

Photosynthetic performance and biochemical adjustments in two co-occurring Mediterranean evergreens, *Quercus ilex* and *Arbutus unedo*, differing in salt-exclusion ability

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Abstract. The responses to mild root zone salinity stress were investigated in two co-occurring Mediterranean woody evergreens, *Quercus ilex* L. and *Arbutus unedo* L., which differ in morpho-anatomical traits and strategies to cope with water deficit. The aim was to explore their strategies to allocate potentially toxic ions at organism level, and the consequential physiological and biochemical adjustments. Water and ionic relations, gas exchange and PSII performance, the concentration of photosynthetic pigments, and the activity of antioxidant defences, were measured. *Q. ilex* displayed a greater capacity to exclude Na^+ and Cl^- from the leaf than *A. unedo*, in part as a consequence of greater reductions in transpiration rates. Salt-induced reductions in CO_2 assimilation resulted in *Q. ilex* suffering from excess of light to a greater extent than *A. unedo*. Consistently, in *Q. ilex* effective mechanisms of nonphotochemical quenching, also sustained by the lutein epoxide-lutein cycle, operated in response to salinity stress. *Q. ilex* also displayed a superior capacity to detoxify reactive oxygen species (ROS) than *A. unedo*. Our data suggest that the ability to exclude salt from actively growing shoot organs depends on the metabolic cost of sustaining leaf construction, i.e. species-specific leaf life-span, and the relative strategies to cope with salt-induced water stress. We discuss how contrasting abilities to restrict the entry and transport of salt in sensitive organs relates with species-specific salt tolerance.

Additional keywords: leaf longevity, net ion fluxes, salt tolerance, stomatal conductance, violaxanthin-cycle pigments, water relations.

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Introduction

Plants inhabiting the Mediterranean basin are frequently faced with a transient increase in soil salinity during summer, especially in coastal areas (Greaver and Sternberg 2010). Acclimation to excess soil salinity in Mediterranean woody species has been shown to depend strongly on their ability to exclude salt from the shoot, possibly as a consequence of their capacity to withstand severe dehydration (Gucci *et al.* 1997; Tattini *et al.* 2002). The ability to properly allocate potentially toxic ions at organism level (Cheeseman 2013) may have great ecological significance for evergreen sclerophylls (which transpire and live for long time: Munns 1993; Tattini *et al.* 2006; Munns and Tester 2008), whose leaf life-span may be severely constrained in the harsh

Mediterranean climate. However, large differences in the control of salt entry and allocation at organism level have been reported for co-occurring Mediterranean species. For example, *Pistacia lentiscus* L. behaves as a salt accumulator whereas *Phyllirea latifolia* L. displays an extraordinary ability to exclude salt from actively growing shoot organs (Tattini *et al.* 2002, 2006; Tattini and Traversi 2008).

Mediterranean maquis ecosystems consist mostly of evergreen sclerophyllous shrubs, of which a large portion of the canopy suffers from excess light stress on both a daily and seasonal basis. The excess of radiant energy reaching the photosynthetic apparatus is exacerbated because of stomatal and biochemical limitations in the usage of photosynthetic

active radiation in photosynthetic processes (Chaves *et al.* 2009). Net carbon gain is indeed severely constrained as a consequence of many stressors, which may occur concomitantly during the long growth season in Mediterranean areas (Fares *et al.* 2009). As a consequence most Mediterranean plants faced with excess soil salinity have the challenge to control the entry and allocation of potentially toxic ions at organism level (while countering salt-induced osmotic unbalance, Munns and Tester 2008) under sunlight irradiance capable of massive generation of reactive oxygen species (ROS, Tattini *et al.* 2006; Cimato *et al.* 2010). Therefore, mechanisms aimed at countering salt-induced drastic alterations in ROS homeostasis have to effectively operate. These primarily involve (1) adjustments in the relative concentrations of photosynthetic pigments, particularly carotenoids, thus, avoiding the generation of ROS (i.e. physical quenching, Müller *et al.* 2001) and then quenching of ROS once they are formed (i.e. chemical quenching by specific carotenoids, e.g. β -carotene and zeaxanthin, Ramel *et al.* 2012); and (2) antioxidant enzymes and low-molecular weight antioxidants such as ascorbic acid (Shalata and Neumann 2001; Apel and Hirt 2004).

Quercus ilex L. and *Arbutus unedo* L. co-occur in most Mediterranean areas, and have long been explored for their acclimation and adaptive strategies to drought stress (Martínez-Ferri *et al.* 2000; Martínez-Vilalta *et al.* 2003). *Q. ilex* and *A. unedo* have distinct strategies to cope with drought stress in their natural habitats (Ogaya *et al.* 2003; Mereu *et al.* 2009): the former species displaying a 'water-saving' and the latter species a 'water-spending' strategy. This conforms to strikingly different species-specific leaf morpho-anatomical traits – *A. unedo*, which is intermediate between evergreen and semi-deciduous sclerophylls, has smaller leaf mass per area (LMA) and leaf density (*sensu* Gratani and Ghia 2002) as compared with *Q. ilex* (De Lillis 1991), a typical Mediterranean sclerophyll evergreen. Leaf life-span indeed ranges from 11 to 12 months in *A. unedo* to 22 to 24 months in *Q. ilex* (Mediavilla and Escudero 2009).

