjustments [i.e. increase in proline content (+41%) and induction of catalase activity (8-fold higher than in AO\_NS)]. The interaction of the two stressors induced responses considerably different to those observed when each stressor was applied independently. An analysis of the antioxidant pool revealed that the biochemical adjustments adopted by *P. granatum* under EO\_S conditions (e.g. reduction of total ascorbate; increased activities of superoxide dismutase and catalase) were not sufficient to ameliorate the O<sub>3</sub>-induced oxidative stress.

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## 1. Introduction

Climate change, encompassing shifts in precipitation, water composition and air quality, represents a moving target for plant acclimation and/or adaptation (Gray and Brady, 2016). In the Mediterranean basin, natural (i.e., elevated average temperature, drought, salinization) and anthropogenic factors, such as increased tropospheric ozone (O<sub>3</sub>), are expected to be harsher than in other areas worldwide (Dayan et al., 2017; Skliris et al., 2017). Among environmental problems, salinity is one of the abiotic stresses that severely limits the productivity of crop plants (Tester and Langridge, 2010). In general terms, the effects of salinity on plants are the result of both water stress (due to a higher osmotic potential in soil as compared to plant tissues) and a toxic effect caused by the influx of ions mainly Na<sup>+</sup> and Cl<sup>-</sup> into plant tissues (Flowers and Flowers, 2005; Verslues et al., 2006; Munns and Tester, 2008). The result of these effects is a spectrum of physiological, biochemical and genomic changes that provoke alterations in photosynthesis, carbohydrate partition, respiration, reactive oxygen species (ROS) production, and unbalanced uptake of other nutrients (Chaves et al., 2009). However, plants possess different degrees of tolerance to salinity, conferred by physiological and biochemical adjustments, which can alleviate the negative effect of salt toxicity (Munns and Gilliam, 2015).

Tropospheric O<sub>3</sub> air-pollution represents a serious concern to plant health either due to direct toxicity or increased plant susceptibility to biotic and abiotic stress (Guidi et al., 2017; Mills et al., 2018). This photo-oxidant pollutant, as other environmental stresses such as salinity, affects plant growth provoking alterations at physiological (i.e. reduction of photosynthesis, stomatal closure; Hoshika et al., 2018), biochemical (i.e. lipid peroxidation, oxidative damage; Cotrozzi et al., 2016) and molecular levels (increase in transcript levels of genes encoding enzymes in the early part of the phenylpropanoid and flavonoid biosynthesis; Pellegrini et al., 2018).

Studies on the combined effects of salt and O<sub>3</sub> documented different responses from those observed when each stressor was applied independently. Results were sometimes contradictory: salt stress either counteracted O<sub>3</sub> impact by reducing stomata opening (Maggio et al., 2007; Gerosa et al., 2014), or further exacerbated O<sub>3</sub>-triggered effects (Zheng et al., 2012; Guidi et al., 2017). In addition to the plant physiological status, analyses of cellular and metabolic rearrangements provide complementing evidence to describe the role(s) of several metabolites (such as compatible solutes, osmoprotectants, low-molecular weight proteins, and antioxidants) in the adaptation/acclimation of plants to harsh environmental conditions, such as the complex interactions between salt and O<sub>3</sub>.

Pomegranate (*Punica granatum* L.) is a deciduous shrub or small tree originally distributed in Iran and Afghanistan (Zhang et al., 2010). It is one of the oldest known edible fruits, and has previously been considered to be a minor crop (Hasanpour et al., 2015). However, pomegranates are increasingly being recognized as attractive fruit trees due to the presence of compounds beneficial to health (Seeram et al., 2006) and their adaptation to a wide range of environmental conditions (i.e., drought and salt; Catola et al., 2016; Mastrogianidou et al., 2016). For this reason, pomegranate cultivation has gained increasing attention in areas where soil salinity and drought are a concern (Sarkhosh et al., 2006). Although salt usually occurs simultaneously

with other stresses, including O<sub>3</sub>, no experimental studies on the combination of these stressors has yet been published in pomegranate.

The aim of this work was to evaluate the interactive effects of salt and O<sub>3</sub> under realistic field conditions on physiological and biochemical parameters of the commercial pomegranate cultivar Dente di Cavallo. We hypothesized that a partial protective effect of salt against O<sub>3</sub> exists, and that the interactive effects of the two factors depends upon physiological and biochemical adjustments.

We asked the following questions: i) Which physiological and biochemical adjustments are induced by salt or O<sub>3</sub> exposure? ii) Can salt treatment trigger a set of plant adaptive responses to O<sub>3</sub>?

## 2. Materials and methods

### 2.1. Plant material and experimental design

One-year-old saplings of *Punica granatum* L. cv. Dente di Cavallo were moved from a local nursery to the O<sub>3</sub>-FACE facility of Sesto Fiorentino, Florence, Italy (43°48'59"N, 11°12'01"E, 55 m a.s.l.), where the experimental activities were conducted. Here, plants were transferred into 18.5 L pots containing peat:soil:sand (1:1:1 in volume) and maintained under field conditions until the beginning of the treatment. Soil was collected in a semi-natural area nearby the experimental site (43°46'56"N, 11°10'24"E), characterized by a slightly acidic sandy-loam texture.

Plants of uniform size (about 90 cm tall) were selected and grown under O<sub>3</sub> [two levels, denoted as ambient O<sub>3</sub> (AO) and elevated O<sub>3</sub> (EO) concentrations, respectively] and salinity (50 mM of NaCl), from 1st June to 30th September 2017 (for a total of 17 weeks). The accumulated exposure over a threshold of 40 ppb (AOT40, sensu Kärenlampi and Skärby, 1996) was 21.51 and 58.74 ppm h in AO and EO, over the experimental period. A detailed description of the O<sub>3</sub> exposure methodology is available in Paoletti et al. (2017). For the salt (S) treatment, 50 mM of NaCl dissolved in 200 ml of distilled water was added to each pot every week. No salt (NS) plants received 200 ml of distilled water. The electrical leakage and pH of the irrigation water (1.5 l per treatment) were 5.5 mS cm<sup>-1</sup> and 7.6, respectively. Three replicated plots (5 × 5 × 2 m) were assigned to each O<sub>3</sub> level, with three plants for each combination of O<sub>3</sub> and NaCl in each plot. Plant positions were changed every month within each plot to avoid positional effects (Potvin and Tardif, 1988). At the end of the experiment, five fully expanded leaves of all plants per plot in each salt × O<sub>3</sub> treatment were gathered, divided into aliquots, immediately frozen in liquid nitrogen and stored at -80 °C until biochemical analyses. Sampling was performed from 9:00 to 12:00 am. The ecophysiological measurements were carried out on two fully expanded sun leaves (5–10th order from the tip of shoots) per plant (3 plants per replicated treatment). The biochemical data were obtained from 4 replicates for each treatment, where each replicate was a bulk of at least two fully expanded sun leaves (5–10th order from the tip of shoots) per plant (3 plants per replicated treatment).

