1 **The harsh life of an urban tree: ozone responses of salt-stressed *Quercus ilex* saplings**

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14 **Running head: O₃ effect on salt-stressed *Quercus ilex* saplings**

16

17 **ABSTRACT**

18 Ozone (O_3) and salinity are usually tested as combined factors on plant performances, but the effect

19 of a single episode of O_3 in plants prior subjected to salinity has never been tested before. For this

20 purpose, three-year-old *Quercus ilex* (holm oak) saplings were exposed to salinity (150 mM NaCl,

21 15 days) and effects on photosynthesis, hydric relations and ion partitioning were evaluated

22 (Experiment I). In Experiment II, salt-treated saplings were exposed to a single peak of O_3 with a

23 realistic concentration in the Mediterranean environment (80 nL L^{-1}, 5 hours). Gas exchanges,

24 chlorophyll fluorescence and antioxidant systems were characterized to test whether the salt-induced

25 stomatal closure limits O_3 uptake and stress or, differently, the pollutant represents an additional

26 stressor for plants. Salt-dependent stomatal closure depressed the photosynthetic process (-71.6% of

27 A_{380}) and strongly enhanced the dissipation of energy *via* xanthophyll cycle. However, salt-treated

28 plants had higher values of A/g_s than controls attributable to a greater g_m/g_s and carboxylation

29 efficiency (higher g_m/V_{cmax}), suggesting no damages to chloroplasts. Accumulation of Na^+ and Cl^-

30 accounted together for 47.1% foliar osmotic potential adjustment. O_3 did not exacerbate the effect of

31 salinity on photosynthesis, but a general enhancement of the Halliwell-Asada components was

32 necessary to counteract the O_3-triggered oxidative stress. Despite the 79.4% g_s reduction in salt-

33 stressed plants, strongly limiting O_3 uptake, a single peak of the air pollutant induced a costly

34 enhancement of the antioxidant system when plants were prior subjected to salinity.

35

Introduction

37 Trees provide a physiological, ecological and social service (Perino et al. 2014) in the urban

38 environment in reason of their ability to remove atmospheric pollution, such as O_3, NO_2 and SO_2

39 (Nowak et al. 2006) and to accumulate airborne particulates (Lorenzini et al. 2006). However, the

40 climate change scenario predicts an exacerbation of different abiotic factors that negatively influence

41 plant life. This is particularly true in the Mediterranean area, where the effects of climate change are

42 expected to be more extreme than the other areas worldwide and human activities are inducing

43 profound environmental alterations resulting in increased habitat fragmentation, deforestation and

44 land abandonment (Matesanz and Valladares 2014).

45   Tropospheric O_3 represents the major air pollutant and frequently its concentration in

46 Mediterranean cities exceeds the European limit value set for the protection of human health and

47 vegetation (EEA 2015). It has since long been documented that this photo-oxidant pollutant affects

48 plant growth causing negative effects at both biochemical and physiological levels (Ainsworth et al.

49 2012, Leisner and Ainsworth 2012). Ozone insult usually occurs simultaneously with other stresses

50 and, among them, soil salinity is considered nowadays the most limiting factor in agriculture (FAO

51 2007) as well as in natural ecosystems (Flexas et al. 2014). In addition, predictions of global climate

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change (IPCC 2014) suggest an increase in aridity for the semi-arid regions of the globe as well as the Mediterranean one in the near future.

The high concentration of ions in soil reduces water uptake by plants, so inducing osmotic stress (Munns and Tester 2008). Moreover, the uptake of Na^+ and Cl^-, the major saline ions, induces ionic stress that can perturb plant metabolism (Munns and Tester 2008, Harris et al. 2010). Photosynthetic process is strongly influenced by salinity, directly or indirectly. Under severe stress conditions, salinity leads to the reduction in CO_2 availability due to diffusional limitations, alterations in photosynthetic metabolism and restrictions of photochemical apparatus (Niinemets and Keenan 2014). Certainly, one of the first responses of plants to this stressor is the stomatal closure that impairs CO_2 uptake (Chaves et al. 2009). However, even though the primary role in photosynthetic CO_2 assimilation is played by stomatal conductance, g_s, this is not the major site of photosynthesis regulation (Lawlor 1995). Many researchers have reported that in the long period, non-stomatal constraints to photosynthesis, namely biochemical limitations, are prevalent than stomatal ones (Sharkey and Seemann 1989, Tezara et al. 1999). Under stress conditions, another factor that can limit CO_2 diffusion into the chloroplasts is the mesophyll conductance, g_m (Centritto et al. 2003), which limits significantly photosynthesis (Loreto et al. 1992, Flexas et al. 2008, von Caemmerer 2013). This implies that CO_2 amount really reaching the chloroplast is overestimated by Ci (intercellular CO_2 concentration). Thus, chloroplastic CO_2 concentration values, Cc, should be considered to calculate the maximal carboxylation efficiency of Rubisco, V_{cmax}, and maximum electron transport rate, J_{max}, that otherwise would be underestimated by A/Ci curves (net CO_2 assimilation rate, A, versus calculated substomatal CO_2 concentration, Ci).

The effects of soil salinity on maquis species are of particular interest as they result well equipped to contrast a plethora of stresses (also occurring simultaneously) typical of Mediterranean areas, such as drought, high irradiances, UV, and air pollutants (Fini et al. 2014, Flexas et al. 2014, Niinemets and Keenan 2014). Among different species typical of the Mediterranean maquis, the woody evergreen sclerophyllous *Quercus ilex* (L.) (holm oak) is widely distributed extending

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longitudinally from Portugal to Syria and latitudinally from Morocco to France (Valladares et al. 2000). Furthermore, it has been successfully used since the sixteenth century in the landscaping of urban and rural parks (Fantozzi et al. 2015). As other woody evergreen sclerophyll plants co-habiting the Mediterranean basin, *Q. ilex* is characterized by long-lived leaves (Gàlmes et al. 2005) with high leaf mass per area (LMA), great thickness of cell wall (Tomás et al. 2013, Flexas et al. 2014) and, in turn, a slow growth rate. These features usually suggest a low efficiency of photosynthetic process. However, Flexas et al. (2014) demonstrated that the slow growth rate of Mediterranean sclerophyllous is mainly due to restricted favorable periods for photosynthesis, while some mechanistic components of photosynthesis allow these species to maintain a photosynthetic rate similar to that of non-Mediterranean ones, despite their large LMA. To allow these species to achieve a high rate of photosynthesis per mass unit, the low g_m is compensated by higher g_m/g_s and larger carboxylation efficiency in the chloroplasts (Flexas et al. 2014). So that, the Mediterranean sclerophyllous present the largest net primary productivities worldwide (Flexas et al. 2014). In addition, it has been reported that *Q. ilex* shows a high ability to respond adequately to environmental constrains by reason of its high plasticity (Valladares et al. 2000, Cotrozzi et al. 2016). To date, many researches have been carried out on the effects of different environmental factors on the photosynthetic process of *Q. ilex* (e.g. Manes et al. 2007, Paoletti et al. 2007, Vitale et al. 2008, Limousin et al. 2010, Vaz et al. 2010, Gallè et al. 2011), but only a few papers describe the effect of salinity on this species (e.g. Fusaro et al. 2013).