Here we hypothesised that mechanisms of 'salt-exclusion' should operate more efficiently in *Q. ilex* than in *A. unedo*, reflected in their different leaf traits (i.e. leaf life span). Indeed, mechanisms aimed at limiting salt load in the leaf are of particular significance for evergreen species that have high metabolic cost in leaf construction. Since salt exclusion from the photosynthetic tissue is also effectively sustained by reductions in transpiration rates (in both glycophytes and halophytes, Flowers and Colmer 2008; Munns and Tester 2008; Flowers *et al.* 2010; Cheeseman 2013), 'salt-exclusion' should operate more efficiently in species that display a water-saving 'conservative' strategy to withstand salt-induced water stress. In turn, differential salt-exclusion capacities will translate in differential reductions in the usage of photosynthetic active radiation in photosynthetic processes, and hence, in the biochemical adjustments aimed at countering salt stress-induced ROS generation (Tattini *et al.* 2006).

Here, relevant physiological and biochemical adjustments operating in *A. unedo* and *Q. ilex* to cope with mild root-zone salinity stress, i.e. 75 mM NaCl, over the summer season were examined. It is conceivable that root-zone NaCl concentrations experienced by *A. unedo* and *Q. ilex* in their natural habitats do not usually exceed 25–100 mM (Alessio *et al.* 2004). Measurements were performed of water and ionic relations (including net ion

fluxes), gas exchange and PSII performance, the concentration of individual photosynthetic pigments, as well as the activity of antioxidant enzymes and the concentration of ascorbic acid. To our knowledge, such a comprehensive analysis of mechanisms adopted by these species to respond to NaCl stress has not been previously reported, although the issue is of key significance for the ecology of woody species inhabiting semiarid Mediterranean areas.

Materials and methods

Plant material and growth conditions

Two-year-old *Quercus ilex* L. and *Arbutus unedo* L. plants were grown in outside in 30 L pots (with a substrate consisting of a mixture of sand, turf and garden soil, 60:20:20, v/v/v) and supplied, from May to August, with 0 mM (control) or 75 mM NaCl (salt-treated), twice a week. Final salinity concentration was reached by the end of a 3 day period by daily increments of 25 mM NaCl.

The experiment was performed at the Centre for Research on Effects of Pollutants on Ecosystems, in Curno, North Italy (45°41'N, 9°37'E). Midday photosynthetic active radiation (PAR, over the 400–700 nm waveband) was on average 1357 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$, over the 12 week experimental period. Minimum/maximum air temperatures were on average 18°C/27°C and RH was on average 55% over the whole experimental period.

Analysis of Na^+ , K^+ , and Cl^- and calculations of their fluxes

Ground dried-tissues (200 mg) were extracted with 20 mL of ultrapure water for 2 h in a boiling water bath, and filtered through paper. Aqueous extract aliquots were injected in a ionic chromatograph (Dionex ICS 90, equipped with a Ionpac AS14 column and an ASRS ULTRA suppressor for the anions analysis, and a Ionpac CS12A column and CSAS ULTRA II suppressor for the cation analysis). Net Na^+ , Cl^- and K^+ fluxes were calculated using the following equation (Tattini and Gucci 1999):

$$J_X = ((X_1 - X_0)/t_1 - t_0 \times \ln(\text{WR}_1/\text{WR}_0))/(\text{WR}_1 - \text{WR}_0), \quad (1)$$

where X is the elemental content (Na^+ , Cl^- or K^+) of the whole plant ($J_{X,\text{plant}}$); WR is the root dry weight; $t_1 - t_0$ is the time interval. Rates of Na^+ , Cl^- or K^+ transport to the shoot ($J_{X,\text{shoot}}$) or to the leaves ($J_{X,\text{leaf}}$) were calculated using the elemental contents in corresponding organs. The elemental content at t_0 was determined on a total of 10 plants per species.

Water relations and osmotic adjustment

Predawn leaf water potential (Ψ_{pd}) and relative water content ($\text{RWC} = \text{FW} - \text{DW}/\text{TW} - \text{DW}$), being FW, DW, and TW the fresh, dry and turgid weights respectively) were measured using standard methodologies (Guidi *et al.* 2008). Leaf osmotic potential (Ψ_π) was measured on expressed sap of frozen and thawed leaves using a boiling-point Wescor VAPRO 5520 osmometer (Wescor Inc., Logan, UT, USA). Leaf turgor potential (Ψ_p) was calculated as the difference

between ψ_{pd} and ψ_{π} . Leaf osmotic potential at full turgor was then calculated as:

$$\psi_{\pi FT} = \psi_{\pi}((RWC - AWF)/(100 - AWF)), \quad (2)$$

where AWF, the apoplastic water fraction, was estimated from the analysis of pressure/volume isotherms at 7% in *A. unedo* and 6% in *Q. ilex* respectively, irrespective of salt-treatment. The contribution of dehydration (D) to osmotic adjustment was calculated as.

$$D = \Delta\psi_{\pi} - \Delta\psi_{\pi FT} (\Delta\psi_{\pi} = \psi_{\pi}^{\text{salt-treated}} - \Delta\psi_{\pi}^{\text{control}}). \quad (3)$$

Osmotic contribution of Na^+ , Cl^- , K^+ and soluble carbohydrates to $\psi_{\pi FT}$ was calculated using the Van't Hoff equation:

$$\psi_{\pi}^i = -0.002479(RDW)C, \quad (4)$$

ψ_{π}^i indicates the contribution (in $-\text{MPa}$) of individual solutes to $\psi_{\pi FT}$; RDW is relative DW at saturation (kg m^{-3}); C is the molar concentration of solutes (mol kg^{-1} DW); $0.002479 \text{ m}^3 \text{ MPa}^{-1} \text{ mol}^{-1}$ is the RT value at 25°C . RDW varied from 0.57 in controls to 0.62 in salt-treated plants, irrespective of species.