### 2.2. Visible injury, element content and water status of leaves

All plants were evaluated weekly by the same two observers to record the first date of visible foliar injury in each individual plant. After

the onset of injury, the assessment of visible injury was conducted on the same randomly selected branch per plant every two weeks until biomass harvest. No visible injury by salt was detected. The number of O<sub>3</sub>-symptomatic leaves was counted and expressed as percentage relative to the total number of leaves of the selected branch (LA). The percentage of injured leaf surface per O<sub>3</sub>-symptomatic leaf (IL) was visually assessed with the help of photoguides (Paoletti et al., 2009) and averaged at the branch level. The Injury Index (II) was calculated by combining the two parameters:  $II = (LA \times IL) / 100$ .

Relative water content (RWC) and electrolytic leakage (EL) were determined on three fully-expanded mature leaves of three plants per treatment, following standard methodologies (Nali et al., 2005).

At harvest, all plant material [leaves, stems, fine roots (diameter  $\leq 2$  mm) and coarse roots (diameter  $> 2$  mm)] was oven dried at 80 °C until a constant weight was achieved and then each organ was grounded into a fine powder. The total C and N content was determined with an element analyser (TruSpec, CNS, LECO, Saint Joseph, MI, USA). About 0.3 g of powder were mineralized with 2 ml of a mixture of ultra-pure concentrated HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> at 190 °C and a pressure of 3.2 MPa in a microwave pressure digestion-closed system (Speedwave MWS-2, Berghof, Germany). The total content of Ca, K, Mg, Mn, Na and P were determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OPS Optima 2100 DV, Perkin Elmer, Waltham, MA, USA). Analytical quality was checked with the Certified Reference Material (Multi-Element standard solution for ICP, CPAChem, Stara Zagora, Bulgaria). All analyses were carried out in triplicate. The accuracy was within 99.999% for all elements. Chloride content was determined spectrophotometrically (Lambda 25 UV-VIS, Perkin Elmer, Waltham, MA, USA) at 463 nm, on the ashes calcined with calcium carbonate at 450 °C for 12 h, and dissolved in water.

### 2.3. Gas exchange and chlorophyll *a* fluorescence

In early August, leaf gas exchange and chlorophyll *a* fluorescence were measured using a portable Infrared Gas Analyser (Model 6400, Li-Cor instruments, Lincoln, NE, USA) with a fluorescence chamber head (Model 6400-40, Li-Cor instruments, Lincoln, NE, USA). Measurements were carried out on two fully expanded sun leaves (5–10th order from the tip of shoots) per plant (3 plants per replicated treatment) at ambient CO<sub>2</sub> concentration (400 ppm), controlled leaf temperature (25 °C) and relative humidity (40–60%) with varying photosynthetic photon flux densities (PPFD: 1500, 400, 300, 200, 100, 80, 60, 40, 20, 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Net photosynthetic rate (*A*) and stomatal conductance to water vapor (*g<sub>s</sub>*), the ratio of the intercellular CO<sub>2</sub> concentration (*C<sub>i</sub>*) of a leaf to the ambient CO<sub>2</sub> concentration (*C<sub>a</sub>*: 400 ppm), i.e., the *C<sub>i</sub>/C<sub>a</sub>* ratio, were recorded, and mesophyll conductance (*g<sub>m</sub>*) was estimated using the variable *J* method (Harley et al., 1992). Respiration rate in the light (*R<sub>d</sub>*) was estimated by the Kok method (Kok, 1948), which is based on extrapolation of the linear relationship between *A* and low light intensity above the light compensation point. Dark respiration (*R<sub>n</sub>*) was measured by switching off the LED light source after the Kok protocol until the efflux of CO<sub>2</sub> from the leaf remained stable. Photorespiration (*R<sub>PR</sub>*) was calculated according to Sharkey (1988), i.e.  $R_{PR} = (A + R_d) / (C_c / \Gamma^* - 1)$  where *C<sub>c</sub>* and  $\Gamma^*$  are the CO<sub>2</sub> concentration at the chloroplast and the CO<sub>2</sub> compensation point in the absence of *R<sub>d</sub>*, respectively. After the gas exchange measurements, chlorophyll *a* fluorescence and the status of the electron transport chain of PSII were measured with a PAM-2000 fluorometer (Walz, Effeltrich, Germany) on dark-adapted leaves. The maximal fluorescence level (*F<sub>m</sub>*) and the maximal PSII photochemical efficiency [*F<sub>v</sub>/F<sub>m</sub>*, where *F<sub>v</sub>* is the variable fluorescence (*F<sub>m</sub>* - minimal fluorescence)] were determined. The saturation pulse method was used for analysis of photochemical (qP) and non-photochemical quenching (qNP) components as described by Schreiber et al. (1986) and Cotrozzi et al. (2017).