Many reports have studied the combined effects of O_3 and abiotic stresses, such as drought (Bohler et al. 2014, Hayes et al. 2015, Cotrozzi et al. 2016), salinity (Welfare et al. 2002, Mereu et al. 2011, Gerosa et al. 2014) or increased CO_2 concentration (Hoshika et al. 2012, Richet et al. 2012, Biswas et al. 2013). However, no works report the effects of a peak of O_3 in plants already suffering for salinity, condition that plants can commonly experience in Mediterranean area, especially in the urban environment. Consequently, the aim of this study was an in-depth characterization of the mechanisms involved in the response of young saplings of *Q. ilex* subjected to a mild salinity stress

104 (Experiment I). Afterwards, plants were subjected to a single pulse of O_3 (80 nL L^{-1}, 5 hours;

105 Experiment II) to explore whether the salt-induced stomatal closure limits the O_3 uptake and,

106 consequently, its negative effects or, conversely, the pollutant represents an additional stressor.

107

108 **Materials and Methods**

109 *Plant material and experimental design*

110 Three-year-old saplings of *Q. ilex* were potted (6.5-L containers) in a growing medium containing a

111 mixture of standard soil Einhetserde Topfsubstrat ED 63 grob (peat and clay, 34% organic C, 0.2%

112 organic N and pH 3.8-6.8) and sand (3.5:1 in volume). Then, uniformly sized plants were placed in a

113 field and irrigated daily with a half-strength Hoagland solution for 1 month before salinization starting

114 point. In the first experiment (Salinity), salt treatments were imposed from 5 to 20 July 2015 and

115 recently developed leaves were marked at the beginning of the treatment. Half of plants were

116 supplied, at 2-day intervals, with increasing concentration of NaCl (0, 25, 50, and 100 mM). Thus,

117 final salinity treatments were reached by the end of a 8-day period, when plants received 150 mM

118 NaCl (+Salt). Salt treatments were imposed until to photosynthetic activity, in light saturated

119 conditions (about 800 μmol quanta m^{-2} s^{-1}) and a CO_2 concentration of 380 μmol mol^{-1}, declined

120 about to 70% of the control values (15 days). Control plants (-Salt) were dealt with an optimal nutrient

121 solution for the entire period. Over the whole experimental period, on average, midday photosynthetic

122 active radiation (PAR, over the range 400-700 nm waveband) was 1644 μmol quanta m^{-2} s^{-1};

123 minimum, medium and maximum air temperatures were 22.5, 28.1 and 33.6 °C, respectively, and

124 relative humidity (RH) was 67%.

125 Throughout the experiment, photosynthetic process was monitored by using gas exchange and

126 chlorophyll fluorescence measurements. At the end of the salt treatment period, photosynthetic and

127 hydric parameters were measured on the current-year leaves that were expanded during the salt

128 treatment. At the same time, sampling for biometric parameters and biochemical analyses were

129 collected.

The second test (Ozone treatment after salinity) was carried out from 5 to 20 September 2015, using the same conditions of the first experiment. On average, midday photosynthetic active radiation (PAR, over the range 400-700 nm waveband) was 1585 μmol quanta m^{-2} s^{-1}; minimum, medium and maximum air temperatures were 17.1, 22.1 and 27.0 °C, respectively, and RH was 71%. When the decline in photosynthetic activity was similar to that reached in the first experiment (-70% of the controls), plants were subjected to a single pulse of O_3. Plants were transferred into four controlled environment fumigation facilities which were ventilated with charcoal filtered air (two boxes of -Salt/-O_3 and +Salt/-O_3) or treated with O_3 (80±3 nL L^{-1}, 5 h) (two boxes of -Salt/+O_3 and +Salt/+O_3. The O_3 exposure was carried out from 09:00 to 14:00 (GMT).The entire methodology of O_3 exposure was performed according to Nali et al. (2004). Ozone was generated by electrical discharge using a Fisher 500 air-cooled apparatus (Zurich, Switzerland) supplied with pure oxygen, and mixed with the inlet air entering the fumigation chambers. Its concentration at plant height was continuously monitored with a photometric analyzer (Monitor Labs, mod. 8810, San Diego, CA, USA) connected to a computer. During the salt treatment, minimum, medium and maximum daily O_3 concentrations were 2.5, 28.4 and 59.0 nL L^{-1}, respectively. The actual O_3 levels in the study area were measured by an automatic photometric analyzer operated by local environmental authorities (ARPAT) and located in Pisa (N:4843724 - E:1612822) in a densely populated area surrounded by car-crowded streets, where several bus and train lines run through (urban/traffic site).

At the end of the fumigation period, photosynthetic process was assessed by using gas exchange and chlorophyll fluorescence measurements. At the same time, pre-dawn leaf water potential (Ψ_{pd}), relative water content (RWC), osmolality, osmotic potential (Ψ_s) and relative contribute of each ion to ionic adjustment were assessed. Then, leaves of all thesis (-Salt/-O_3, -Salt/+O_3, +Salt/-O_3 and +Salt/+O_3) were divided into aliquots, pulverized in liquid nitrogen (N_2) and stored at -80 °C until biochemical analyses.

Pre-dawn leaf water potential and relative water content measurements

In six leaves per sapling, Ψ_{pd} was measured using a pressure chamber (PMS model 600, PMS Instrument Company, Albany, OR, USA). All measurements were completed before sunrise.

The relative water content (RWC) was determined in three leaf discs ($\varnothing$ 1 cm) per replication removed from top, left and right interveinal areas in a leaf and calculated as follows: RWC = (FW-DW)/(TW-DW)$\times$100; FW, TW and DW means fresh, turgid and dry weight, respectively. To determine TW of the leaf discs, these were kept in distilled water in the darkness at 4 °C to minimize respiration losses, until they reached a constant weight (full turgor, typically after 24 h). DW was obtained after 48 h at 60 °C in an oven. Six replicates per treatment were obtained.

To calculate the leaf mass per unit area (LMA, g m^{-2}), six leaves per treatment were kept and their DW and area were measured. The leaf succulence was determined using the equation: succulence = (FW/disc area) and expressed as mg H_2O cm^{-2}.

Osmolality, osmotic potential and relative contribute of each ion to ionic adjustment

Small segments from six marked leaves were macerated in a mortar with liquid N_2. After extract filtration in miracloth membrane, the sap was centrifuged at 2700g for 10 min at 4 °C. After that, 10 µL of supernatant was utilized to determine the osmolality using a vapor pressure osmometer (Digital Osmometer Roebling, Berlin, Germany). According to the Van't Hoff equation, the osmotic potential was determined using the formula: Ψ_s (MPa) = - osmolality (mosmol kg^{-1}) $\times$ 2.58 $\times$ 10^{-3}.

According to Silveira et al. (2009), the relative contribution of each ion to the osmotic potential (OA) was estimated as the percentage of the osmolality calculated by the following ratio: solute concentration (mmol kg^{-1} water tissue)/osmolality (mmol kg^{-1} solvent).

Gas exchange and chlorophyll fluorescence measurements

Measurements of gas exchange and chlorophyll fluorescence were simultaneously carried out on three leaves from three plants per treatment, using a portable Li-Cor 6400 (Li-Cor, Lincoln, NE,

USA) infrared gas analyzer equipped with a 6400-06 PAM2000 adapter (Walz, Effeltrich, Germany).
They were carried out in field conditions; to avoid the effect of fluctuating environments on gas exchange measurements, all plants were measured between 9.30 and 16.30.