Gas exchange, PSII photochemistry

Leaf gas-exchange performances were estimated at saturating light ($1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ photons over the photosynthetic active radiation (PAR) waveband) by measuring net CO_2 assimilation rate (P_n), stomatal conductance (g_s) and transpiration rate (E) and calculating the ratio of internal to ambient CO_2 concentration (C_i/C_a) and water use efficiency (WUE), using as a CIRAS 2 infrared-gas analyser (PP Systems, Hitchin, UK). Modulated chlorophyll *a* (Chl *a*) fluorescence analysis was conducted under laboratory conditions, using a PAM-2000 fluorometer (Walz, Effeltrich, Germany) connected to a Walz 2030-B leaf-clip holder through a Walz 2010-F trifurcated fibre optic, on attached, dark-adapted leaves (over a 40-min period). The maximum efficiency of PSII photochemistry was calculated as $F_v/F_m = (F_m - F_0)/F_m$, where F_v is the variable fluorescence and F_m is the maximum fluorescence of dark-adapted leaves. The minimal fluorescence, F_0 , was measured using a light pulse $<1 \mu\text{mol m}^{-2} \text{ s}^{-1}$, to avoid appreciable variable fluorescence. The maximum fluorescence in light conditions (F_m and F_m') were measured at 20 kHz using a 0.8-s saturating light pulse of white light at $8000 \mu\text{mol m}^{-2} \text{ s}^{-1}$. We determined F_m' at $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ photons over the PAR waveband, i.e. at irradiance at which saturation of photosynthesis occurred. PSII quantum yield in the light (Φ_{PSII}) and nonphotochemical quenching ($\text{NPQ} = (F_m/F_m') - 1$) were estimated using the saturation pulse method described by Schreiber *et al.* (1986) and by Bilger and Björkman (1990) respectively.

Analysis of nonstructural carbohydrates, photosynthetic pigments, antioxidant enzymes and ascorbate

Soluble carbohydrates were identified and quantified as previously reported (Tattini *et al.* 1996). In brief, 20–30 mg of freeze-dried tissue was extracted for 24 h in 20 mL of 75% ethanol (adjusted to pH 7 with 0.01 M KOH). The ethanol fraction was

evaporated to dryness under vacuum and the residue dissolved in 1 mL of ultrapure water. The aqueous extract was then purified by solid-liquid extraction through -CH and -SAX pre-packed Bond-Elute cartridges (Varian, Harbor City, CA, USA), and the eluate reduced to dryness under vacuum. Samples were rinsed with ultrapure (pH 7) water and injected in a Perkin Elmer Series 200 chromatograph equipped with an LC 200 RI detector (Perkin-Elmer, Bradford, CT, USA). Soluble carbohydrates were separated on an $8 \times 300 \text{ mm}$ SC1011 column (Showa Denko, Tokyo, Japan) maintained at $88 \pm 1^{\circ}\text{C}$ and equipped with a $6 \times 50 \text{ mm}$ SC1011 pre-column. Eluent was ultra-pure water at a flow rate of 0.8 mL min^{-1} during a 22 min run. In both species, sucrose, glucose, galactose and fructose constituted the major proportion of the soluble carbohydrate pool in control leaves (92% in *Q. ilex* and 85% in *A. unedo*). In *A. unedo* appreciable amounts of mannitol were also detected.

Individual carotenoids were identified and quantified as reported by Beckett *et al.* (2012). Fresh leaf material (120–150 mg) was extracted with $2 \times 4 \text{ mL}$ acetone (added with $0.5 \text{ g L}^{-1} \text{ CaCO}_3$) and $15 \mu\text{L}$ aliquots were injected in a Perkin Elmer Flexar chromatograph equipped with a quaternary 200Q/410 pump and a LC 200 diode array detector (DAD) (all from Perkin Elmer). Photosynthetic pigments were separated in a $250 \times 4.6 \text{ mm}$ Waters Spherisorb ODS1 ($5 \mu\text{m}$) column operating at 30°C , eluted with a linear gradient solvent system, at a flow rate of 1.2 mL min^{-1} , consisting of $\text{CH}_3\text{CN}/\text{MeOH}/\text{H}_2\text{O}$ (8.4/0.8/0.7) and $\text{MeOH}/\text{ethyl acetate}$ (6.8/3.2) during a 18 min run. Violaxanthin cycle pigments, lutein and β -carotene were identified using visible spectral characteristics and retention times. The compounds were calibrated as such: violaxanthin (V) and antheraxanthin (A) with the calibration curve of lutein in the lower concentration range; lutein with the calibration curve of lutein; zeaxanthin (Z) with the calibration curve of zeaxanthin; and β -carotene with the calibration curve of β -carotene (all from Extrasynthese, Lyon-Nord, Genay, France). Chlorophyll *a* and *b* were quantified by spectrophotometric analysis as reported by Beckett *et al.* (2012).

Antioxidant enzyme activities were determined in fresh leaf material, which was extracted as described previously (Guidi *et al.* 2008). Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured spectrophotometrically at 560 nm, based on the inhibition by SOD of nitroblue tetrazolium (NBT) reduction. One unit of SOD was defined as the amount needed to achieve 50% inhibition of the NBT reduction state. Catalase (CAT, EC 1.11.1.6) activity was measured spectrophotometrically at 270 nm by determining the rate of conversion of H_2O_2 to O_2 . Total ascorbate peroxidase (APX, EC 1.11.1.11) was measured as the decrease in absorbance at 290 nm, resulting from ascorbate oxidation. The concentration of ascorbic acid (ASC) was determined spectrophotometrically as described by Kampfenkel *et al.* (1995).