### 2.4. ROS production and oxidative damage

The content of anion superoxide (O<sub>2</sub><sup>•-</sup>) was determined according to Cotrozzi et al. (2017) by the reduction of tetrazolium dye sodium by O<sub>2</sub> to soluble formazan. After extraction with Tris-HCl (50 mM, pH 7.5), the reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 53 mM tetrazolium dye sodium, 3'-(1-[phenylamino-carbonyl]-3,4-tetrazolium)bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate and 50  $\mu\text{l}$  of supernatant in a final volume of 1 ml. O<sub>2</sub><sup>•-</sup> was determined with a spectrophotometer (6505 UV-Vis, Jenway, UK) at 470 nm. The amount of O<sub>2</sub><sup>•-</sup> was calculated using the molar extinction coefficient 21.6  $\text{mM}^{-1} \text{cm}^{-1}$ . Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content was measured using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life Technologies Corp., Carlsbad, CA, USA), according to Cotrozzi et al. (2017). After extraction with potassium-phosphate buffer (20 mM, pH 6.5), H<sub>2</sub>O<sub>2</sub> was determined with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA) at 510 and 590 nm (excitation and emission of resorufin fluorescence, respectively). Each outcome was plotted against a H<sub>2</sub>O<sub>2</sub> standard curve (from 0 to 20  $\mu\text{M}$ ). Oxidative damage was estimated in terms of lipid peroxidation by determining the malondialdehyde (MDA) by-product accumulation, according to the method of Hodges et al. (1999) with minor modifications, as reported by Pellegrini et al. (2012). After extraction with ethanol (80%, v/v), MDA was determined with the same fluorescence/absorbance microplate reader reported above at 532 and 600 nm. The amount of MDA was calculated using the molar extinction coefficient of 155  $\text{mM}^{-1} \text{cm}^{-1}$ .

### 2.5. Non-enzymatic and enzymatic antioxidant compounds

After extraction with trichloroacetic acid (6%, w/v), reduced (AsA) and oxidized (DHA) ascorbate were determined with the same spectrophotometer reported above at 525 nm. Supernatants were also used for the determination of reduced (GSH) and oxidized (GSSG) glutathione content. Further details of AsA and GSH determinations are available in Pellegrini et al. (2018). After extraction with 100% HPLC-grade methanol (v/v), pigment determination was performed with a High Performance Liquid Chromatography (HPLC; P680 Pump, UVD170U UV-VIS detector, Dionex, Sunnyvale, CA, USA) at room temperature with a reverse-phase Dionex column (Acclaim 120, C18, 5  $\mu\text{m}$  particle size, 4.6 mm i.d.  $\times$  150 mm length) according to Cotrozzi et al. (2017). The flow rate was 1  $\text{ml min}^{-1}$ . The pigments were detected by their absorbance at 445 nm.

After extraction with potassium/phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone (PVP), 0.1% (v/v) Triton X-100 and 10% (v/v) glycerol, catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1) activities were determined with the same spectrophotometer reported above at 240 and 560 nm, following the methods of Aebi (1984) and Zhang and Kirkham (1996), respectively. After extraction with Na-phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol and 5 mM ascorbate, ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined with the same spectrophotometer reported above at 290 nm, following the method of Mittler and Zilinskas (1993). For all assays, a previous protein precipitation with ammonium sulphate was carried out following the method of Wingfield (2016) for crude extract purification. One unit of SOD was described as the amount of enzyme required to inhibit the reduction of nitroblue tetrazolium by 50%. One unit of CAT was defined as the amount of enzyme required for the dismutation of 1  $\mu\text{mol H}_2\text{O}_2/\text{min}$ . One unit of APX was defined as the amount of enzyme required to oxidize or reduce 1  $\mu\text{mol}$  of AsA/min. The whole procedures are described by Pellegrini et al. (2018). The protein content was measured by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard.



## 2.6. Abscisic acid and osmolytes

Abscisic acid (ABA) was estimated by an indirect Enzyme-Linked Immunosorbent Assay (ELISA), using DBPA1 monoclonal antibody, raised against S(+)-ABA (Trivellini et al., 2011) according to the method of Walker-Simmons (1987), with some modifications. After extraction with deionized water (water: tissue ratio = 1000:1 v/w), ABA was determined with an absorbance microplate reader (MDL 680, Perkin-Elmer, USA) at 415 nm. After extraction with ethanol (70%, v/v), proline was determined spectrophotometrically at 520 nm, according to Carillo and Gibbon (2011). Starch content was quantified using K-TSTA kit (Megazyme, Wicklow, Ireland), respectively. After extraction with ethanol (80%, v/v), soluble sugars were determined spectrophotometrically at 340 nm, according to Lo Piccolo et al. (2018). The residual pellet was used for the determination of starch at 510 nm.

## 2.7. Statistical analyses

Normal distribution of data was analysed following the Shapiro-Wilk test. As time was a random factor, the effects of salt, O<sub>3</sub> and time on II values were tested with a repeated two-way ANOVA test using R version 3.4.3 (R Core Team, 2017). For all the other parameters, the significance of treatments was determined using Tukey's honestly significant difference (HSD) or Dunn's post test following two-way ANOVA (parametric analysis) or Kruskal-Wallis (non-parametric analysis) test. All analyses were performed in JMP 13 (SAS Institute, Cary, NC, USA).

## 3. Results

### 3.1. Visible foliar injury

The typical foliar injury due to salt stress (e.g. tip yellow-brown lesions) was not observed in plants grown under salt conditions (independently of O<sub>3</sub> concentrations), confirming that a mild stress occurred. The repeated two-way ANOVA measurements of II values revealed that the interaction “salt × O<sub>3</sub>” was not significant. Starting from 21st August (12 weeks after the beginning of the exposure), the typical foliar injury due to O<sub>3</sub> stress (e.g. reddish stipples, homogeneously distributed in the interveinal adaxial leaf area, Fig. 1) was recorded in plants of all treatments, independently from the salt treatment. Such type of visible injury was strongly increased in plants grown under EO conditions compared to AO ones, as confirmed by II values ( $1.13 \pm 0.43$  vs  $0.20 \pm 0.07$ ,  $P \leq 0.001$ ).

### 3.2. Element content and water status of leaves

Under AO\_S conditions, salt induced a significant increase of Na<sup>+</sup> content in fine roots (+27% compared to AO\_NS, Table 1). A similar response was observed for Cl<sup>-</sup> content in stems and roots of plants grown under AO\_S (+212 and +53%, respectively) and EO\_S conditions (+104 and +53%, respectively). Elevated O<sub>3</sub> concentrations induced a significant decrease of Na<sup>+</sup> content in fine roots (-60% compared to AO\_NS). By contrast, an increase of Cl<sup>-</sup> content was observed in the roots of individuals grown under EO\_NS conditions (about 2-fold higher than AO\_NS). Differential partitioning of ions was observed in relation to biomass allocation: Na<sup>+</sup> was accumulated more belowground than in the aboveground parts ( $1.71 \pm 0.23$  vs  $0.48 \pm 0.05$  mg g<sup>-1</sup> dry weight, DW;  $P \leq 0.001$ ). An opposite trend was observed for Cl<sup>-</sup> ( $2.19 \pm 0.17$  vs  $3.65 \pm 0.37$  mg g<sup>-1</sup> DW;  $P \leq 0.05$ ). No significant differences were observed among treatments regarding other macro- and microelements, RWC and EL (data not shown).