For each plant, a marked current-year leaf was chosen and placed inside the cuvette, where temperature, leaf-to-air vapor pressure deficit (VPD) and flow rate were maintained at 25 °C, below 1 kPa and 300 μmol mol^{-1}, respectively. The leaf was left to equilibrate inside the chamber for 10 min at about 380 μmol mol^{-1} CO_2 concentration and saturating irradiance (about 1800 μmol quanta m^{-2} s^{-1}) before measuring the response of net assimilation rate (A) to intercellular CO_2 concentration (Ci). Once steady-state was reached, CO_2 concentration was decreased stepwise to 50 μmol CO_2 mol^{-1} air. Upon completion of measurements, CO_2 concentration was returned to 380 μmol mol^{-1} and, then, was increased stepwise to 1800 μmol mol^{-1}. Measurements were recorded after values of A, Ci and stomatal conductance became stable at each step within the sequence. At each value of Ci, measurements of chlorophyll fluorescence for the light-adapted leaf were made simultaneously to the gas exchange. After the leaf reached the steady-state conditions, steady-state fluorescence and maximum fluorescence with a light-saturating pulse of 8000 μmol quanta m^{-2} s^{-1} were recorded. Values of F and F_m' (the steady and maximal fluorescence in light conditions, respectively) were utilized to calculate the photochemical efficiency of photosystem II (PSII) in light conditions (Φ_{PSII}) as follows: (F_m'-F)/F_m'. The electron transport rate (J_{flu}) was then calculated as $J_{flu} = \Phi_{PSII} \times PPFD \times 0.5 \times \alpha$, where α is the leaf absorption and 0.5 is the partitioning of absorbed quanta between PSII and photosystem I (PSI). The absorption coefficient α over the 400-700 nm for *Q. ilex* was 0.926 measured with a spectroradiometer equipped with an integrating sphere (LI-Cor 1800-12S, LI-Cor, Lincoln, NE, USA),

The photosynthesis model described by Farquhar et al. (1980) was used to describe the rate of photosynthesis: $A_c = V_{cmax} [(C_i - \Gamma^*)/(C_i + K_c(1 + O_i/K_o))] - R_d^*$, where A_c is the photosynthetic rate limited by Rubisco carboxylation, V_{cmax} is the maximum rate of Rubisco carboxylation under

206   saturating ribulose-1,5*bis*phosphate (RuBP) and CO_2, C_i and O_i (210 mmol mol^{-1}) are mol fractions

207   of CO_2 and O_2 at the site of carboxylation, and K_c (405 μmol mol^{-1}) and K_o (278 μmol mol^{-1}) are

208   Michaelis-Menten constants of Rubisco for CO_2 and O_2, respectively (Dubois et al. 2007). The

209   mitochondrial respiration in the light R_d* and the CO_2 compensation point in the absence of

210   respiration Γ* were determined for each sample using the Laisk method (von Caemmerer 2000).

211   Briefly, the A/Ci response was measured at three levels of low irradiance (50, 200 and 400 μmol

212   quanta m^{-2} s^{-1}) for five increasing values of ambient CO_2 concentrations (from 25 to 400 μmol CO_2

213   mol^{-1}). Linear relationships between A and Ci were fitted and the point of intersection of the three

214   lines was taken as Rd (y-axis) and Γ* (x-axis) (see **Supplementary Figure S1**). This model was fitted

215   to the A/Ci curves by non-linear least square regression and values of V_{cmax} were estimated from the

216   lower part of A/Ci curve (Ci lower than 200 μmol mol^{-1}).

217   The response of net assimilation to irradiance (light curves) was measured at increasing light

218   irradiances with ambient CO_2 concentration maintained at 380 μmol mol^{-1} using a CO_2 mixer. Light

219   response curves were determined on three leaves per treatment. Light-saturated rate of photosynthesis

220   (A_{380}), apparent maximum quantum yield of CO_2 assimilation ($Φ_{CO2}$) and the photosynthetic photon

221   flux density (PPFD) at which photosynthesis saturated were estimated by fitting a non-rectangular

222   hyperbola function to individual A/PPFD curves. Similarly, transpiration rate (E), stomatal

223   conductance to water vapor (g_s) and the intrinsic water use efficiency (A/g_s) were estimated at

224   saturated light conditions. The rate of mitochondrial respiration at zero irradiance (R_{dark}) was

225   determined early in the morning in dark-adapted leaves.

226   Chlorophyll fluorescence was determined even by using modulated PAM-2000 fluorometer

227   (Walz, Effeltrich, Germany) on dark-adapted leaves for 40 min to determine values of maximum

228   fluorescence (F_m) and ground fluorescence (F_0), used for the calculation of the maximum quantum

229   yield of PSII (F_v/F_m ratio). For details, see Degl'Innocenti et al. (2002). The saturation pulse method

230   was used for the analysis and calculation of quenching components (Schreiber et al. 1986):

photochemical quenching coefficient (q_P), non-photochemical quenching (NPQ) and efficiency of excitation energy capture by open PSII reaction centres (F_v'/F_m'). Measurements of F_0' were carried out in the presence of far-red light (7 μmol quanta m^{-2} s^{-1}) in order to fully oxidize the PSII acceptor side.

Mesophyll conductance and values of V_{cmax} and J_{flu}

The variable J method described by Harley et al. (1992) was used to calculate mesophyll conductance, g_m, and chloroplastic CO_2 concentration (Cc), combining gas exchange and chlorophyll fluorescence: $Cc = \Gamma^* (J_{flu} + 8 (A + R_d^*)/J_{flu} - 4 (A + R_d^*)$; $g_m = A/(Ci-Cc)$, where Γ^* represents the CO_2 compensation point in the absence of respiration and R_d^* the day respiration. Thereafter, we calculate the A/Ci curves into A/Cc curves and the maximum carboxylation rate (V_{cmax}) and the maximum electron transport rate (J_{flu}) by fitting the equation of the Farquhar model (Farquhar et al. 1980).

Quantitative limitation analysis

The A/Ci response curves and values of g_s and g_m were used to partition stomatal, mesophyll and biochemical limitations to photosynthesis. Values of stomatal conductance to CO_2 transfer were calculated by $g_s = A/(C_a-C_i)$, dividing values by 1.64 (Jones 1992).

The relative limitations to assimilation by stomatal conductance (L_s), mesophyll diffusion (L_m) and biochemical processes (L_b) were separated utilizing the limitation analysis reported by Jones (1985) and implemented by Grassi and Magnani (2005). The limitation of the different components were calculated as follows: $L_s = [(g_{tot}/g_s) \times \delta A/\delta Cc]/(g_{tot} + \delta A/\delta Cc)$; $L_m = [(g_{tot}/g_m) \times \delta A/\delta Cc]/(g_{tot} + \delta A/\delta Cc)$; $L_b = g_{tot}/(g_{tot} + \delta A/\delta Cc)$, where g_s was the stomatal conductance to CO_2, g_m was the mesophyll conductance according to Harley et al. (1992), g_{tot} was the total conductance to CO_2 from ambient air to chloroplast (sum of the inverse serial conductance g_s and g_m), $\delta A/\delta Cc$ was calculated

255 as the slope of A/Cc response curves over a range of 50-100 μmol mol^{-1}. At the least three curves per

256 treatments were used and average estimates of the limitation were calculated.

257

258 ***Determination of inorganic solutes***

259 Dried samples of roots, leaves and stems were finely ground. Cations (Na^+ and K^+) were extracted

260 with concentrated HNO_3 and determined by atomic absorption spectrophotometer while Cl^- anion

261 determination was performed on water extract of material. A 200 mg sample was incubated in 5 mL

262 for 30 min at 60 °C and following centrifugation supernatant was collected. This procedure was

263 repeated two times. The supernatants were pooled, evaporates to dryness and the residues re-

264 suspended in 2 mL of water. Chloride contents were determined by using ion chromatograph (D-X

265 100 ion chromatograph, Dionex, Sunnyvale, CA, USA), as reported in Tarchoune et al. (2012).

