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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ABSTRACT

Plants are exposed to a broad range of environmental stresses, such as salinity and ozone (O_3), 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 of were exposed to O_3 [two levels denoted as ambient (AO) and elevated (EO) O_3 concentrations] and salinity [S (salt, 50 mM NaCl)]

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for three consecutive months. No salt (NS) plants received distilled water. Despite the accumulation of Na^+ and Cl^- in the aboveground biomass, no evidence of visible injury due to salt (e.g. tip yellow-brown necrosis) 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_3, as confirmed by the reduction of the CO_2 assimilation rate (-21%), accumulation of reactive oxygen species (+10 and +225% of superoxide anion and hydrogen peroxide) and malondialdehyde 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_3-induced oxidative stress.

Keywords

Pomegranate, oxidative damage, stomatal regulation, osmoprotectants, antioxidants, multiple stress

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_3), 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^+ and Cl^- 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 Gilliham, 2015).

Tropospheric O_3 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_3 documented different responses from those observed when each stressor was applied independently. Results were sometimes contradictory: salt stress either counteracted O_3 impact by reducing stomata opening (Maggio et al., 2007; Gerosa et al., 2014), or further exacerbated O_3-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_3.

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; Mastrogiannidou 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_3, 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_3 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_3 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_3 exposure? ii) Can salt treatment trigger a set of plant adaptive responses to O_3?

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_3-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_3 [two levels, denoted as ambient O_3 (AO) and elevated O_3 (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_3 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^{-1} and 7.6, respectively. Three replicated plots (5 × 5 × 2 m) were assigned to each O_3 level, with three plants for each combination of O_3 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_3 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_3-symptomatic leaves was counted and

121 expressed as percentage relative to the total number of leaves of the selected branch (LA). The
122 percentage of injured leaf surface per O_3-symptomatic leaf (IL) was visually assessed with the help
123 of photoguides (Paoletti et al. 2009) and averaged at the branch level. The Injury Index (II) was
124 calculated by combining the two parameters: II = (LA × IL)/100.

125 Relative water content (RWC) and electrolytic leakage (EL) were determined on three fully-
126 expanded mature leaves of three plants per treatment, following standard methodologies (Nali et al.
127 2005).

128 At harvest, all plant material [leaves, stems, fine roots (diameter ≤ 2 mm) and coarse roots
129 (diameter > 2 mm)] was oven dried at 80 °C until a constant weight was achieved and then each
130 organ was grounded into a fine powder. The total C and N content was determined with an element
131 analyser (TruSpec, CNS, LECO, Saint Joseph, MI, USA). About 0.3 g of powder were mineralized
132 with 2 ml of a mixture of ultrapure concentrated HNO_3 and H_2O_2 at 190 °C and a pressure of 3.2
133 MPa in a microwave pressure digestion-closed system (Speedwave MWS-2, Berghof, Germany).
134 The total content of Ca, K, Mg, Mn, Na and P were determined by Inductively Coupled Plasma-
135 Optical Emission Spectroscopy (ICP-OPS Optima 2100 DV, Perkin Elmer, Waltham, MA, USA).
136 Analytical quality was checked with the Certified Reference Material (Multi-Element standard
137 solution for ICP, CPAChem, Stara Zagora, Bulgaria). All analyses were carried out in triplicate.
138 The accuracy was within 99.999% for all elements. Chloride content was determined
139 spectrophotometrically (Lambda 25 UV-VIS, Perkin Elmer, Waltham, MA, USA) at 463 nm, on the
140 ashes calcined with calcium carbonate at 450 °C for 12 h, and dissolved in water.

141 *2.3. Gas exchange and chlorophyll* a *fluorescence*

142 In early August, leaf gas exchange and chlorophyll *a* fluorescence were measured using a portable
143 Infrared Gas Analyser (Model 6400, Li-Cor instruments, Lincoln, NE, USA) with a fluorescence
144 chamber head (Model 6400-40, Li-Cor instruments, Lincoln, NE, USA). Measurements were
145 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_2 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 μmol m^{-2} s^{-1}). Net photosynthetic rate (A) and stomatal conductance to water vapour (g_s), the ratio of the intercellular CO_2 concentration (C_i) of a leaf to the ambient CO_2 concentration (C_a: 400 ppm), i.e., the C_i/C_a ratio, were recorded, and mesophyll conductance (g_m) was estimated using the variable J method (Harley et al., 1992). Respiration rate in the light (R_d) 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_n) was measured by switching off the LED light source after the Kok protocol until the efflux of CO_2 from the leaf remained stable. Photorespiration (R_{PR}) was calculated according to Sharkey (1988), i.e. $R_{PR} = (A+R_d)/(C_c/ \Gamma^*-1)$ where C_c and Γ^* are the CO_2 concentration at the chloroplast and the CO_2 compensation point in the absence of R_d, 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_m) and the maximal PSII photochemical efficiency [F_v/F_m, where F_v is the variable fluorescence (F_m - minimal fluorescence)] were determined. The saturation pulse method was used for analysis of photochemical (qP) and no-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 ($O_2^{\bullet-}$) was determined according to Cotrozzi et al. (2017) by the reduction of tetrazolium dye sodium by O_2 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 μl of supernatant in a final volume of 1 ml. $O_2^{\bullet-}$ was determined with a spectrophotometer (6505 UV-Vis, Jenway, UK) at 470 nm. The amount of $O_2^{\bullet-}$ was calculated using