Experimental design and statistics

The experiment was a completely randomised design with 48 plants per species. All measurements were performed on fully-developed medial leaves. Water relations and elemental content were analysed on four replicate samples, each constituted by two plants. Gas exchange was measured in the field between 1030 and

1230 hours on 2 consecutive days, and modulated Chl *a* fluorescence under laboratory conditions on six replicate plants. Biochemical analyses were performed on leaves collected between 1100 and 1300 hours on four replicate plants. Leaves sampled for biochemical analyses were immediately frozen in liquid nitrogen and kept at -80°C until analysis. Data were subjected, when appropriate, to a two-way ANOVA, with salt and species as fixed factors, with their interaction factor, using the Statistica ver. 8.0 software (StatSoft, Inc., Tulsa, OK, USA).

Results

Ionic and water relations

The examined species greatly differed for the Na^+ and Cl^- fluxes at both whole-plant (J_{plant}) and shoot level (J_{shoot} and J_{leaf}), at the end of three months of exposure to 75 mM external NaCl (Fig. 1). *Q. ilex* displayed a smaller J_{plant} for Cl^- (–25%), and particularly for Na^+ (–41%) as compared with *A. unedo* (Fig. 1*a, b*). These species-specific differences were much greater for the transport rates of potentially toxic ions. J_{shoot} and J_{leaf} were on average (mean of Na^+ and Cl^-) 54% or 71% smaller in *Q. ilex* than in *A. unedo*. Furthermore, $J_{\text{Na}^+, \text{leaf}}$ accounted for 36 or 52% of $J_{\text{Na}^+, \text{shoot}}$ in *Q. ilex* and *A. unedo*, respectively. Species-specific variations for $J_{\text{K}^+, \text{plant}}$ and $J_{\text{K}^+, \text{shoot}}$ were not observed, whereas $J_{\text{K}^+, \text{leaf}}$ was 37% greater in *Q. ilex* than in *A. unedo* (Fig. 1*c*). The ratio of $J_{\text{K}^+, \text{leaf}}$ to $J_{\text{Na}^+, \text{leaf}}$ was indeed much greater in *Q. ilex* (4.92 ± 0.52 , mean $\pm$ s.d., $n = 4$) than in *A. unedo* (1.07 ± 0.19). Consistently the leaf water molar concentration of potentially toxic ions in *A. unedo* largely exceeded (+44%) that in *Q. ilex* (see insets in Fig. 1*a, b*).

Salt treatment affected leaf water potential (ψ_{pd}) and relative water content (RWC), irrespective of species (Table 1). On the contrary, leaf bulk osmotic potential (ψ_{π}) decreased to a greater degree in *A. unedo* than in *Q. ilex* in response to salinity stress. Leaf osmotic potential (ψ_{p}) was consequentially higher in salt-treated *A. unedo* (2.10 MPa) than in corresponding *Q. ilex* (1.75 MPa), at the end of the experiment (Table 1). Salt-induced decrease in $\psi_{\pi\text{FT}}$ in *A. unedo* was mostly due to a massive accumulation of Na^+ and Cl^- (–0.33 MPa) and much less to soluble carbohydrate accumulation (from –0.11 MPa). In *Q. ilex* Na^+ and Cl^- contributed little to salt-induced changes in $\psi_{\pi\text{FT}}$ (–0.12 MPa), as also observed for soluble carbohydrates (–0.02 MPa). In *Q. ilex* the concentration of soluble carbohydrates was indeed unaffected by salinity (from 230 in controls to 227 mM in salt-treated plants, on a leaf tissue water basis), whereas in *A. unedo* it increased as much as 50 mM (from 324 in controls to 374 mM in salt-treated plants). This increase was exclusively due to a salt-induced enhancement in the concentration of mannitol (from 75 to 122 mM, data not reported). Dehydration ($D = \Delta\psi_{\pi} - \Delta\psi_{\pi\text{FT}}$) contributed much more in *Q. ilex* (39%) than in *A. unedo* (26%) to $\Delta\psi_{\pi}$ ($\Delta\psi_{\pi} = \psi_{\pi}^{\text{salt-treated}} - \psi_{\pi}^{\text{control}}$). Despite a greater contribution of K^+ to $\psi_{\pi\text{FT}}$, *Q. ilex* displayed a smaller osmotic adjustment (i.e. net solute accumulation) in response to root zone salinity than *A. unedo*.

Gas exchange and PSII performance

Gas-exchange performances were significantly affected by root zone salinity, with much greater reductions observed in *Q. ilex*