266

267 ***Photosynthetic pigments analysis***

268 Leaves for pigment analysis were collected between 11.00 and 13.00. Pigments were determined by

269 high pressure liquid chromatography (HPLC, P680 HPLC Pump, UVD170U Uv-Vis detector,

270 Dionex, Sunnyvale, CA, USA), according to Döring et al. (2014) with some minor modifications.

271 Fifty mg (FW) of sample were homogenized in 1 mL of 100% HPLC-grade methanol and incubated

272 overnight at 4 °C in the dark. Samples were centrifuged for 15 min at 16000g at 5 °C and the

273 supernatant was filtered through 0.2 μm Minisart® SRT 15 aseptic filters (Sartorius AG, Goettingen,

274 Germany) and immediately analysed. HPLC separation was performed at room temperature with a

275 Dionex column (Acclaim 120, C18, 5 μm particle size, 4.6 mm internal diameter x 150 mm length;

276 Thermo Fisher Scientific, Waltham, MA, USA). The pigments were eluted using 100% solvent A

277 (acetonitrile/methanol, 75/25, v/v) for the first 14 min to elute all xanthophylls, also the separation of

278 lutein (Lut) from zeaxanthin (Zea), followed by a 1.5 min linear gradient to 100% solvent B

279 (methanol/ethylacetate, 68/32, v/v), 15 min with 100% solvent B, which was pumped for 14.5 min to

280 elute chlorophyll (Chl) *b* and Chl *a* and β-carotene (β-car), followed by 2 min linear gradient to 100%

11

281 solvent A. The flow-rate was 1 ml min^{-1}. The column was allowed to re-equilibrate in 100% solvent

282 A for 10 min before the next injection. The pigments were detected by their absorbance at 445 nm.

283 To quantify the pigment contents [Chl *a*, Chl *b*, neoxanthin (Neo), Lut, xanthophyll cycle pigments

284 (violaxanthin, Vio, antheraxanthin, Ant, and Zea) and β-Car], known amounts of pure standards were

285 injected into the HPLC system and equations, correlating peak area to pigment concentration, were

286 formulated. Pigment concentrations other than total Chl were normalized to total Chl. The data were

287 evaluated by Dionex Chromeleon software (Thermo Fisher Scientific, Waltham, MA, USA).

288

289 *Proline content and metabolism*

290 Proline content was determined according to the method of Bates et al. (1973) with some minor

291 modifications. Plant material (100 mg FW) was ground in an ice-cold mortar with 2 mL of 3%

292 sulfosalicylic acid. Homogenates were centrifuged for 30 min at 10000*g* at 4 °C. The supernatant was

293 filtered through 0.2 μm Minisart® SRT 15 aseptic filters (Sartorius AG, Goettingen, Germany) and

294 1 mL of the filtrate was mixed with equal volumes of glacial acetic acid and of ninhydrin reagent

295 (1.25 g ninhydrin, 30 mL of glacial acetic acid, 20 mL of 6 M H_3PO_4), and was incubated for 1 h at

296 100 °C. The reaction was stopped by placing test tubes in ice-cold water. Samples were vigorously

297 mixed with 2 mL toluene. After 20 min, the light absorption of the toluene phase was estimated at

298 520 nm using a spectrophotometer (Ultrospec 2100 Pro UV–Vis spectrophotometer; GE Healthcare

299 Ltd, Little Chalfont, England), with toluene used as blank. The proline concentration was determined

300 with a standard curve and calculated on a FW basis.

301 Proline dehydrogenase (PHD; EC 1.5.1.2) and Δ^1-pyrroline-5-carboxylate synthetase (P5CS;

302 EC 1.2.2.41) were extracted as described by Wang et al. (2011) with minor modifications. Fresh leaf

303 material (0.2 g) was extracted with 8 mL 100 mM sodium phosphate buffer (pH 7.4) containing 1

304 mM ethylenediaminetetraacetic acid (EDTA), 10 mM β-mercaptoethanol, 1% (w/v)

305 polyvinylpolypyrrolidone (PVPP), 5 mM $MgCl_2$ and 60 mM KCl. The homogenate was centrifuged

306 at 12000*g* for 20 min at 4° C and the resulting supernatant was kept at -20 °C until enzyme assay.

PDH activity was assayed as described by Lutts et al. (1999). Fifty µL of crude enzyme extract were added to 950 µL of 0.15 M Na_2CO_3-HCl buffer (pH 10.3) containing 15 mM L-proline and 1.5 mM NAD^+. The reduction of NAD^+ was followed at 340 nm for 2 min. PDH activity was expressed as µmol NADH mg protein^{-1} min^{-1}.

P5CS activity was determined as described by Saibi et al. (2015) based on a modified version of the original method of Hayzer and Leisinger (1980). The amount of ɣ-glutamyl hydroxamate complex produced was estimated from the molar extinction coefficient (250 M^{-1} cm^{-1}) reported for the Fe^{3+}-hydroxamate-ɣ-glutamyl hydroxamate complex. P5CS activity was expressed as µmol ɣ-glutamyl hydroxamate mg protein^{-1} min^{-1}

Abscisic acid determination

Abscisic acid (ABA) was measured after an extraction of 100 mg (FW) of leaves in distilled water (water:tissue ratio = 10:1 v/w) overnight at 4 °C. Then, ABA was determined by an indirect enzyme-linked immunosorbent assay based on the use of the DBPA1 monoclonal antibody raised against S(+)-ABA (Vernieri et al. 1989), as described previously in Trivellini et al. (2011).

Lipid peroxidation

Oxidative damage was estimated in terms of lipid peroxidation measuring the MDA by-products accumulation (Hodges et al. 1999), which takes into account the possible influence of interfering compounds in the assay (e.g. phenols) for the 2-thiobarbituric acid (TBA) - reactive substances. MDA levels were expressed as nmol g^{-1} FW.

Antioxidant enzymes

Enzymes were extracted from 0.2 g fresh leaves material with 1 mL of 66 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.1% PVPP. The extract was then centrifuged for 15

min at 11000g at 4 °C, and the supernatant was used for all the enzyme assays, while the protein

determinations were performed with the Protein Assay Kit II (Bio Rad®, Hercules, CA, USA).

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured at 560 nm, based on the

inhibition of NitroBlue Tetrazolium (NBT) reduction by SOD (Beyer and Fridovich 1987). One unit

of SOD was defined as the enzymatic amount required to reduce the NBT reduction state by 50%.

Catalase (CAT; EC 1.11.1.6) activity was measured at 240 nm by determining the rate of

conversion of H_2O_2 into O_2 and water, as described by Cakmak and Marschner (1992). Catalase

activity was expressed as µmol H_2O_2 mg protein^{-1} min^{-1}.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined following the H_2O_2-

dependent oxidation of ascorbic acid at 290 nm in a reaction mixture composed of 50 µM reduced

ascorbate (ASA), 90 µM H_2O_2, 50-100 µg proteins and 0.1 M phosphate buffer (pH 6.4) (Nakano

and Asada 1981). APX activity was expressed as µmol ASA mg protein^{-1} min^{-1}.