Fig. 1. Visible injury on *Punica granatum* cv. Dente di cavallo leaf exposed to ambient air ozone concentrations. Bar = 1 cm.

### 3.3. Gas exchange and chlorophyll *a* fluorescence analyses

With the exception of “salt” stress, the two-way ANOVA test of gas exchange and chlorophyll *a* fluorescence parameters revealed a significant increase in the R<sub>PR</sub>/A ratio in the “O<sub>3</sub>” and in the interactions “salt × O<sub>3</sub>” (Fig. 2). Salt per se induced a slight increase of the R<sub>PR</sub>/A ratio and R<sub>n</sub> only under AO conditions (+28 and +26% compared to NS, respectively). Similarly, O<sub>3</sub> (alone or in combination with salt) significantly increased R<sub>PR</sub>/A compared to AO\_NS. A similar pattern was observed for R<sub>n</sub> in plants grown under EO\_NS conditions (+17%). No significant interactions were observed regarding the other gas exchange parameters such as A, g<sub>s</sub>, C<sub>i</sub>/C<sub>a</sub> and g<sub>m</sub> (Fig. S1). However, an evident decrease of A values was observed in plants grown under EO\_NS (-22% compared to AO\_NS;  $P \leq 0.01$ ) and EO\_S conditions (-25%;  $P \leq 0.01$ ; Fig. S1A). A similar response was observed for g<sub>s</sub> in AO\_S (-25% relative to AO\_NS;  $P \leq 0.01$ ), EO\_NS (-35%;  $P \leq 0.01$ ) and EO\_S conditions (-41%;  $P \leq 0.01$ ; Fig. S1B). Only under EO\_S conditions, a significant reduction of C<sub>i</sub>/C<sub>a</sub> ratio and g<sub>m</sub> values was observed (-7 and -27%;  $P \leq 0.05$ ; Fig. S1C–D).

Regarding the chlorophyll *a* fluorescence parameters, salt per se induced a decrease of F<sub>v</sub>/F<sub>m</sub> ratio and qP values (-17 and -3% compared to AO\_NS, respectively; Fig. 3). An opposite trend was observed regarding qNP (+5%). Increasing O<sub>3</sub> (alone and in combination with salt) induced a reduction of F<sub>v</sub>/F<sub>m</sub> ratio (-9 and -14% compared to AO\_NS, respectively), qP (-11%, only under EO\_S conditions) and qNP values (-11 and -32%).

### 3.4. ROS production and oxidative damage

The Kruskal-Wallis test of ROS and MDA content revealed that the interaction “salt × O<sub>3</sub>” and the effects of each factor were significant

**Table 1**

Sodium ( $\text{Na}^+$ ) and chloride ( $\text{Cl}^-$ ) contents (expressed as  $\text{mg g}^{-1}$  dry weight) in different organs of pomegranate plants treated with  $\text{O}_3$  [two levels denoted as ambient  $\text{O}_3$  (AO) and elevated  $\text{O}_3$  (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Data are shown as means  $\pm$  standard error. Asterisks show the significance of factors/interaction following the two-way ANOVA with salt and  $\text{O}_3$  as factors: ns  $P > 0.05$ , \* $P \leq 0.05$ , \*\*\* $P \leq 0.001$  ( $N = 3$ ). Different letters indicate significant differences among treatments following the Tukey's post-hoc test ( $P \leq 0.05$ ).

	Organ	AO_NS	AO_S	EO_NS	EO_S	Salt	$\text{O}_3$	Salt $\times$ $\text{O}_3$
$\text{Na}^+$	Leaves	0.27 $\pm$ 0.01	0.51 $\pm$ 0.07	0.27 $\pm$ 0.01	0.76 $\pm$ 0.12	***	ns	ns
	Stems	0.36 $\pm$ 0.01	0.47 $\pm$ 0.01	0.26 $\pm$ 0.03	0.45 $\pm$ 0.02	***	*	ns
	Fine roots	2.11 $\pm$ 0.10 b	2.68 $\pm$ 0.07 c	0.85 $\pm$ 0.04 a	2.30 $\pm$ 0.06 b	***	***	***
	Coarse roots	0.88 $\pm$ 0.10 a	0.81 $\pm$ 0.08 a	1.69 $\pm$ 0.09 b	1.01 $\pm$ 0.12 a	***	***	*
$\text{Cl}^-$	Leaves	3.84 $\pm$ 0.23	3.78 $\pm$ 0.57	3.65 $\pm$ 0.23	4.57 $\pm$ 0.21	ns	ns	ns
	Stems	1.19 $\pm$ 0.05 a	3.72 $\pm$ 0.08 c	1.45 $\pm$ 0.14 bc	1.82 $\pm$ 0.09 b	***	***	***
	Fine roots	2.56 $\pm$ 0.08 b	1.53 $\pm$ 0.12 a	2.48 $\pm$ 0.18 b	2.93 $\pm$ 0.03 b	*	***	***
	Coarse roots	1.22 $\pm$ 0.06 a	2.50 $\pm$ 0.05 c	1.32 $\pm$ 0.02 a	1.87 $\pm$ 0.05 b	***	***	***