171 the molar extinction coefficient 21.6 mM^{-1} cm^{-1}. Hydrogen peroxide (H_2O_2) content was measured
172 using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Life
173 Technologies Corp., Carlsbad, CA, USA), according to Cotrozzi et al. (2017). After extraction with
174 potassium-phosphate buffer (20 mM, pH 6.5), H_2O_2 was determined with a fluorescence/absorbance
175 microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA) at 510 and
176 590 nm (excitation and emission of resofurin fluorescence, respectively). Each outcome was plotted
177 against a H_2O_2 standard curve (from 0 to 20 µM). Oxidative damage was estimated in terms of lipid
178 peroxidation by determining the malondialdehyde (MDA) by-product accumulation, according to
179 the method of Hodges et al. (1999) with minor modifications, as reported by Pellegrini et al. (2012).
180 After extraction with ethanol (80%, v/v), MDA was determined with the same
181 fluorescence/absorbance microplate reader reported above at 532 and 600 nm. The amount of MDA
182 was calculated using the molar extinction coefficient of 155 mM^{-1} cm^{-1}.

183 *2.5. Non-enzymatic and enzymatic antioxidant compounds*

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

193 After extraction with potassium/phosphate buffer (50 mM, pH 7.0) containing 1 mM EDTA,
194 1% (w/v) polyvinylpyrrolidone (PVP), 0.1% (v/v) Triton X-100 and 10% (v/v) glycerol, catalase
195 (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1) activities were determined with

196 the same spectrophotometer reported above at 240 and 560 nm, following the methods of Aebi
197 (1984) and Zhang and Kirkham (1996), respectively. After extraction with Na-phosphate buffer (50
198 mM, pH 7.0) containing 1 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol and 5 mM
199 ascorbate, ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined with the same
200 spectrophotometer reported above at 290 nm, following the method of Mittler and Zilinskas (1993).
201 For all assays, a previous protein precipitation with ammonium sulphate was carried out following
202 the method of Wingfield (2016) for crude extract purification. One unit of SOD was described as
203 the amount of enzyme required to inhibit the reduction of nitroblue tetrazolium by 50%. One unit of
204 CAT was defined as the amount of enzyme required for the dismutation of 1 μmol H_2O_2 per minute.
205 One unit of APX was defined as the amount of enzyme required to oxidize or reduce 1 μmol of
206 AsA per minute. The whole procedures are described by Pellegrini et al. (2018). The protein content
207 was measured by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard.

208 *2.6. Abscisic acid and osmolytes*

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

219 *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_3 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 Kruskall-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 necrosis) was not observed in plants grown under salt conditions (independently of O_3 concentrations), confirming that a mild stress occurred. The repeated two-way ANOVA measurements of II values revealed that the interaction "salt × O_3" was not significant. Starting from 21st August (12 weeks after the beginning of the exposure), the typical foliar injury due to O_3 stress (e.g. reddish stipples, homogenously 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±0.43 *vs* 0.20±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^+ content in fine roots (+27% compared to AO_NS, Table 1). A similar response was observed for Cl^- 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_3 concentrations induced a significant decrease of Na^+ content in fine roots (-60% compared to AO_NS). By contrast, an increase of Cl^- 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^+ was accumulated more belowground than in the aboveground parts (1.71±0.23 *vs* 0.48±0.05 mg g^{-1} dry weight, DW; $P \leq 0.001$). An opposite trend was observed for Cl^- (2.19±0.17 *vs* 3.65±0.37 mg g^{-1} DW; $P \leq 0.05$). No significant differences were observed among treatments regarding other macro- and micro-elements, RWC and EL (*data not shown*).