Glutathione reductase (GR, E.C 1.6.4.2) was assayed with the method of Schaedle and

Bassham (1977) with minor modifications. The reaction mixture contained 850 µL of 50 mM

potassium phosphate buffer (pH 7.5), 3 mM $MgCl_2$, 0.15 mM NADPH and 50 µL enzyme extract

(containing 0.15-0.30 mg protein). Reaction was initiated by adding 100 µL of 10 mM oxidized

glutathione (GSSG). The increase in absorbance at 340 nm was monitored for 5 min. Enzyme activity

was determined using the molar extinction coefficient for NADPH (6.2 mM^{-1} cm^{-1}) and expressed as

µmol NADPH mg protein^{-1} min^{-1}.

Antioxidant molecules: ascorbate and glutathione

Total ascorbate (ASA$_T$), dehydroascorbate (DHA) and ASA were determined as described by

Kampfenkel et al. (1995). Ascorbate content (ASA$_T$, DHA and ASA) was expressed as µg g^{-1} FW.

Total glutathione (GSH$_T$) and reduced glutathione (GSH) were determined as described by Ryang et

al. (2009) with minor modifications. Raw material (0.2 g FW) was ground in 1 mL of HCl 0.1 M

containing 0.1% of PVPP and centrifuged for 20 min at 15000g at 4 °C to prepare the supernatant.

358   Calibration curve was realized with glutathione disulphide from 4.5 µM to 90 µM and the absorbances

359   were read at 412 nm against a blank. Glutathione content (GSH_T and GSH) was expressed as nmol g⁻

360   ¹ FW.

361

362   *Statistical analysis*

363   Both the experiments were set up with a completely randomized design with 20 plants for each

364   treatment. Recorded parameters were tested with Bartlett's test for normality and homogeneity of

365   variance. Data were subjected to Student's *t* test to distinguish between control and salt-treated

366   samples with a confidence level of $P \leq 0.05$ in Experiment I (Salinity). For the second test (Ozone

367   treatment after salinity), data were subjected to a two-way ANOVA with salt and ozone treatment as

368   factors of variability. Means (±SD) were separated after the least significant difference (LSD) post-

369   hoc test ($P \leq 0.05$). Linear regression was used to determine the light mitochondrial respiration (R_d*),

370   the CO_2 compensation point in the absence of respiration Γ*, quantum yield to CO_2 uptake Φ_{CO2}, the

371   carboxylation efficiency of Rubisco, V_{cmax}. Non-linear least square regression was utilized to

372   interpolate the response of CO_2 assimilation to light (light curves) or to Ci (A/Ci curves). Percentage

373   data were angularly transformed prior statistical analysis. All statistical analysis were conducted using

374   GraphPad (GraphPad, La Jolla, CA, USA).

375

376   **Results**

377   *Experiment I (Salinity)*

378   *Water status, proline metabolism and abscisic acid*

379   No significant differences in biomass production were observed in plants subjected to salt stress as

380   compared to -Salt and no visible leaf injury were detected at the end of the treatment (*data not shown*).

381   However, significant differences in other physiological and biochemical traits were shown. **Table 1**

382   reports some variables frequently used to evaluate water status of plants. In +Salt plants Ψ_{pd} and Ψ_S

383   (calculated from osmolality values) significantly decreased as well as the RWC. As already reported

384 (Gallè et al. 2011), most likely related to leaf structure, RWC is high in sclerophyllous leaves of holm

385 oak. RWC values were generally high since in -Salt (around 89%) and in individuals subjected to

386 salinity were also maintained at favorable values (around 83%). No significant differences between

387 treatments were recorded for LMA. Yet, succulence increased significantly in response to salt

388 application (**Table 1**).

389   The Na^+ concentration in current-year leaves significantly increased (4.2-fold higher than –

390 Salt plants) as well as in stems and roots (about 3.7 fold) (**Figure 1**). Even the Cl^- concentration

391 significantly increased in all organs of +Salt plants as compared to -Salt ones: 4-, 3- and 2-fold higher

392 in leaves, roots, and stems, respectively (**Figure 1**). On the other hand, the K^+ concentration

393 significantly increased in +Salt leaves, but no changes were observed in stems and roots (**Figure 1**).

394 However, the Na^+/K^+ ratio significantly increased in all organs.

395   Na^+ and Cl^- ions accounted for most osmolality adjustment in +Salt leaves where Na^+ and Cl^-

396 accounted for 33% and 14%, respectively (**Table 2**). On the other hand, the relative contribution of

397 K^+ did not change as a consequence of salt stress.

398   At constitutive level, the proline content averaged 90 $\mu g\ g^{-1}$ FW and its contribute as

399 osmoliticum was thus negligible (*data not shown*); in addition, it did not change as a consequence of

400 salt stress (**Table 3**). P5CS activity was not affected by salinity and PHD one increased (**Table 3**).

401 Salt stress induced a reduction of ABA (519 *vs* 285 ng g^{-1} FW in -Salt and +Salt leaves, respectively)

402 (**Table 3**).

403

Photosynthetic parameters

405 Light response curves were very different between -Salt and +Salt leaves even for very low levels of

406 light intensities (**Supplementary material Figure S2**). Indeed, Φ_{CO2} (the slope of the light response

407 curve in the linear trait) was significantly lower in +Salt leaves than in -Salt ones, and a strong

408 reduction due to salinity was observed also in A_{380} (-71.6%) (**Table 4**). Differently, R_{dark} was 1.3 and

409 1.5 $\mu mol\ CO_2\ m^{-2}\ s^{-1}$ in -Salt and +Salt leaves, respectively, evidencing no significant differences

between treatments, while E and g_s decreased significantly following salt stress (-61.1 and -79.4%, respectively) (**Table 4**). No differences were found in Ci between thesis, while A/g_s significantly increased following the salt stress (**Table 4**).

The response of photosynthetic rate at increasing Ci changed strongly between -Salt and +Salt leaves (**Supplementary material Figure S3** and **Table 5**). CO_2 assimilation in saturated light and CO_2 averaged 20 and 4.87 μmol m^{-2}s^{-1} in –Salt and +Salt leaves, respectively (Table 5). Actually, V_{cmax} was lowered following salt stress, and its values on a Cc basis were greater than those on a Ci one (**Table 5**). Salt treatment had a greater effect on V_{cmax} than on J_{flu}, resulting in an increase in the J_{flu}/V_{cmax} ratio when calculated using Cc (**Table 5**).

Also g_{sCO2} and g_m were significantly lower in +Salt as compared with -Salt leaves (-61.6 and -76.7%, respectively, **Table 5**), determining a decrease in CO_2 assimilation in non-limited conditions for light and CO_2. The g_m/g_s ratio was significantly higher in +Salt leaves as compared with -Salt ones (**Table 5**). Values of g_m in this study were lower than those reported by Gallè et al. (2011), but similar to that reported by Flexas et al. (2008) for *Quercus* genus. On the other hand, a large variability in g_m is present within groups, genus and even species, suggesting that g_m is a rapidly adapting trait likely being involved in the differences of photosynthetic efficiency found among species and cultivars (Flexas et al. 2008). Differences between ambient CO_2 and intercellular CO_2 concentrations were relatively small compared to the differences between Ci and Cc (see **Table 4** and **5**). In fact, in *Q. ilex* plants a strong drawdown of CO_2 was observed following salt stress, but also in leaves of untreated plants. The consequence of this drawdown of CO_2 implies that relative limitations imposed by mesophyll conductance to photosynthesis are much greater than those induced by stomata. Mesophyll limitations in -Salt and +Salt leaves were 0.59 and 0.71, respectively (**Table 5**). In +Salt leaves, biochemical limitations were even lower as compared to -Salt ones, whereas no differences were recorded for stomatal constrains. Values of g_m/V_{cmax} (Cc) were significantly lower in +Salt plants as compared to -Salt ones (**Table 5**).