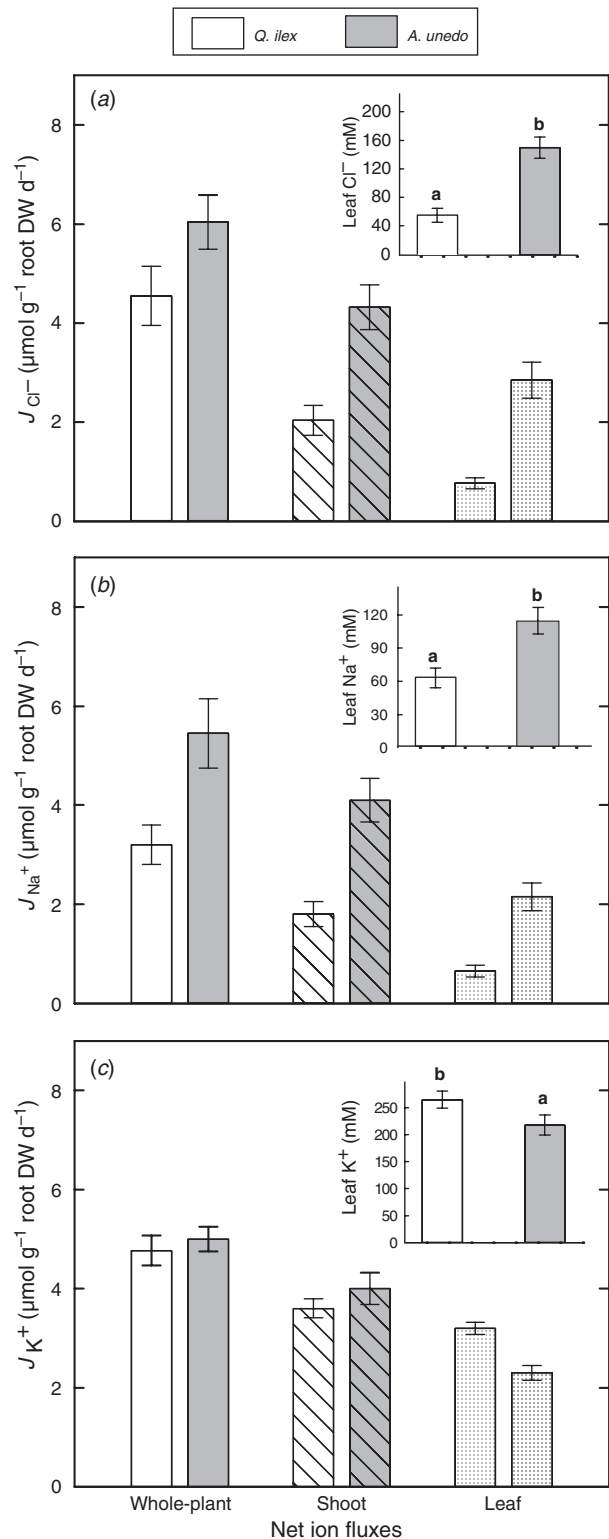


Fig. 1. Net Cl^- (a), Na^+ (b), and K^+ (c) fluxes in salt-treated *Quercus ilex* and *Arbutus unedo* over a 3 month period of treatment with 75 mM NaCl at the root zone. Data are means $\pm$ s.d. ($n = 4$). Graphs in the insets denote the leaf tissue water molar concentration of Cl^- (a), Na^+ (b) or K^+ (c). Bars denote s.d. ($n = 4$) and those not accompanied by the same letter are significantly different at $P < 0.05$, using a least significant difference (l.s.d.) test.

Table 1. Water and osmotic relations in *Arbutus unedo* and *Quercus ilex* supplied with 0 (control) or 75 mM NaCl (salt-treated) over a 3 month period

Ψ_{pd} , predawn water potential, Ψ_{π} bulk osmotic potential, RWC, relative water content, $\Psi_{\pi FT}$ osmotic potential at full turgor. Ψ_{π}^i denotes the contribution of individual osmolites to $\Psi_{\pi FT}$. Data are means $\pm$ s.d. ($n=4$) and those in a row not accompanied by the same letter are significantly different at $P<0.05$, using a least significant difference (l.s.d.) test

Parameter	<i>Arbutus unedo</i>		<i>Quercus ilex</i>	
	Control	Salt-treated	Control	Salt-treated
Ψ_{pd} (–MPa)	$0.45 \pm 0.03a$	$0.88 \pm 0.05b$	$0.46 \pm 0.05a$	$0.89 \pm 0.04b$
Ψ_{π} (–MPa)	$2.21 \pm 0.10a$	$3.02 \pm 0.11c$	$2.27 \pm 0.07c$	$2.63 \pm 0.09b$
RWC	$0.92 \pm 0.02b$	$0.88 \pm 0.02ab$	$0.91 \pm 0.01b$	$0.86 \pm 0.02a$
$\Psi_{\pi FT}$ (–MPa)	$2.02 \pm 0.07a$	$2.63 \pm 0.13c$	$2.05 \pm 0.06a$	$2.24 \pm 0.10b$
Ψ_{π}^{Cl-} (–MPa)	$0.03 \pm 0.006a$	$0.22 \pm 0.023c$	$0.02 \pm 0.005a$	$0.08 \pm 0.012b$
Ψ_{π}^{Na+} (–MPa)	$0.03 \pm 0.004a$	$0.17 \pm 0.012c$	$0.03 \pm 0.006a$	$0.09 \pm 0.009b$
Ψ_{π}^{K+} (–MPa)	$0.35 \pm 0.021a$	$0.32 \pm 0.016a$	$0.41 \pm 0.027b$	$0.45 \pm 0.019b$
Ψ_{π}^{sugars} (–MPa)	$0.45 \pm 0.015b$	$0.56 \pm 0.018c$	$0.35 \pm 0.009a$	$0.37 \pm 0.023a$

(on average -56% for P_n , g_s and E) than in *A. unedo* (on average -26% , Fig. 2). Great depression in gas-exchange performance was observed early in salt-treated *Q. ilex*: P_n decreased as much as 35% (from 9.9 in controls to $6.4 \mu\text{mol m}^{-2} \text{s}^{-1}$ in salt-treated, $P=0.019$) and g_s by 48% (from 182 to $95 \mu\text{mol m}^{-2} \text{s}^{-1}$, $P=0.007$) over the first 2 months of treatment (data not shown). In *A. unedo*, P_n declined from 8.9 in controls to $7.8 \mu\text{mol m}^{-2} \text{s}^{-1}$ in salt-treated plants ($P=0.047$) and g_s from