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_{PR}/A ratio in the "O_3" and in the interactions "salt × O_3" (Fig. 2). Salt *per se* induced a slight increase of the R_{PR}/A ratio and R_n only under AO conditions (+28 and +26% compared to NS, respectively). Similarly, O_3 (alone or in combination with salt) significantly increased R_{PR}/A compared to AO_NS. A similar pattern was observed for R_n in plants grown under EO_NS conditions (+17%). No significant interactions were observed regarding the other gas exchange parameters such as A, g_s, C_i/C_a and g_m (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_s 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_i/C_a ratio and g_m 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_v/F_m ratio and qP values (-17 and -3% compared to AO_NS, respectively; Fig. 3). An opposite trend was observed regarding qNP (+5%). Increasing O_3 (alone and in combination with salt) induced a reduction of F_v/F_m 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 Kruskall-Wallis test of ROS and MDA content revealed that the interaction "salt × O_3" and the effects of each factor were significant (Fig. 4 A-C). Salt alone did not alter the content of $O_2^{\bullet-}$, H_2O_2 and MDA in comparison to plants grown under AO_NS conditions. Conversely, O_3 (alone and in combination with salt) significantly induced ROS accumulation (+10 and +23% of $O_2^{\bullet-}$ in comparison to AO_NS; +225 and +67% of H_2O_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 Kruskall-Wallis test of total AsA and total GSH content revealed that the interaction "salt × O_3" and the effects of each factor (except "salt" for total AsA) were significant (Fig. 5 A-B). Salt *per se* induced a significant increase of total GSH content (+27% in comparison to AO_NS). 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, O_3 (alone and in combination with salt) significantly increased the total GSH content (+25 and +21% in comparison to AO_NS, respectively).

The Kruskall-Wallis test of the enzymatic antioxidant compounds involved in H_2O_2 metabolism revealed that the interaction "salt × O_3" and the effects of each factor were significant (Fig. 6 A-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 Kruskall-Wallis test of ABA, proline and starch content revealed that the interaction "salt × O_3" and the effects of each factor (except "salt" for proline) were significant (Fig. 7 A-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 regarding the content of proline (+65 and +41% in comparison to AO_NS) and starch (+78% only under EO_S conditions).

4. Discussion

Punica gratanum 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 (Apse 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

316 grown under AO_S conditions, which could be one reason for their unchanged RWC values.
317 According to the literature, the unchanged photosynthetic performance and the maintenance of leaf-
318 root growth and functions for diluting toxic ions could be considered a conservative water-use
319 strategy and an adaptive mechanism against salt stress (Munns and Tester, 2008). Chlorophyll *a*
320 fluorescence measurements revealed that no alterations occurred in the biochemical chloroplast
321 processes. The significant decrease of the F_v/F_m ratio (although no changes in F_0 were detected)
322 indicates that the severe reduction in potential PSII photochemical efficiency observed in plants
323 under AO_S conditions was due to photoprotective processes and not photoinhibitory damage
324 (Maxwell and Johnson, 2000). This is confirmed by the enhancement of thermal dissipation in the
325 PSII antennae (i.e. increase of qNP values and decrease of qP values) that can prevent possible
326 photodamage to PSII due to the excess of excitation energy (Demmig-Adams et al. 1996; Guidi et
327 al., 2017). The efficiency of this mechanism involved in photoprotection was confirmed by the
328 maintenance of membrane integrity (i.e. unchanged MDA by-product values) and the lack of ROS
329 production (i.e. unchanged H_2O_2 and O_2^- levels). It is well established that this strategy is usually
330 associated with enzymatic and non-enzymatic antioxidant components to further counteract the salt-
331 triggered oxidative stress (Penella et al., 2016; Guidi et al., 2017). In our experiment, the activity of
332 the primary antioxidant enzymes involved in removing and/or scavenging ROS (i.e. SOD, CAT and
333 APX) was stimulated in plants under AO_S conditions. The concomitant enhancement of low-
334 molecular-weight antioxidants (i.e. AsA and GSH content) confirms that they were actively
335 involved in response to salt toxicity. We conclude that the simultaneous involvement of
336 physiological adjustments, osmoprotectants and antioxidant compounds is necessary to obtain an
337 adaptive response in pomegranate plants against salt stress.

338 The physiological responses in plants exposed to increased O_3 concentrations were quite
339 different from those induced by the salt treatment. Under EO_NS conditions, a significant reduction
340 of the CO_2 assimilation rate was a result of decreased CO_2 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 qNP values; Demmig-Adams and Adams, 1992) and generation of ROS (i.e. H_2O_2 and $O_2^{\bullet-}$ 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 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 H_2O_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 O_3?

The combination of salt and 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 O_3 by limiting stomatal O_3 flux into the leaf and reducing mesophyll conductance (Iyer et al., 2013). However, plants grown