17

Changes in PSII photochemistry were first investigated in dark-adapted state in +Salt leaves: a significant decrease in F_v/F_m was observed (**Table 6**). In addition, parameters derived from quenching analysis were negatively altered by the salt stress, including a strong and significant increase in NPQ was detected (2 fold as compared with -Salt leaves). In particular, reductions in Φ_{PSII}, F_v'/F_m' and q_P were recorded.

Photosynthetic pigments

Leaves grown under salinity showed a similar concentration of total Chl as compared to -Salt leaves and a lower carotenoid to Chl_{tot} ratio (-10.9%) (**Table 7**). The composition of carotenoids varied significantly in response to salinity even though no differences were found for Neo concentration. Vio and Ant decreased significantly whereas increases in Zea and Lut were observed in +Salt leaves (**Table 7**). However, no differences in VAZ/Chl_{tot} were found and β-car significantly decreased. The conversion state of Vio (DES = Ant+Zea on total VAZ) increased significantly following the salt stress (+16.1%) (**Table 7**).

The changes in DES were significantly related to changes in F_v'/F_m' ($R^2 = 0.882***$), Φ_{PSII} ($R^2 = 0.850**$) and NPQ ($R^2 = 0.881**$) (**Figure 2**). Thus, the decrease in Φ_{PSII} that was caused by the decrease in F_v'/F_m' can be explained by the de-epoxidation state of Vio to Zea.

Experiment II (Ozone treatment after salinity)

At the end of salt treatment and after O_3 fumigation, plants did not show neither symptoms nor alterations in growth parameters (*data not shown*). Following salt stress (+Salt/-O_3) current-year leaves showed similar decreases in A_{380} and g_s as compared with -Salt/-O_3 leaves at experiment 1 (-67% and -79%, respectively) (**Figure 3; Supplementary material Table S1**). The effect of O_3 on these parameters was negligible (**Figure 3**). Even chlorophyll fluorescence derived parameters showed in +Salt/-O_3 plants trends similar to that recorded in the first experiment with the exception

of F_v/F_m which did not change (**Figure 3**). Ozone had no effects on chlorophyll fluorescence parameters. No interaction between salt and O_3 was found for all parameters (**Figure 3; Supplementary material Table S1**).

Antioxidant enzyme activities, ascorbic acid and glutathione content and MDA by-products content

Results concerning SOD, CAT, APX and GR are summarized in **Figure 4** and statistical analysis is reported in **Supplementary material Table S1**. Differences in SOD and CAT activity were attributable only to the effect of O_3, while no interactive effects of O_3 and salinity were found for these parameters. In particular, -Salt/+O_3 and +Salt/+O_3 leaves showed a reduced activity of SOD and CAT. Differently, the activities of APX and GR were significantly enhanced by O_3 when applied alone or imposed after the salt treatment. For both enzymes, the activity found in +Salt/+O_3 leaves was significantly higher than that in -Salt/+O_3 ones (18.5% and 30.5%, respectively).

The content of ASA_T increased significantly following all the treatments (302.6 µg g^{-1} FW in -Salt/-O_3 plants vs 366.3 µg g^{-1} FW as the average of values found in +Salt/-O_3, -Salt/+O_3 and +Salt/+O_3 plants) (**Table 8**; **Supplementary material Table S1**). Ozonated plants held remarkable higher values of ASA/ASA_T as compared to unfumigated ones (84% vs 76.5%, respectively).

As shown in **Supplementary material Table S1**, no interactive effect between salinity and O_3 was found for GSH_T as its content increased only following salt treatment. The GSH/GSH_T ratio significantly decreased only because of O_3 treatment.

When compared to -Salt/-O_3 leaves, levels of MDA increased significantly in plants subjected to salt alone and/or in combination with O_3 (on average +49%). MDA by-products increased significantly even in plants exposed to O_3 alone (+31% as compared with -Salt/-O_3 leaves) (**Figure 5**).

Discussion

Holm oak photosynthetic activity under salinity stress

486   *Q. ilex* is a typical Mediterranean evergreen sclerophyllous that, in altered conditions of water

487   potential, exhibits the decrease of the leaf one, allowing the extraction of water from soil (Salleo and

488   Lo Gullo 1990, Tomàs et al. 2013). It is also reported how this species is characterized by different

489   morphological and physiological traits when living in contrasting climates (Gratani et al. 2003,

490   Sanchèz-Villas and Retuerto 2007, Camarero et al. 2012, Cotrozzi et al. 2016).

491   Water and osmotic potentials of *Q. ilex* leaves became more negative under NaCl treatment

492   as widely reported in other species (Parida and Das 2005, Melgar et al. 2009, Gallè et al. 2011,

493   Tounekti et al. 2012). However, although a decrease in RWC due to salinity was found, its values

494   remained higher than 80% also in treated plants. This indicates a mild stress condition (Rzigui et al.

495   2013) and an ability of holm oak to retain water in leaf, as also evidenced by the increase in leaf

496   succulence (Han et al. 2013). The strong decrease in Ψ_{pd} under salt stress, associated with few related

497   changes in tissue hydration, is regarded as an adaptive strategy of evergreen sclerophyllous trees

498   (Noitsakis and Tsiouvaras 1990, Tomás et al. 2013).

499   The osmotic adjustment necessary under salinity could lead to an accumulation of compatible

500   solutes and ions in the vacuole, thus increasing the turgor potential as widely reported (Munns 1993,

501   Romero Aranda et al. 2001). Na^+ and Cl^- uptake was found to be considerable in both roots and shoots

502   of salt-treated plants. However, it is noteworthy that, despite the large accumulation of salt ions in

503   leaves, no leaf visible injury occurred, which provides an indirect evidence for the effective vacuolar

504   compartmentalization of Na^+ and Cl^-. The involvement of these ions (that accounted together for 47%

505   of osmotic adjustment in salt-treated plants) is important to maintain turgor and water uptake from

506   saline soils (Gucci et al. 1997). Furthermore, it is well known how K^+ plays a key role as osmolite in

507   maintaining cellular turgor (Shabala and Cuin 2008, Silva et al. 2010). In our plants, despite a high

508   increase in Na^+/K^+ ratio in all the organs, the unbalance of this ratio was mainly attributable to the

509   strong accumulation of Na^+ rather than to the K^+ uptake inhibition (K^+ accumulation was even higher

510   in +Salt leaves, while unchanged values were found in stems and roots). Thus, the K^+ contribute to

511   osmotic potential remained substantial even under salinity.

20

512   Forcing ions into vacuole against their gradient requires the facilitated H^+-chemical gradient

513   generated by H^+-ATPase (Niu et al. 1993). Despite it occurs at expense of energy, this type of osmotic

514   adjustment is certainly less energetic and carbon consuming when compared to the biosynthesis of

515   organic osmolites (Wyn Jones 1981). Since we speculate that saline ions accumulated into vacuoles

516   of +Salt plants, compatible solutes had to be synthetized and accumulated in cytosols to maintain

517   water potential equilibrium within cell. Despite many reports have found the accumulation of proline

518   as key osmoliticum under salt stress (e.g. Iqbal et al. 2014), we did not found any significant change

519   in this amino acid level. The activity of enzymes involved in its metabolism confirmed this point,

520   since those of PC5CS was unchanged and those of PHD increased slightly. The regulation of proline

521   accumulation is mainly dependent to the up-regulated activity of PC5CS (Szekely et al. 2008), rather

522   than to the down-regulation of PHD activity (Iqbal et al. 2014). It is well known how PC5CS is up

523   regulated by ABA (Abraham et al. 2003), while ABA does not appear to play a major role neither in

524   the regulation of PHD transcription (Sharma and Verslues 2010) nor in its activity (Dallmier and

525   Stewart 1992). In our experimental conditions, ABA decreased significantly in +Salt leaves,

526   consistently with no changes in proline content. Our results contrast with some reports in which

527   proline play a key role as compatible osmolytes (Hasegawa et al. 2000, Szabados and Savouré 2010).