160 in controls to $124 \mu\text{mol m}^{-2} \text{s}^{-1}$ in salt-treated plants ($P=0.032$) over the same period (data not shown). Reductions in net photosynthesis were mostly due to stomatal limitations at the end of the experiment: C_i/C_a declined as much as 30 or 18% in *Q. ilex* or *A. unedo*, respectively, because of root-zone salinity (Fig. 2d). WUE was inherently (i.e. in control plants) greater in *Q. ilex* (4.44 ± 0.27 mean $\pm$ s.d., $n=6$) than in *A. unedo* (3.81 ± 0.22 , data not shown, but see Fig. 2a, b), and increased

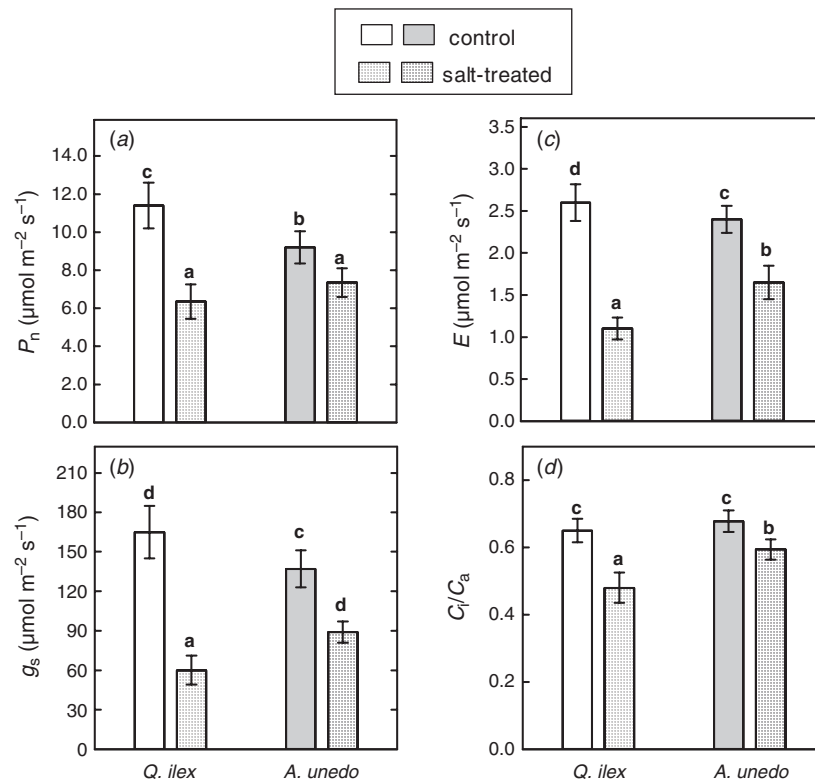


Fig. 2. Net CO_2 assimilation rate (P_n , a), stomatal conductance (g_s , b), transpiration rate (E , c) and the ratio of internal to external CO_2 concentration (C_i/C_a , d) in control and salt-treated *Quercus ilex* and *Arbutus unedo*. Measurements were performed between 1030 and 1230 hours, on 2 consecutive days. Data are means $\pm$ s.d. ($n=6$) and bars not accompanied by the same letter are significantly different at $P<0.05$, using a least significant difference (l.s.d.) test.

much more in *Q. ilex* ($\text{WUE}=5.68 \pm 0.44$) than in *A. unedo* ($\text{WUE}=4.35 \pm 0.39$) as a consequence of root zone salinity (Fig. 2a, b).

Following salt-induced declines in P_n , adjustments aimed at preserving the photosynthetic apparatus from excess radiant energy effectively operated in *A. unedo*, and particularly in *Q. ilex* (Fig. 3). *A. unedo* displayed an inherently greater capacity to dissipate radiant energy through nonphotochemical quenching than *Q. ilex*. However, both NPQ increased and Φ_{PSII} decreased to a markedly greater degree in *Q. ilex* (+105% for NPQ and –30% for Φ_{PSII}) than in *A. unedo* (+36% or –14% for NPQ and Φ_{PSII} respectively) in response to root zone salinity (Fig. 3b, c). Salt-treated *Q. ilex* suffered from excess excitation energy to the photosynthetic apparatus, here estimated in terms of the reduction state of primary acceptors ($1 - q_P$), to a much greater degree than *A. unedo* (Fig. 3d). Salt-induced impairments in the maximal efficiency of PSII (F_v/F_m) were not observed in our experiment (Fig. 3a).

Photosynthetic pigments, antioxidant enzymes and ascorbate

Q. ilex and *A. unedo* displayed marked differences in their carotenoid concentration and composition in control plants (Table 2). The carotenoid to Chl_{tot} ratio was significantly greater in *Q. ilex* (0.45) than in *A. unedo* (0.37) mostly due

to a greater concentration of lutein, lutein epoxide, and violaxanthin-cycle pigments (VAZ). The de-epoxidation state of VAZ ($\text{DES}=(A+Z)/(V+A+Z)$) was instead greater in *A. unedo* than in *Q. ilex* under well watered conditions (0.38 vs 0.22), conforming to species-specific NPQs. Salt-induced changes in the concentration and composition of the carotenoid pool were more pronounced in *Q. ilex* than in *A. unedo*. Carotenoid (Car) to Chl_{tot} ratio increased as much as 25 or 13% and DES by 259 or 103% in *Q. ilex* or *A. unedo*, respectively, in response to root zone salinity. The concentration of zeaxanthin in salt-treated *Q. ilex* exceeded by 53% that in *A. unedo*. We noted that the salt-induced increase in lutein concentration was detected only in *Q. ilex* and was paralleled by a significant decrease in the concentration of lutein epoxide.