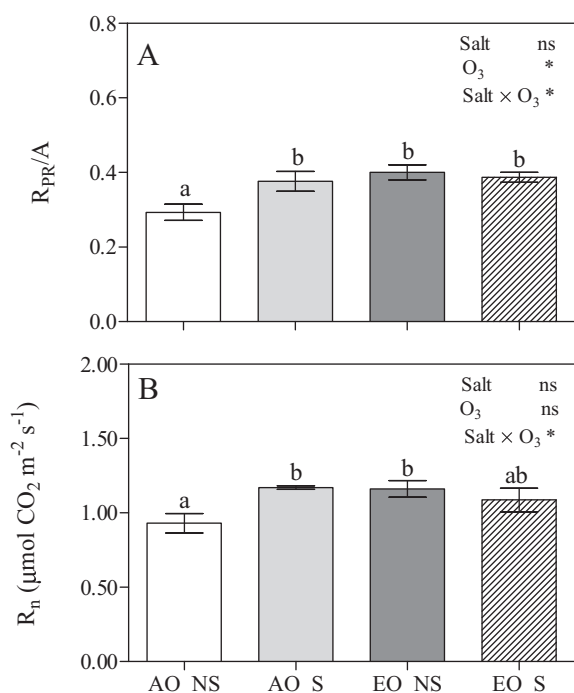
(Fig. 4A–C). Salt alone did not alter the content of  $\text{O}_2^{\bullet-}$ ,  $\text{H}_2\text{O}_2$  and MDA in comparison to plants grown under AO\_NS conditions. Conversely,  $\text{O}_3$  (alone and in combination with salt) significantly induced ROS accumulation (+10 and +23% of  $\text{O}_2^{\bullet-}$  in comparison to AO\_NS; +225 and +67% of  $\text{H}_2\text{O}_2$ ) and MDA production (about 2-fold higher than AO\_NS, respectively), with significant differences between salt treatment.

### 3.5. Non-enzymatic and enzymatic antioxidant compounds

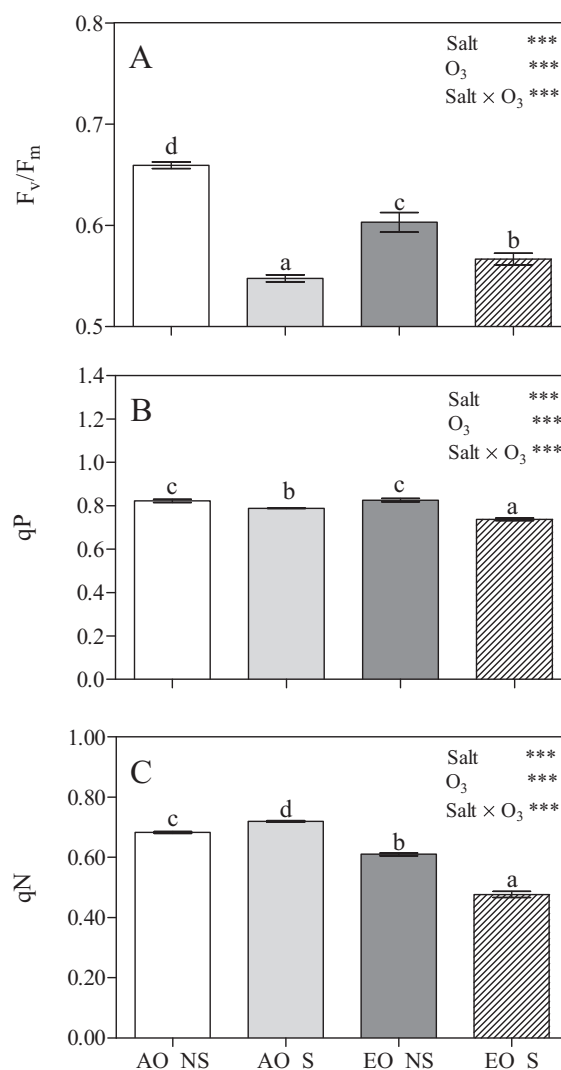
The Kruskal-Wallis test of total AsA and total GSH content revealed that the interaction “salt  $\times$   $\text{O}_3$ ” and the effects of each factor (except “salt” for total AsA) were significant (Fig. 5A–B). Salt per se induced a significant increase of total GSH content (+27% in comparison to AO\_NS).  $\text{O}_3$  (alone and in combination with salt) induced a slight reduction of total AsA content, with significant differences between salt treatments (−7 and −44% in comparison to AO\_NS, respectively). Conversely,  $\text{O}_3$  (alone and in combination with salt) significantly

increased the total GSH content (+25 and +21% in comparison to AO\_NS, respectively).

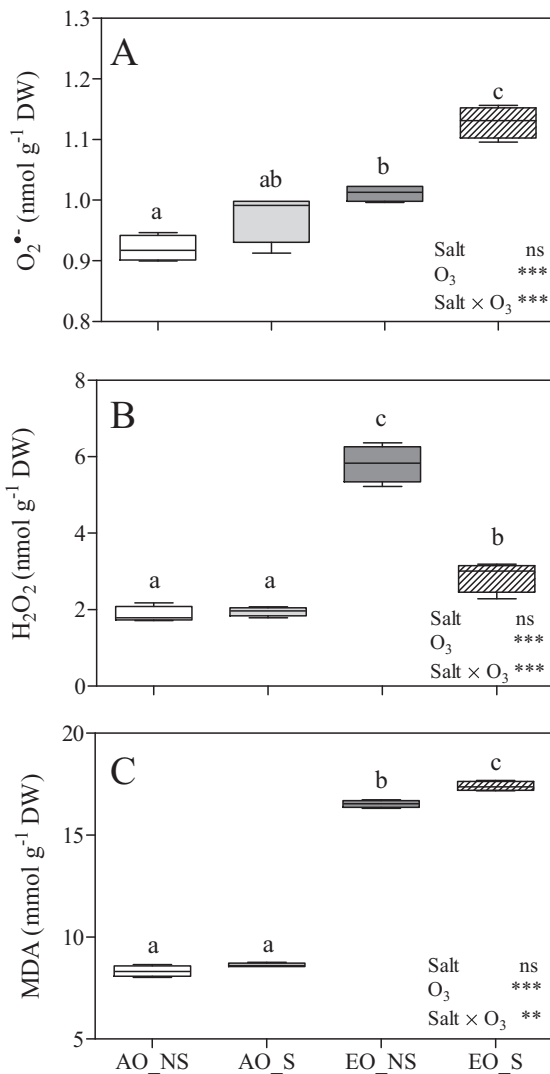
The Kruskal-Wallis test of the enzymatic antioxidant compounds involved in  $\text{H}_2\text{O}_2$  metabolism revealed that the interaction “salt  $\times$   $\text{O}_3$ ” and



**Fig. 2.** Photorespiration/net photosynthesis ratio ( $R_{PR}/A$ , A) and dark respiration rate ( $R_n$ , B) in pomegranate plants treated with  $\text{O}_3$  [two levels denoted as ambient  $\text{O}_3$  (AO) and elevated  $\text{O}_3$  (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Data are shown as mean  $\pm$  standard error. According to a two-way ANOVA with salt and  $\text{O}_3$  as factors, different letters indicate significant differences among bars ( $P \leq 0.05$ ,  $N = 3$ ). Asterisks show the significance of factors/interaction following the Tukey test: \* $P \leq 0.05$ ; ns  $P > 0.05$ .