366 under EO_S conditions were not able to counteract the O_3-triggered oxidative stress. The failure of
367 these leaf-intrinsic adjustments was confirmed by the alteration of membrane integrity (i.e. dramatic
368 increase in the MDA by-products level) and the presence of visible injury (as confirmed by II
369 values). After penetration through stomatal openings, O_3 causes a general ionic and metabolic
370 disturbance within the cell or the formation of toxic products, in a dose-dependent manner (Van
371 Breusegem and Dat, 2006). In our experiment, the production of ROS (i.e. significant increase of
372 H_2O_2 and $O_2^{\bullet-}$ content), the photoinhibitory damage of PSII (as confirmed by the concomitant
373 reduction of F_v/F_m ratio, qP and qNP values) and the substantial decrease of CO_2 assimilation rate in
374 the combined treatment suggests that salt did not play an antagonistic role against O_3. According to
375 Deinlein et al. (2014), the investment of energy and carbon reserves, and the consequent
376 osmoregulation, is a crucial process underlying plant tolerance to salt and influencing the
377 interaction of salt with other stressors. In our experiment, the significant increase of proline and
378 starch content confirmed that osmotic adjustments occurred to maintain the water potential
379 equilibrium within cells (Ackerson, 1981; as confirmed by unchanged RWC and EL values).
380 However, ABA seemed not to be involved in osmotic regulation (as confirmed by the significant
381 decrease), but may have been involved in O_3-eliciting adaptive mechanisms, such as the regulation
382 of phenolic compounds biosynthesis (González-Villagra et al., 2018; Pellegrini et al., 2018). An
383 analysis of the antioxidant pool revealed that further adjustments were not able to maintain control
384 of O_3-induced oxidative stress. The dramatic reduction of Tot AsA content in plants grown under
385 EO conditions indicated that the Halliwell-Asada cycle (the first line of defence against an oxidative
386 load induced by O_3; Noctor and Foyer, 1998) was not sufficient to counteract the negative effects of
387 O_3. In fact, O_3 (irrespective of the presence of salt) induced a strong alteration of the AsA pool (as
388 confirmed by the significant decrease of APX activity). Similarly, the increased activities of SOD
389 and CAT did not efficiently sustain ROS scavenging in relation to O_3-triggered ROS production,
390 confirming the failure of these metabolic adjustments. We thus conclude that the ecophysiological
391 and biochemical adjustments adopted by *P. granatum* to preserve leaf functionality under salt

stress, did not ameliorate 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.

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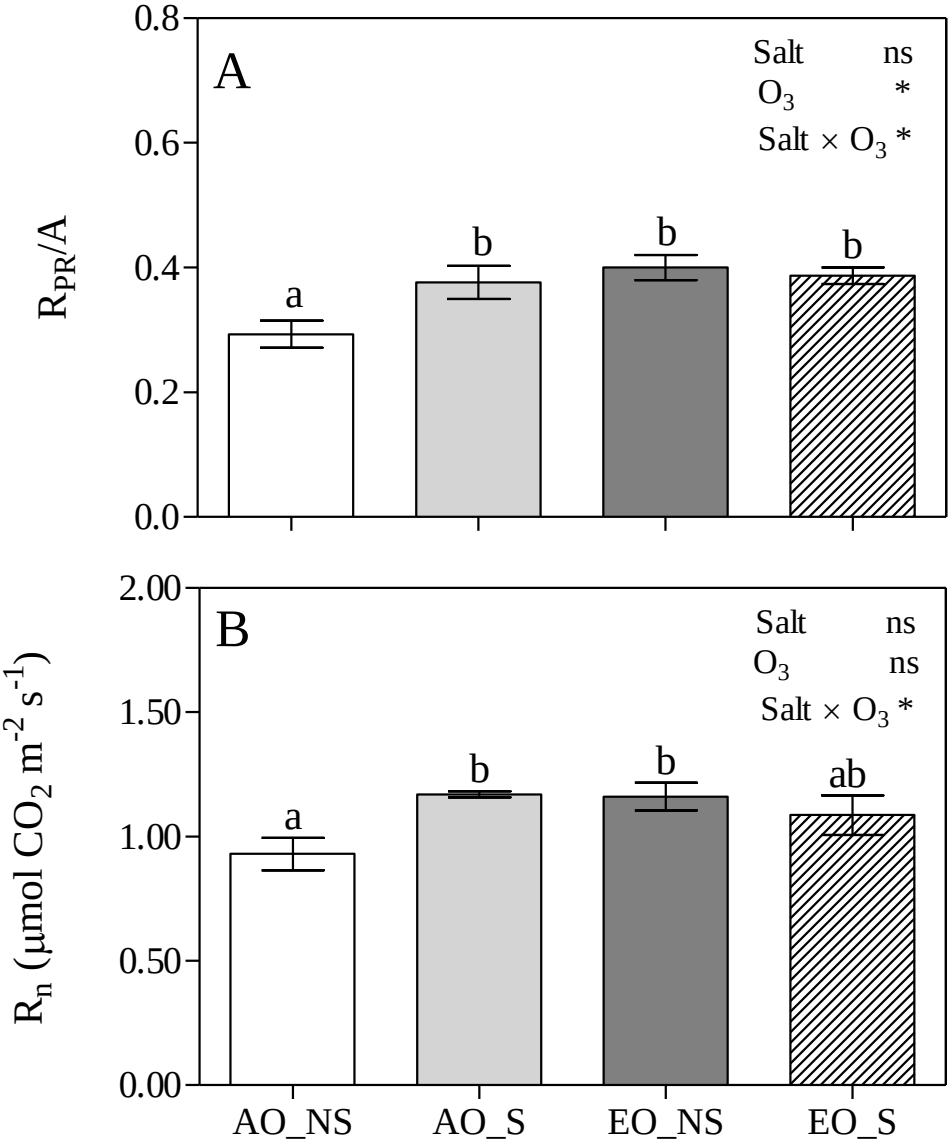
555 Table 1 Sodium (Na^+) and chloride (Cl^-) contents (expressed as mg g^{-1} dry weight) in different organs of pomegranate plants treated with O_3 [two
556 levels denoted as ambient O_3 (AO) and elevated O_3 (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received
557 distilled water. Data are shown as means ± standard error. Asterisks show the significance of factors/interaction following the two-way ANOVA
558 with salt and 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
559 following the Tukey's post-hoc test ($P \leq 0.05$).