Control plants of *Q. ilex* displayed a greater ability than *A. unedo* to detoxify ROS (Fig. 4). In general, the activities of antioxidant enzymes and the concentration of ascorbic acid in *Q. ilex* exceeded by 100 and 25%, respectively, those detected in *A. unedo*. However the activities of both SOD (Fig. 4a) and CAT (Fig. 4b) declined in *Q. ilex* under salinity stress (on average –42%). Instead SOD increased as much as 73% and CAT was unaffected by the salt treatment in *A. unedo*. APX activity (Fig. 4c) increased as a consequence of salt stress in both species (on average +60%), particularly in *A. unedo* (+87%). The concentration of total ASA (Fig. 4d) increased more in *Q. ilex* (+46%) than in *A. unedo* (+14%) because of salinity stress, mostly

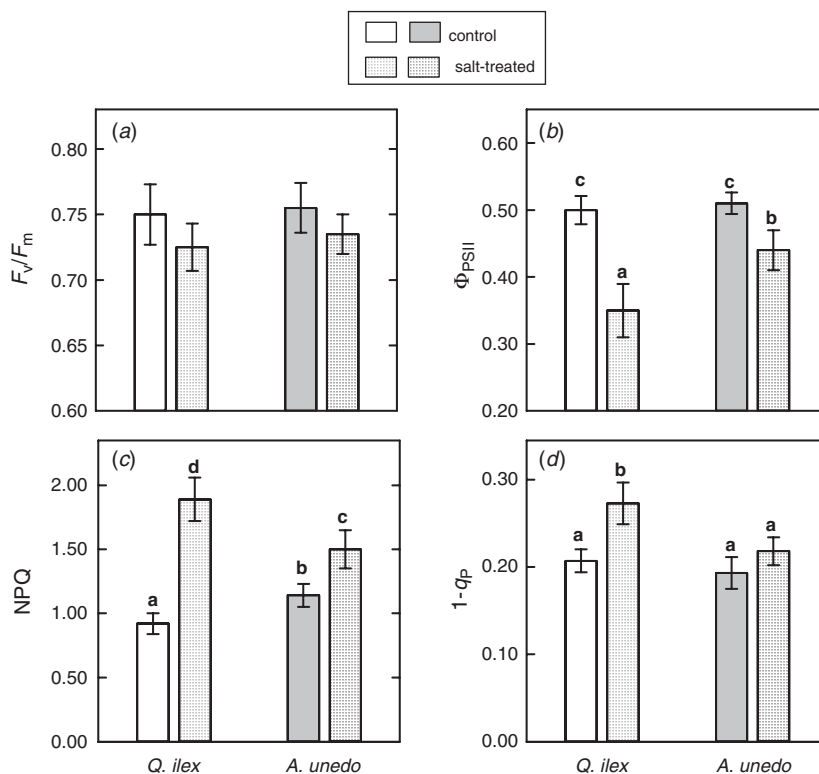


Fig. 3. Maximal (a) and actual (b) efficiency of PSII photochemistry, non-photochemical quenching (NPQ, c) and the reduction state of primary acceptors ($1 - q_P$, d) in leaves of well watered or salt-treated *Quercus ilex* and *Arbutus unedo*. Data are means $\pm$ s.d. ($n=6$) and bars not accompanied by the same letter are significantly different at $P < 0.05$, using a least significant difference (l.s.d.) test.

Table 2. The concentration of photosynthetic pigments in *Arbutus unedo* and *Quercus ilex* supplied with 0 (control) or 75 mM NaCl (salt-treated) over a 3 month period

Measurements were conducted on leaves collected between 1100 and 1300 hours. Data are means $\pm$ s.d. ($n=4$) and those in a row not accompanied by the same letter differ significantly at $P<0.05$, using a least significant difference (l.s.d.) test. Chl_{tot}, total chlorophyll; Vio, violaxanthin; Ant, antheraxanthin; Zea, zeaxanthin; Lut ep_x, lutein epoxide; Lut, lutein; β -car, β -carotene; Neo, neoxanthin; VAZ, V + A + Z; DES, de-epoxidation state of violaxanthin-cycle pigments. n.d., not detected

Pigment	<i>Arbutus unedo</i>		<i>Quercus ilex</i>	
	Control	Salt-treated	Control	Salt-treated
Chl _{tot} ($\mu\text{mol g}^{-1}$ DW)	1.93 $\pm$ 0.13ab	1.80 $\pm$ 0.14a	2.12 $\pm$ 0.09b	1.79 $\pm$ 0.11a
Vio (V, $\mu\text{mol g}^{-1}$ DW)	0.29 $\pm$ 0.01c	0.08 $\pm$ 0.01a	0.21 $\pm$ 0.01b	0.07 $\pm$ 0.01a
Ant (A, $\mu\text{mol g}^{-1}$ DW)	0.03 $\pm$ 0.01a	0.05 $\pm$ 0.01b	0.04 $\pm$ 0.01a	0.07 $\pm$ 0.01c
Zea (Z, $\mu\text{mol g}^{-1}$ DW)	0.05 $\pm$ 0.01a	0.26 $\pm$ 0.02d	0.09 $\pm$ 0.01b	0.17 $\pm$ 0.01c
Lut ep _x ($\mu\text{mol g}^{-1}$ DW)	0.04 $\pm$ 0.01b	0.02 $\pm$ 0.01a	n.d.	n.d.
Lut ($\mu\text{mol g}^{-1}$ DW)	0.23 $\pm$ 0.01a	0.41 $\pm$ 0.04b	0.20 $\pm$ 0.01a	0.22 $\pm$ 0.03a
β -car ($\mu\text{mol g}^{-1}$ DW)	0.14 $\pm$ 0.02a	0.14 $\pm$ 0.02a	0.22 $\pm$ 0.01b	0.20 $\pm$ 0.02b
Neo ($\mu\text{mol g}^{-1}$ DW)	0.07 $\pm$ 0.01b	0.07 $\pm$ 0.01b	0.05 $\pm$ 0.01a	0.05 $\pm$ 0.01a
Carotenoid Chl _{tot} ⁻¹	0.45 $\pm$ 0.03b	0.57 $\pm$ 0.02c	0.39 $\pm$ 0.02a	0.43 $\pm$ 0.02ab
VAZ Chl _{tot} ⁻¹ (mmol mol ⁻¹)	195 $\pm$ 18bc	217 $\pm$ 16c	160 $\pm$ 11a	174 $\pm$ 8ab
DES (A + Z/V + A + Z)	0.26 $\pm$ 0.03a	0.79 $\pm$ 0.03c	0.34 $\pm$ 0.02b	0.77 $\pm$ 0.05c