528   However, Chen and Kao (1993) demonstrated that in detached rice leaves only a reduction in water

529   potential induced proline and ABA accumulation, whereas no effects were induced by an osmotic

530   stress (500 mM NaCl). Moreover, Silveira et al. (2009) demonstrated that in salt-adapted *Atriplex*

531   *nummularia* leaf proline content decreased strongly at increasing salt concentrations (ranging from 0

532   to 600 mM), and major cytosolic osmotic adjustments were due to other compounds such as glycine

533   betaine and soluble sugars.

534   The maintenance of a good leaf water status and an effective osmotic adjustment under salinity

535   could represent adaptive mechanisms; this strategy is usually associated with a strong stomatal

536   closure to further protect leaves from water loss. It is well known how many Mediterranean species,

537   such as *Q. ilex*, are characterized by a low diffusion of CO_2 to chloroplast (Flexas et al. 2014) due to

high LMA and low g_m. However, these species show a larger biochemical efficiency to compensate the CO_2 drawdown. In this way, they can maintain an high A/g_s (Flexas et al. 2014). Intrinsic water use efficiency is therefore considered as a valid indicator of salt tolerance (Gleick et al. 2011), since usually increases in tolerant species subjected to salt stress (Ashraf 2001, Reynolds et al. 2005). In our experiment, the decrease in g_s of +Salt leaves influenced negatively the photosynthetic activity that decreased. However, the contraction of A was proportionally less than that of g_s, thus inducing an increase of their ratio. This increment of A/g_s was associated with an improvement in biochemical capacity of holm oak plants subjected to salt stress (see reduction of biochemical limitation, L_b). Although the apparent V_{cmax} decreased significantly on +Salt leaves when calculated on both Ci and Cc basis, this reduction was less than that found for g_m (lower g_m/V_{cmax} Cc), suggesting a higher efficiency in CO_2 utilization. In conditions where CO_2 and light were not limiting factors (A/Ci), -Salt plants showed an increase in photoassimilation of about 100% as compared to A_{380} whereas in +Salt ones it was of 60%. The gap between these raises (~40%) appeared less severe than g_s and g_m depressions (-79 and -77%, respectively). This confirms that low chloroplast CO_2 concentration determined by both low stomatal and mesophyll conductances was the main limitation of photosynthesis in salt-stressed leaves (Centritto et al. 2003, Loreto et al. 2003). Conversely, salinity did not depress the biochemical capacity to assimilate CO_2 as previously reported (for a review, see Flexas et al. 2004).

The imbalance between light energy absorption and utilization for CO_2 assimilation, as suggested for instance by the higher values of J_{flu}/V_{cmax} found in +Salt plants, could increase the susceptibility to photoinhibition (Demmig-Adams and Adams 1992). Really, F_v/F_m decreased significantly even though no changes in F_0 were detected (0.36 and 0.42 in -Salt and +Salt leaves, respectively; $P>0.05$). This indicates that the mild reduction of F_v/F_m observed in holm oak following salt stress was due to photoprotective processes and not to photoinhibitory damages (Maxwell and Johnson 2000).

A crucial mechanism involved in photoprotection is represented by NPQ that strongly increased in +Salt leaves. NPQ is associated with ΔpH, PsbS protein and the xanthophyll-cycle (Ort 2001). Actually, the increase of NPQ in +Salt leaves was accompanied by that of DES as previously reported in other species under salinity (Fini et al. 2014). Furthermore, there was a good relationship between the de-epoxidation state of xanthophylls and Φ_{PSII}, F_v'/F_m' and NPQ. In +Salt leaves, the photoprotective role of xanthophyll cycle was also reflected by the slight reduction in photochemical quenching (-31% as compared with -Salt leaves) as well as the decrease in Φ_{PSII} and in F_v'/F_m'. All these events are associated with the enhancement of thermal dissipation in the PSII antennae such that PSII photochemistry can be regulated with the decreased CO_2 assimilation to avoid a possible photodamage to PSII due to the excess of excitation energy (Demmig-Adams et al. 1996).

Carotenoids, beyond their role in xanthophyll cycle, were directly involved in protection of chloroplasts in multiple ways. Firstly, during the central hours of the day VAZ/Chl_{tot} ratio was high, indicating the presence of a free VAZ pool in thylakoids. This VAZ amount does not bound to the light-harvesting protein complex, and hence is not involved in NPQ (Esteban et al. 2015). This indicates an action of zeaxanthin as antioxidant to preserve Chl and, indeed, no oxidation was observed in +Salt leaves. Secondly, the ability of Lut to quench the excited Chl states (e.g. Pogson et al. 1998, Jahns and Holzwarth 2012) could further photo-protect photosynthetic pigments. Thirdly, the degradation of β-car may be the result of the scavenging action of singlet oxygen in thylakoids as already reported in salt-stressed leaves of *Salvia officinalis* (Tounekti et al. 2012) and *Fraxinus ornus* (Fini et al. 2014). It is well known as β-car represents the second defensive line of chloroplast and it is an efficient scavenger of triplet chlorophyll and singlet oxygen in light harvesting antennae (Bassi et al. 1993, Asada 1999, Mittler 2002).

In conclusion, the results of this first experiment indicate that evergreen schlerophyllous as *Q. ilex*, being characterized by long-lived leaves, have a low photosynthetic efficiency on mass basis because these species invest preferentially in vascular and cell wall formation. This induces these

588 species to decrease intercellular spaces and increase cell wall thickness, increasing CO_2 drawdown

589 but also maintaining high foliar relative water content (Tomás et al. 2013) and osmotic stress

590 tolerance. These features are particularly relevant in plants under salt stress, which counteract the

591 excess of excitation energy (due to the reduction in carbon assimilation) also improving further photo-

592 protective mechanisms as i.e. xanthophyll cycle (Peguero-Piňa et al. 2008, Remorini et al. 2009,

593 Flexas et al. 2014) and/or the biosynthesis of other radical-scavenger carotenoids (Sultana et al. 1999,

594 Melgar et al. 2009). These mechanisms are consistent with a conservative strategy adopted by *Q. ilex*

595 to preserve their long-lived leaves against different abiotic stresses in the era of Global Climate

596 Change (Camarero et al. 2012).

597

598 *Does salinity enhance the effect of ozone?*

599 In experimental conditions, a chronic O_3 treatment has been usually applied concomitantly with other

600 stresses (Welfare et al., 2002; Mereu et al., 2011; Hoshika et al., 2012; Richet et al., 2012; Biswas et

601 al., 2013; Bohler et al., 2014; Gerosa et al., 2014; Hayes et al., 2015; Cotrozzi et al., 2016). However,

602 no research group explored the behaviour of plants subjected to a single realistic O_3 event when

603 already suffering for salinity stress. In view of (i) the low (but realistic) concentration of the single

604 pulse of O_3 applied in this experiment, (ii) the lack of consequences induced by O_3 alone on the

605 photosynthetic apparatus, (iii) the restricted O_3 flux due to salt-induced stomatal closure; (iv) the

606 unchanged values of MDA found in +Salt/+O_3 plants as compared to -Salt/+O_3, we discuss below

607 how, unexpectedly, +Salt/+O_3 plants needed further adjustments (with respect to +Salt/-O_3) of the

608 antioxidant system components to counteract the O_3-triggered oxidative stress.