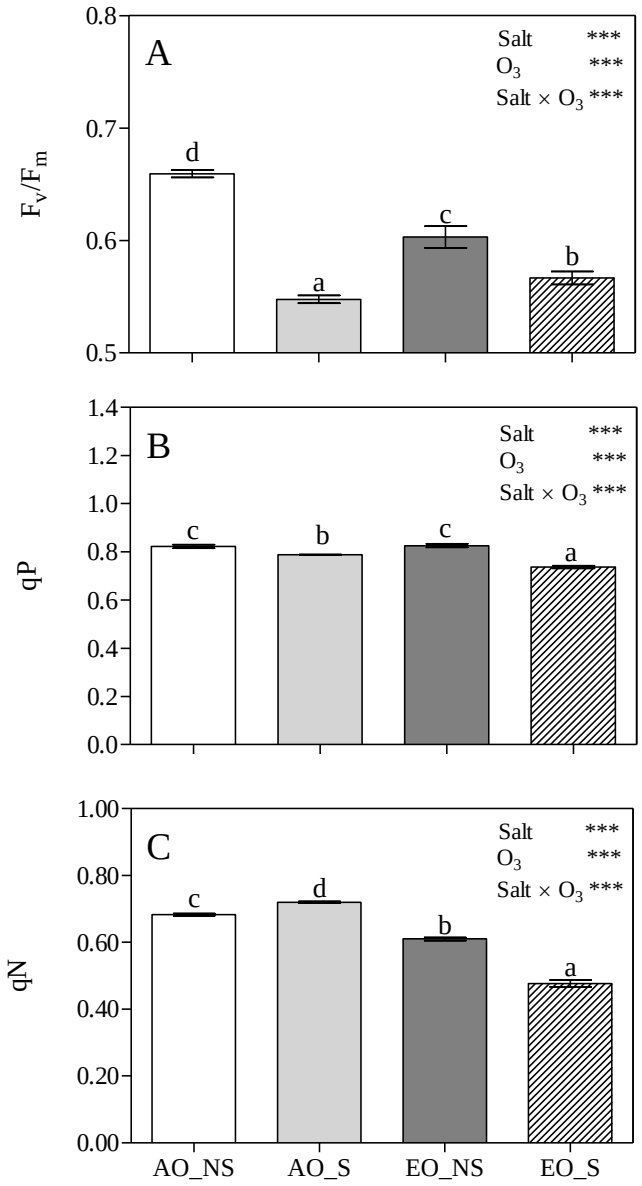
	Organ	AO_NS	AO_S	EO_NS	EO_S	Salt	O_3	Salt × O_3
Na^+	Leaves	0.27±0.01	0.51±0.07	0.27±0.01	0.76±0.12	***	ns	ns
	Stems	0.36±0.01	0.47±0.01	0.26±0.03	0.45±0.02	***	*	ns
	Fine roots	2.11±0.10 b	2.68±0.07 c	0.85±0.04 a	2.30±0.06 b	***	***	***
	Coarse roots	0.88±0.10 a	0.81±0.08 a	1.69±0.09 b	1.01±0.12 a	***	***	*
Cl^-	Leaves	3.84±0.23	3.78±0.57	3.65±0.23	4.57±0.21	ns	ns	ns
	Stems	1.19±0.05 a	3.72±0.08 c	1.45±0.14 bc	1.82±0.09 b	***	***	***
	Fine roots	2.56±0.08 b	1.53±0.12 a	2.48±0.18 b	2.93±0.03 b	*	***	***
	Coarse roots	1.22±0.06 a	2.50±0.05 c	1.32±0.02 a	1.87±0.05 b	***	***	***