**Fig. 3.** Maximum efficiency of PSII photochemistry ( $F_v/F_m$ , A), non-photochemical quenching (qNP, B) and photochemical quenching (qP, C) in pomegranate plants treated with  $\text{O}_3$  [two levels denoted as ambient  $\text{O}_3$  (AO) and elevated  $\text{O}_3$  (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Data are shown as mean  $\pm$  standard error. According to a two-way ANOVA with salt and  $\text{O}_3$  as factors, different letters indicate significant differences among bars ( $P \leq 0.05$ ,  $N = 3$ ). Asterisks show the significance of factors/interaction following the Tukey test: \*\*\* $P \leq 0.001$ .

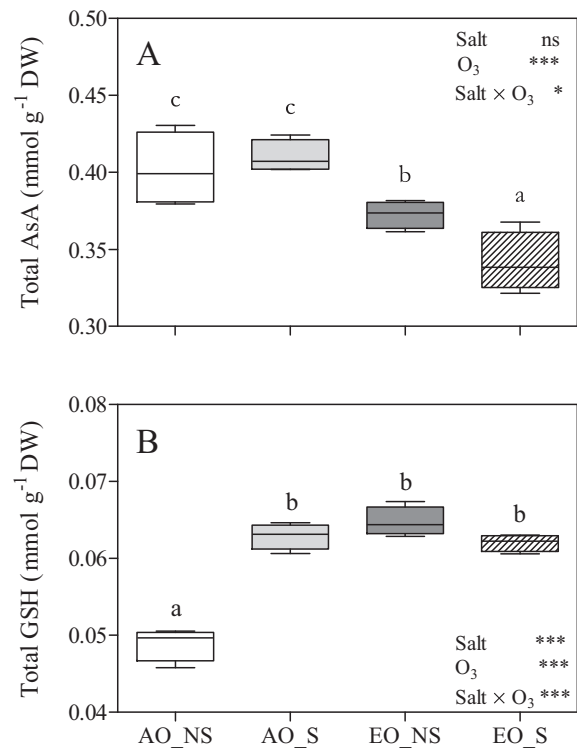


**Fig. 4.** Box and whiskers representation of the content of superoxide anion radical ( $O_2^{\bullet-}$ , A), hydrogen peroxide ( $H_2O_2$ , B) and malondialdehyde (MDA, C) in pomegranate plants treated with  $O_3$  [two levels denoted as ambient  $O_3$  (AO) and elevated  $O_3$  (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Different letters indicate significant differences among treatments ( $P \leq 0.05$ , Dunn's post test,  $N = 3$ ). Asterisks show the significance of factors/interaction following the Kruskal-Wallis test: \*\*\* $P \leq 0.001$ ; \*\* $P \leq 0.01$ ; ns  $P > 0.05$ . Abbreviations: dry weight, DW.

the effects of each factor were significant (Fig. 6A–C). Salt per se increased SOD, CAT and APX activity (+2, +333 and +26% in comparison to AO\_NS, respectively). Similarly,  $O_3$  (alone and in combination) stimulated the activity of these enzymes, with significant differences between salt treatment (+9 and +2% for SOD, 4- and 8-fold higher than AO\_NS for CAT, +55% for APX, only under EO\_NS conditions). A decrease was observed regarding the activity of APX in plants grown under EO\_S conditions (–60%).

### 3.6. Abscisic acid, proline and starch content

The Kruskal-Wallis test of ABA, proline and starch content revealed that the interaction “salt  $\times$   $O_3$ ” and the effects of each factor (except “salt” for proline) were significant (Fig. 7A–C). Salt per se significantly increased ABA (2-fold higher than AO\_NS), proline and starch content (+65 and +59%, respectively). Conversely,  $O_3$  (alone and in combination) induced a marked decrease of ABA content, without significant differences between the salt treatments. An increase was observed



**Fig. 5.** Box and whiskers representation of the content of total ascorbate (Total AsA, A) and total glutathione (Total GSH, B) in pomegranate plants treated with  $O_3$  [two levels denoted as ambient  $O_3$  (AO) and elevated  $O_3$  (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Different letters indicate significant differences among treatments ( $P \leq 0.05$ , Dunn's post test,  $N = 4$ ). Asterisks show the significance of factors/interaction following the Kruskal-Wallis test: \*\*\* $P \leq 0.001$ ; \* $P \leq 0.05$ ; ns  $P > 0.05$ . Abbreviations: dry weight, DW.

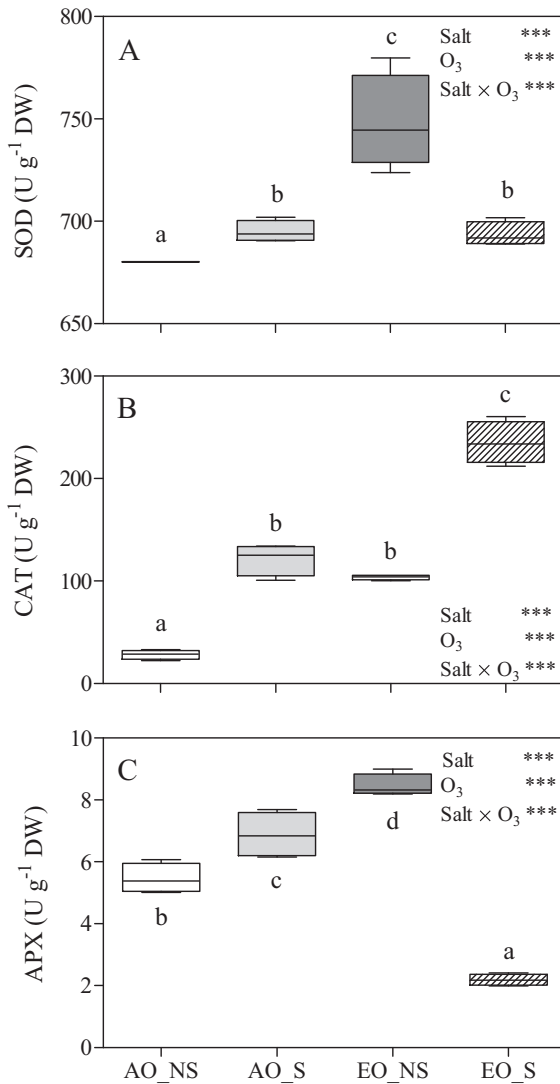
regarding the content of proline (+65 and +41% in comparison to AO\_NS) and starch (+78% only under EO\_S conditions).