609 Ozone (alone or supplied to salt-stressed plants) did not affect the main photosynthetic

610 parameters as values of -Salt/+O_3 and +Salt/+O_3 were statistically similar to those recorded in -Salt/-

611 O_3 and +Salt/-O_3, respectively. It is therefore unlikely that the photosynthetic machinery could have

612 suffered directly for the additive stress induced by O_3. It is noteworthy, in this second experiment that

613 we found no reductions of F_v/F_m independently of the treatments, while in the first experiment in

24

+Salt plants this ratio decreased slightly for photo-protective rather than photo-inhibitory mechanisms. This could be attributable to the lower cumulative irradiance burden to the photosynthetic apparatus in September (II experiment) as compared to that of July (I experiment). The incremented levels of MDA found in -Salt/+O_3 could be attributed to the direct effect of O_3 on the oxidation of cellular lipid bilayer after entering the stomatal cavities. It is well known how the effects of O_3 depend on a number of events, starting with the gas uptake through open stomata. Once in the sub-stomatal chamber, O_3 can directly react with plasma membrane through ozonolysis, stimulating lipid peroxidation and impairing membrane fluidity, or it can be spontaneously converted into reactive oxygen species (ROS) (Caregnato et al. 2013).

It has been reported how ASA represents the first line of defence against oxidative load induced by O_3 (Noctor and Foyer 1998, Conklin and Barth 2004). However, ascorbate seems not to be sufficient to contrast the negative effects of O_3 (van Hove et al. 2001). Other molecules able to regenerate ASA are determinant in O_3 protection, such as GSH, the predominant non-proteic thiol. The metabolic status of ascorbate is dependent on total thiol groups redox state, especially GSH (Caregnato et al. 2013, Kronfuß et al. 1998). ASA_T content increased significantly following salt stress or O_3 applied alone (even when O_3 was supplied after salt treatment) while only salinity (independently on the presence of O_3) induced a strong accumulation of GSH_T. Thus, the main effect on the enhancement of low-molecular-weight antioxidants seems related to salt treatment whereas O_3 significantly influenced the redox state of antioxidants even though in a different way. ASA/ASA_T increased significantly with O_3 while a reduction of GSH/GSH_T ratio was found. These data suggest that the glutathione turnover was probably necessary to sustain the conversion of DHA to ASA in ozonated plants where an extra amount of reducing power (NADPH) is available due to limited CO_2 assimilation (Polle 1996). In confirmation of this, the increased activity of GR represented the attempt to sustain the regeneration of GSH necessary, in turn, to regenerate the ascorbic acid utilized by APX. In conclusion, O_3 did not exacerbate the oxidative stress observed in salt-treated plants even though a further conspicuous enhancement of the Halliwell-Asada cycle components was necessary to

contrast the O_3-induced damages when leaf status was already compromised by a previous period of salt exposure. This harmonic response is an extra cost for plants and growth can pay the price in a long-term base (Fusaro et al. 2016), if these O_3 episodes take place repeatedly as predicted for the future global change scenarios ~~and that is exacerbated in urban environment~~.

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Figure legends

Figure 1. Na^+ (white bars), Cl^- (black bars) and K^+ (grey bars) contents and Na^+/K^+ ratio of control

(-Salt) and salt-treated (150 mM NaCl for 15 days; +Salt) of *Quercus ilex* tissues (leaf, stem and root).

Bars represent means of three replicates (±SD). -Salt *vs* +Salt plants were compared with a Student's

t-test. ns: $P>0.05$; ***: $P\leq0.001$.

Figure 2. Relationship between the de-epoxidation state (DES) of xantophylls and the efficiency of

excitation capture by open PSII reaction centres (F_v'/F_m'; closed circle), the actual PSII efficiency

(Φ_{PSII}; closed square) and non-photochemical quenching coefficient (NPQ; open square) measured at

midday in control or salt-treated (150 mM NaCl for 15 days) leaves of *Quercus ilex*.

Figure 3. Light-saturated rates of photosynthesis (A_{380}), stomatal conductance to water vapour (g_s),

maximal and optimal photochemical efficiency of PSII (F_v/F_m and Φ_{PSII}, respectively), proportion of

open reaction centres (q_P) and non-photochemical quenching (NPQ) measured in *Quercus ilex* plants

(i) regularly irrigated and exposed to charcoal filtered air (-Salt/-O_3), (ii) salt-treated and exposed to

charcoal filtered air (+Salt/-O_3), (iii) regularly irrigated and O_3 fumigated (-Salt/+O_3); and (iv) salt-

treated and O_3 fumigated (+Salt/+O_3). +Salt/-O_3 and +Salt/+O_3 plants were treated with 150 mM NaCl

for 15 days; -Salt/+O_3 and +Salt/+O_3 plants were exposed to 80 nL L^{-1} of O_3 for 5 h. Bars represent

means of five replicates (±SD). For each parameter, the lack of letters above bars indicates the absence

of significant interaction between salt and O_3 factors ($P = 0.05$), following two-way ANOVA.

Figure 4. Activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and

glutathione reductase (GR) in *Quercus ilex* plants (i) regularly irrigated and exposed to charcoal

filtered air (-Salt/-O_3), (ii) salt-treated and exposed to charcoal filtered air (+Salt/–O_3), (iii) regularly

irrigated and O_3 fumigated (-Salt/+O_3); and (iv) salt-treated and O_3 fumigated (+Salt/+O_3). +Salt/-O_3

and +Salt/+O_3 plants were treated with 150 mM NaCl for 15 days; -Salt/+O_3 and +Salt/+O_3 plants

were exposed to 80 nL L^{-1} of O_3 for 5 h. Bars represent means of five replicates (±SD). For each

parameter, bars keyed with different letters indicate significant differences among treatments ($P =$

962 0.05), following two-way ANOVA. The lack of letters above bars indicates the absence of significant

963 interaction between salt and O_3 factors.

964 **Figure 5.** Values of malondialdehyde (MDA) by-products measured in *Quercus ilex* plants (i)

965 regularly irrigated and exposed to charcoal filtered air (-Salt/-O_3), (ii) salt-treated and exposed to

966 charcoal filtered air (+Salt/-O_3), (iii) regularly irrigated and O_3 fumigated (-Salt/+O_3); and (iv) salt-

967 treated and O_3 fumigated (+Salt/+O_3). +Salt/-O_3 and +Salt/+O_3 plants were treated with 150 mM NaCl

968 for 15 days; -Salt/+O_3 and +Salt/+O_3 plants were exposed to 80 nL L^{-1} of O_3 for 5 h. Bars represent

969 means of five replicates (±SD). Bars keyed with different letters indicate significant differences

970 among treatments ($P = 0.05$), following two-way ANOVA.

971

972 **Figure S1**. Response of CO_2 assimilation rate (A) to intercellular CO_2 concentration (Ci) at three

973 irradiances levels (50, 200 and 400 µmol m^{-2} s^{-1}, 400-700 nm) determined in control (-Salt, above)

974 and salt-treated (150 mM NaCl for 15 days; +Salt, below) leaves of *Quercus ilex*.

975 **Figure S2.** Response of net CO_2 assimilation to photosynthetically active radiation determined in

976 control (-Salt, open circles) and salt-treated (150 mM NaCl for 15 days; +Salt, closed circles) leaves

977 of *Quercus ilex*.