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



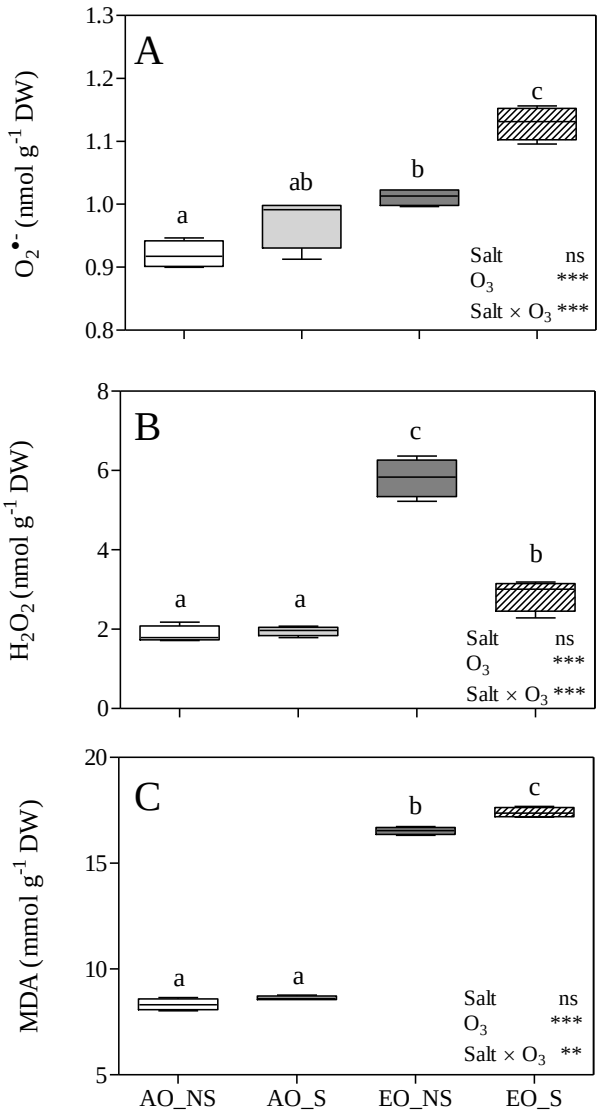
571

572 Fig. 2. Photorespiration/net photosynthesis ratio (R_{PR}/A, A) and dark respiration rate (R_n, B) in
573 pomegranate plants treated with O_3 [two levels denoted as ambient O_3 (AO) and elevated O_3 (EO)
574 concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled
575 water. Data are shown as mean ± standard error. According to a two-way ANOVA with salt and O_3
576 as factors, different letters indicate significant differences among bars ($P \leq 0.05$, N = 3). Asterisks
577 show the significance of factors/interaction following the Tukey test: * $P \leq 0.05$.



578

579 Fig. 3. Maximum efficiency of PSII photochemistry (F_v/F_m, A), non-photochemical quenching
580 (qNP, B) and photochemical quenching (qP, C) in pomegranate plants treated with O_3 [two levels
581 denoted as ambient O_3 (AO) and elevated O_3 (EO) concentrations, respectively], and salt (S: 50 mM
582 NaCl). No salt (NS) plants received distilled water. Data are shown as mean ± standard error.
583 According to a two-way ANOVA with salt and O_3 as factors, different letters indicate significant
584 differences among bars ($P \leq 0.05$, N = 3). Asterisks show the significance of factors/interaction
585 following the Tukey test: *** $P \leq 0.001$.



586

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

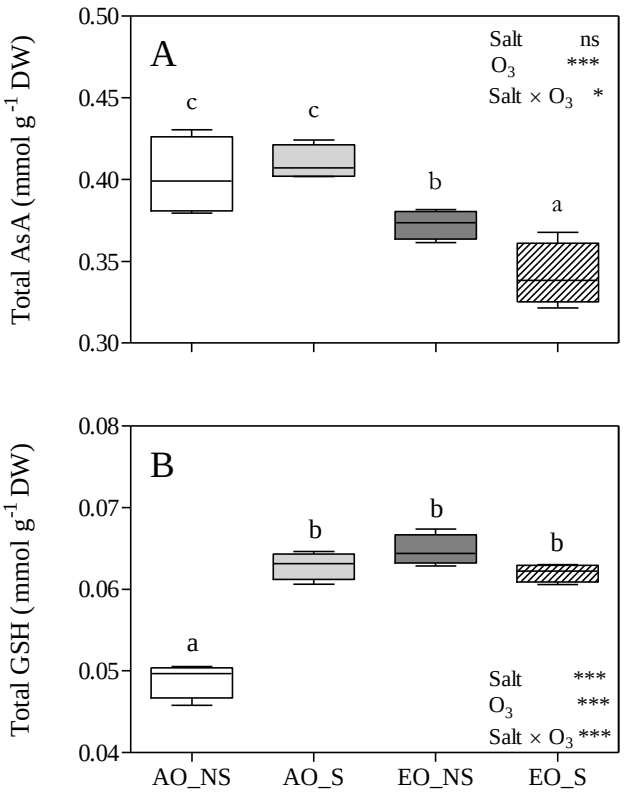


Fig. 5. Box and Whiskers representation of the content of total ascorbate (Total AsA, A) and total glutathione (Total GSH, B) in pomegranate plants treated with O_3 [two levels denoted as ambient O_3 (AO) and elevated O_3 (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Data are shown as mean ± standard error. 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.