## 4. Discussion

*Punica granatum* is an important commercial fruit tree, which exhibits reduced growth in saline soils (Khayyat et al., 2014). However, in comparison to other fruit trees this species is considered to be moderately tolerant to salt at least in terms of visible injury (Tavousi et al., 2015).

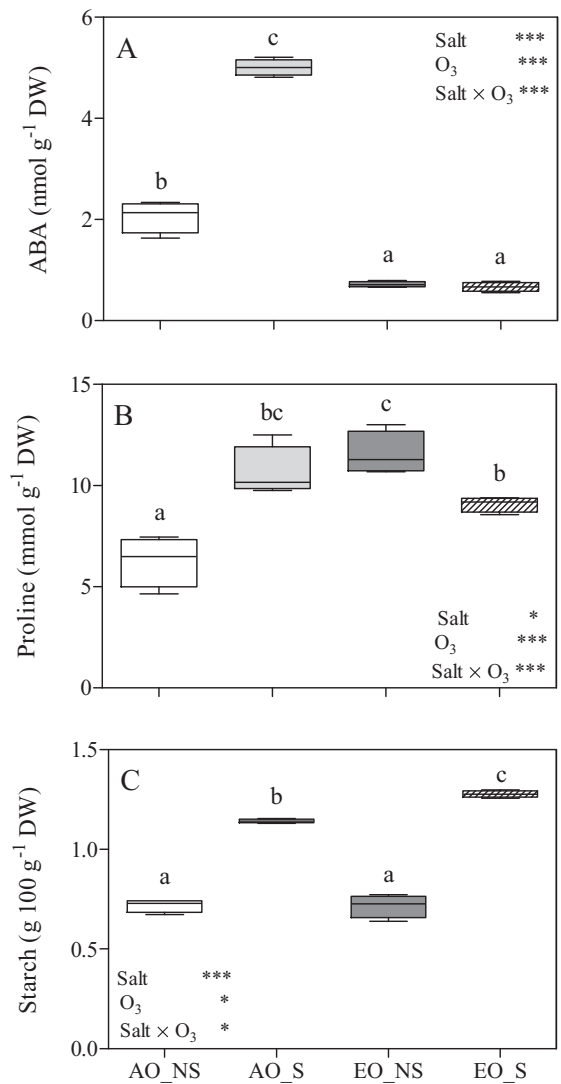
### 4.1. Which physiological and biochemical adjustments are induced by salt treatment or $O_3$ exposure?

Despite the large accumulation of  $Na^+$  in the roots and  $Cl^-$  in the leaves, no evidence of visible injury due to salt (e.g. tip yellow-brown necrosis) was found in plants grown under AO\_S and EO\_S conditions. This result suggests that the harmful effects of saline ions are prevented, likely by accumulation in the vacuoles (Ape and Blumwald, 2007; Teakle and Tyerman, 2010), where they may contribute to osmotic adjustments, as confirmed by the significant increase of ABA and proline. The observed accumulation of osmoprotectants could play a key role in the maintenance of water potential equilibrium within cells (Munns and Tester, 2008; Acosta-Motos et al., 2018). The maintenance of leaf water status (i.e. unchanged RWC and EL values) and effective osmotic adjustments could be considered as adaptive mechanisms against salt (Iqbal et al., 2014; Acosta-Motos et al., 2018). It is well established that this strategy is usually associated with effective stomatal closure to further protect leaves from water loss (“water saving strategy”; Munns and Tester, 2008). In our experiment, this is confirmed by the decrease of  $g_s$  values observed in plants grown under AO\_S conditions,



**Fig. 6.** Box and whiskers representation of the activity of superoxide dismutase (SOD, A), catalase (CAT, B) and ascorbate peroxidase (APX, C) in pomegranate plants treated with O<sub>3</sub> [two levels denoted as ambient O<sub>3</sub> (AO) and elevated O<sub>3</sub> (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Different letters indicate significant differences among treatments ( $P \leq 0.05$ , Dunn's post test,  $N = 4$ ). Asterisks show the significance of factors/interaction following the Kruskal-Wallis test: \*\*\* $P \leq 0.001$ . Abbreviations: dry weight, DW.

which could be one reason for their unchanged RWC values. According to the literature, the unchanged photosynthetic performance and the maintenance of leaf-root growth and functions for diluting toxic ions could be considered a conservative water-use strategy and an adaptive mechanism against salt stress (Munns and Tester, 2008). Chlorophyll *a* fluorescence measurements revealed that no alterations occurred in the biochemical chloroplast processes. The significant decrease of the  $F_v/F_m$  ratio (although no changes in  $F_0$  were detected) indicates that the severe reduction in potential PSII photochemical efficiency observed in plants under AO\_S conditions was due to photoprotective processes and not photoinhibitory damage (Maxwell and Johnson, 2000). This is confirmed by the enhancement of thermal dissipation in the PSII antennae (i.e. increase of  $q_{NP}$  values and decrease of  $q_P$  values) that can prevent possible photodamage to PSII due to the excess of excitation energy (Demmig-Adams and Adams, 1992; Guidi et al., 2017). The efficiency of this mechanism involved in photoprotection was confirmed by the maintenance of membrane integrity (i.e. unchanged MDA by-product values) and the lack of ROS production (i.e. unchanged H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup> levels). It is well established that this strategy is usually