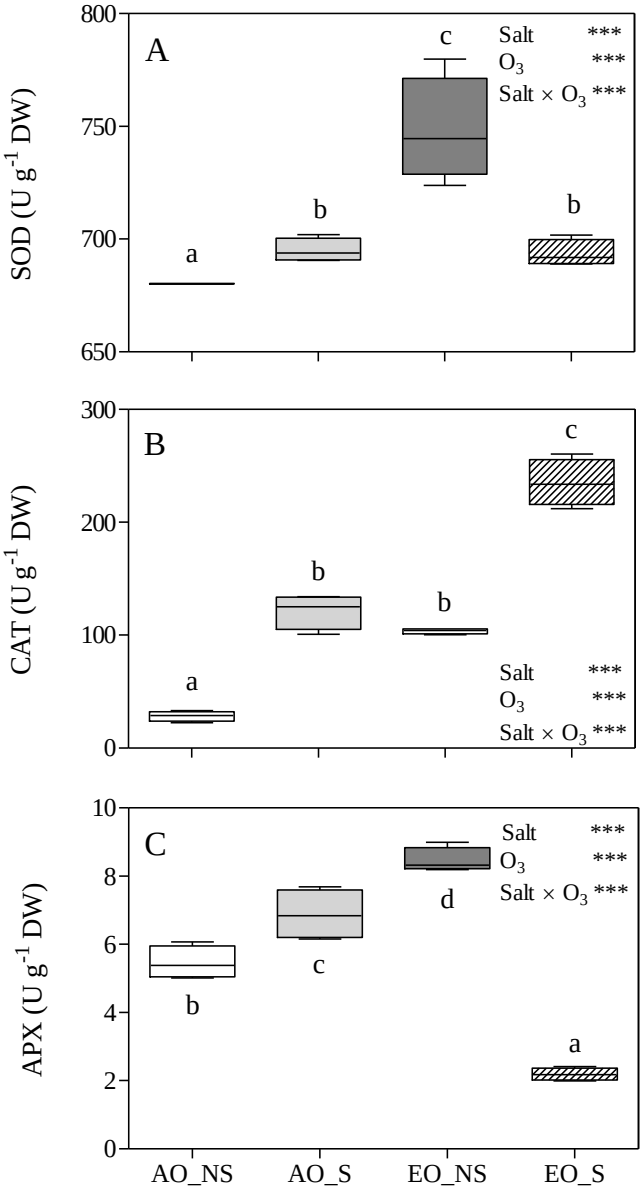
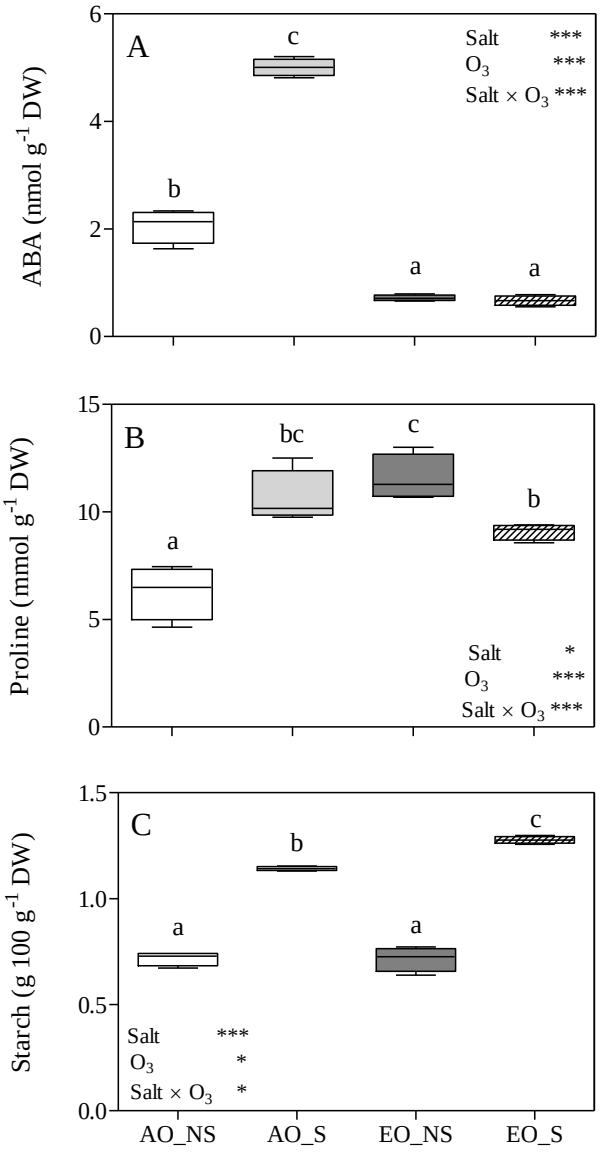


Fig. 6. Box and Whiskers representation of the activity of superoxide dismutase (SOD, A), catalase (CAT, B) and ascorbate peroxidase (APX, C) in pomegranate plants treated with O_3 [two levels denoted as ambient O_3 (AO) and elevated O_3 (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Data are shown as mean ± standard error. 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.