**Fig. 7.** Box and whiskers representation of abscisic acid (ABA, A), proline (B) and starch (C) content in pomegranate plants treated with O<sub>3</sub> [two levels denoted as ambient O<sub>3</sub> (AO) and elevated O<sub>3</sub> (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Different letters indicate significant differences among treatments ( $P \leq 0.05$ , Dunn's post test,  $N = 4$ ). Asterisks show the significance of factors/interaction following the Kruskal-Wallis test: \*\*\* $P \leq 0.001$ ; \* $P \leq 0.05$ . Abbreviations: dry weight, DW.

associated with enzymatic and non-enzymatic antioxidant components to further counteract the salt-triggered oxidative stress (Penella et al., 2016; Guidi et al., 2017). In our experiment, the activity of the primary antioxidant enzymes involved in removing and/or scavenging ROS (i.e. SOD, CAT and APX) was stimulated in plants under AO\_S conditions. The concomitant enhancement of low-molecular-weight antioxidants (i.e. AsA and GSH content) confirms that they were actively involved in response to salt toxicity. We conclude that the simultaneous involvement of physiological adjustments, osmoprotectants and antioxidant compounds is necessary to obtain an adaptive response in pomegranate plants against salt stress.

The physiological responses in plants exposed to increased O<sub>3</sub> concentrations were quite different from those induced by the salt treatment. Under EO\_NS conditions, a significant reduction of the CO<sub>2</sub> assimilation rate was a result of decreased CO<sub>2</sub> diffusion to the chloroplast (as confirmed by the reduction of stomatal conductance). Consequently, a reduction in photochemical energy usage was observed by that lead to photoinhibitory damage (as confirmed by the concomitant reduction of  $F_v/F_m$  ratio and  $q_{NP}$  values; Demmig-Adams and Adams,



1992) and generation of ROS (i.e.  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\bullet-}$  production; Kangasjärvi et al., 2012). It is well established that the accumulation of excessive ROS under stress conditions (Asada, 2006) occurs when the reduction of photosynthesis is much higher than the extent of the reduction in actual PSII efficiency (Baker et al., 2007). In our experiment, the unchanged qP values confirm that non-reductive processes aimed at preserving the photosynthetic apparatus against damage induced by high excitation pressure, were not activated according to Cotrozzi et al. (2016). These results indicate that ROS act as damaging agents that cause cell death through excessive oxidation of cellular components (Choudhury et al., 2017), as confirmed by the significant increase in the MDA by-products levels and the presence of visible injury. Although the low efficiency of the Halliwell-Asada cycle (as confirmed by the decrease of AsA content and the unchanged GSH values) did not protect cells against the negative impact of increased  $\text{O}_3$  concentrations, plants tried to preserve themselves from further oxidative damage. In fact, the strong increase in proline content and the induction of enzymes involved in  $\text{H}_2\text{O}_2$  metabolism suggest that plants activated several cellular processes (i.e. phenolic and anthocyanin synthesis, González-Villagra et al., 2018; Pellegrini et al., 2018). As a consequence, more extensive oxidative damage of other components of the photosynthetic electron transfer chain was avoided, as confirmed by the increase of  $R_{PR}/A$  ratio, the unchanged starch content and the concomitant decrease of ABA.

#### 4.2. Can salt treatment trigger a set of plant adaptive responses to $\text{O}_3$ ?

The combination of salt and  $\text{O}_3$  did not affect stomatal regulation, as  $g_s$  values of plants grown under EO\_S conditions were statistically similar to those recorded under EO\_NS. In contrast, osmotic stress was expected to partially reduce the negative effects of  $\text{O}_3$  by limiting stomatal  $\text{O}_3$  flux into the leaf and reducing mesophyll conductance (Iyer et al., 2013). However, plants grown under EO\_S conditions were not able to counteract the  $\text{O}_3$ -triggered oxidative stress. The failure of these leaf-intrinsic adjustments was confirmed by the alteration of membrane integrity (i.e. dramatic increase in the MDA by-products level) and the presence of visible injury (as confirmed by II values). After penetration through stomatal openings,  $\text{O}_3$  causes a general ionic and metabolic disturbance within the cell or the formation of toxic products, in a dose-dependent manner (Van Breusegem and Dat, 2006). In our experiment, the production of ROS (i.e. significant increase of  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\bullet-}$  content), the photoinhibitory damage of PSII (as confirmed by the concomitant reduction of  $F_v/F_m$  ratio, qP and qNP values) and the substantial decrease of  $\text{CO}_2$  assimilation rate in the combined treatment suggests that salt did not play an antagonistic role against  $\text{O}_3$ . According to Deinlein et al. (2014), the investment of energy and carbon reserves, and the consequent osmoregulation, is a crucial process underlying plant tolerance to salt and influencing the interaction of salt with other stressors. In our experiment, the significant increase of proline and starch content confirmed that osmotic adjustments occurred to maintain the water potential equilibrium within cells (Ackerson, 1981), as confirmed by unchanged RWC and EL values. However, ABA seemed not to be involved in osmotic regulation (as confirmed by the significant decrease), but may have been involved in  $\text{O}_3$ -eliciting adaptive mechanisms, such as the regulation of phenolic compounds biosynthesis (González-Villagra et al., 2018; Pellegrini et al., 2018). An analysis of the antioxidant pool revealed that further adjustments were not able to maintain control of  $\text{O}_3$ -induced oxidative stress. The dramatic reduction of Tot AsA content in plants grown under EO conditions indicated that the Halliwell-Asada cycle (the first line of defence against an oxidative load induced by  $\text{O}_3$ ; Noctor and Foyer, 1998) was not sufficient to counteract the negative effects of  $\text{O}_3$ . In fact,  $\text{O}_3$  (irrespective of the presence of salt) induced a strong alteration of the AsA pool (as confirmed by the significant decrease of APX activity). Similarly, the increased activities of SOD and CAT did not efficiently sustain ROS scavenging in relation to  $\text{O}_3$ -triggered ROS production,

confirming the failure of these metabolic adjustments. We thus conclude that the ecophysiological and biochemical adjustments adopted by *P. granatum* to preserve leaf functionality under salt stress, did not ameliorate  $\text{O}_3$ -induced oxidative stress. These abrupt global change factors pose new challenges for tree species that have already adapted to gradual climate fluctuations. Further studies may be useful to investigate the molecular mechanisms underlying the differences in *P. granatum* between acclimation and early adaptation to these stressors (single and/or combined) and possible processes favoring or limiting metabolic adjustments.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.11.402>.

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