610

611 Fig. 7. Box and Whiskers representation of abscisic acid (ABA, A), proline (B) and starch (C)
612 content in pomegranate plants treated with O_3 [two levels denoted as ambient O_3 (AO) and elevated
613 O_3 (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received
614 distilled water. Data are shown as mean ± standard error. Different letters indicate significant
615 differences among treatments ($P \leq 0.05$, Dunn's post test, N = 4). Asterisks show the significance
616 of factors/interaction following the Kruskal-Wallis test: *** $P \leq 0.001$; * $P \leq 0.05$. Abbreviations:
617 dry weight, DW.

618

Supplemental material

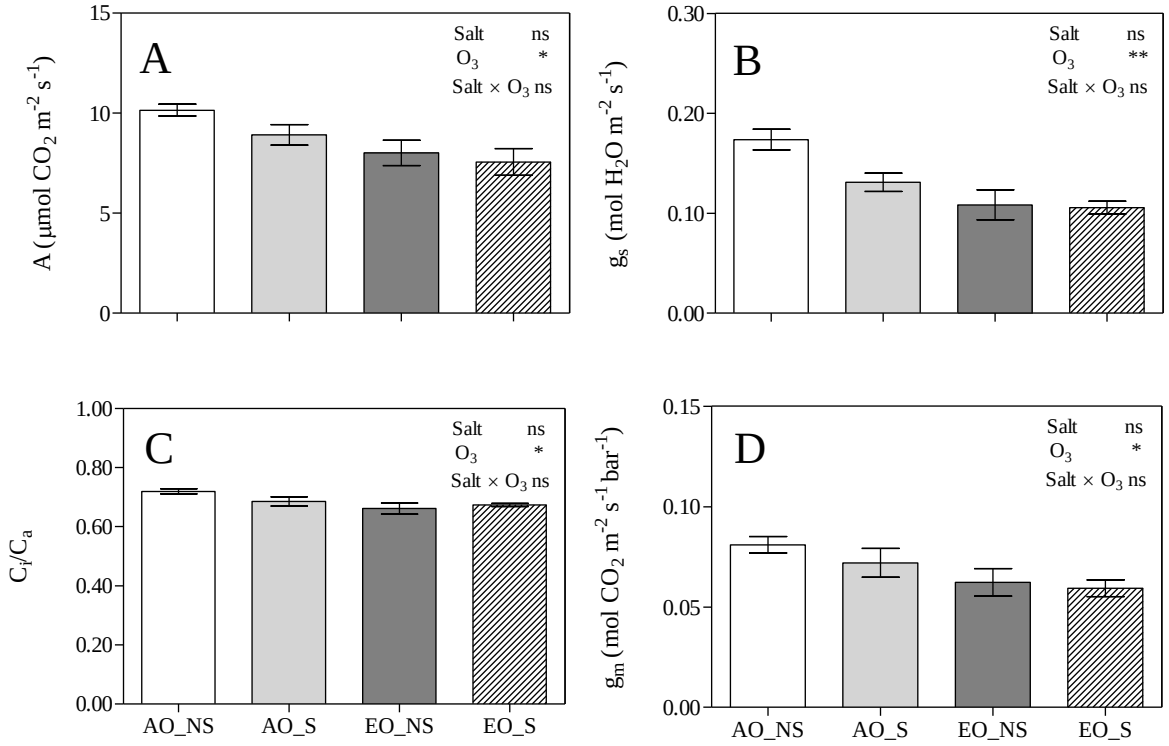


Fig. S1. Net photosynthesis (A, A), stomatal conductance to water vapor (g_s, B), internal CO_2 concentration/ambient CO_2 concentration (C_i/C_a, C) and mesophyll conductance (g_m, D) in pomegranate plants treated with O_3 [two levels denoted as ambient O_3 (AO) and elevated O_3 (EO) concentrations, respectively], and salt (S: 50 mM NaCl). No salt (NS) plants received distilled water. Data are shown as mean ± standard error. According to a two-way ANOVA with salt and O_3 as factors, the interactions were not significant (N = 3). Asterisks show the significance of factors following the Tukey test: ** $P \leq 0.01$; * $P \leq 0.05$.