as a consequence of a greater salt-induced enhancement in the concentration of DHA (+126% in *Q. ilex* vs +42% in *A. unedo*, Fig. 4e). The ratio of DHA/ASA_{tot} indeed varied from 0.33 in controls to 0.50 in salt-treated *Q. ilex*, and from 0.37 to 0.43 in corresponding plants of *A. unedo* (Fig. 4f).

Discussion

The ability to retain potentially toxic ions in the roots, thus allowing a preferential transport of K⁺ over Na⁺ to actively growing organs of the shoot is a key component of salt tolerance, particularly in glycophytes (Munns and Tester 2008; Cheeseman 2013). This may have particular value for Mediterranean evergreens that suffer from transient periods of excess soil salinity (Alessio *et al.* 2004; Cimato *et al.* 2010). In species with slow returns from investments of nutrient and dry mass in leaves (e.g. many evergreen shrubs and trees with high LMA, *sensu* Wright *et al.* 2005) salt-exclusion mechanisms are crucial in preserving leaf functionality (Tattini and Traversi 2008).

Q. ilex displayed a superior capacity to exclude salt from the shoot as compared with *A. unedo*. It is worth noting that the transport of Na⁺ and Cl⁻ to the leaf was on average 65% smaller in *A. unedo* despite transpiration was just 24% smaller in the former than in the latter species. Therefore, in *Q. ilex* salt-exclusion seems to be also supported by an effective ability to exclude Na⁺ from the transpiration stream, and by an effective K⁺/Na⁺ exchange operating in the stem, thus allowing a preferential transport of K⁺ to the leaf (Shabala and Cuin 2008). The 'low-Na⁺ strategy' (*sensu* Flowers and Yeo 1995), is effectively sustained by reductions in transpiration rates, which are of adaptive value to excess soil salinity for perennials inhabiting semiarid/arid regions worldwide (Yakir and Yechieli 1995; Tattini *et al.* 2002). Water use strategy may have a pivotal role in salt-exclusion mechanisms. Capacity to limit transpiration losses, and, accordingly ion fluxes, to photosynthetic organs may indeed prolong survival during the dry summer and allows prompt recovery in gas exchange and whole-plant

performance when good-quality water becomes newly available to the roots (Tattini *et al.* 1995, 2002). At one extreme, perennials survive in hypersaline (up to 2.2 M NaCl concentration) sea-shores of the Dead Sea (halophytes *sensu stricto*) as capable of using only rain water during their life cycle (Yakir and Yechieli 1995).

Like most *Quercus* spp., *Q. ilex* has a very conservative resource use strategy to cope with adverse environmental conditions. (Martínez-Ferri *et al.* 2000). The severe restrictions in water flow displayed by *Q. ilex* to cope with salt-induced drought stress is consistent with greater capacity to control both stomatal opening and water loss under severe water deprivation as compared with *A. unedo* (Martínez-Vilalta *et al.* 2003). This behaviour is typical of evergreen sclerophylls, such as *Q. ilex* and *P. latifolia*, which display a 'water-saving' strategy as compared with the 'water-spending' strategy usually adopted by semi-deciduous sclerophylls, such as *A. unedo* (Gratani and Ghia 2002). Therefore, we suggest that species-specific abilities to exclude potentially toxic ions from sensitive shoot organs tightly depend on their strategy to use water, rather than on their 'capacity to withstand water stress' *sensu lato*, as reported previously for other Mediterranean evergreens (Tattini *et al.* 1996, 2002). This may have great ecological significance, as it allows long-living species inhabiting semiarid or arid zones worldwide to survive during transient periods of excess soil salinity concentration (Yakir and Yechieli 1995; Munns and Tester 2008).

Despite the mild salinity stress (*sensu* Munns and Tester 2008) at which plants were exposed in our experiment, the 'low-Na⁺ strategy' adopted by *Q. ilex* resulted in smaller declines in leaf ψ_{π} as compared with *A. unedo*. In *Q. ilex* dehydration indeed contributed greatly in countering salt-induced osmotic unbalance to sustaining water uptake from the salty soil. It is worth noting that soluble carbohydrates did not contribute in *Q. ilex* (and contributed little in *A. unedo*) to salt-induced changes in leaf $\Psi_{\pi\text{FT}}$. This finding contrasts with those previously reported for other salt excluding sclerophylls, such as *Olea europaea* and *Phillyrea latifolia*, in which leaf $\Psi_{\pi\text{FT}}$ markedly declined as a consequence of massive accumulation of soluble carbohydrates,

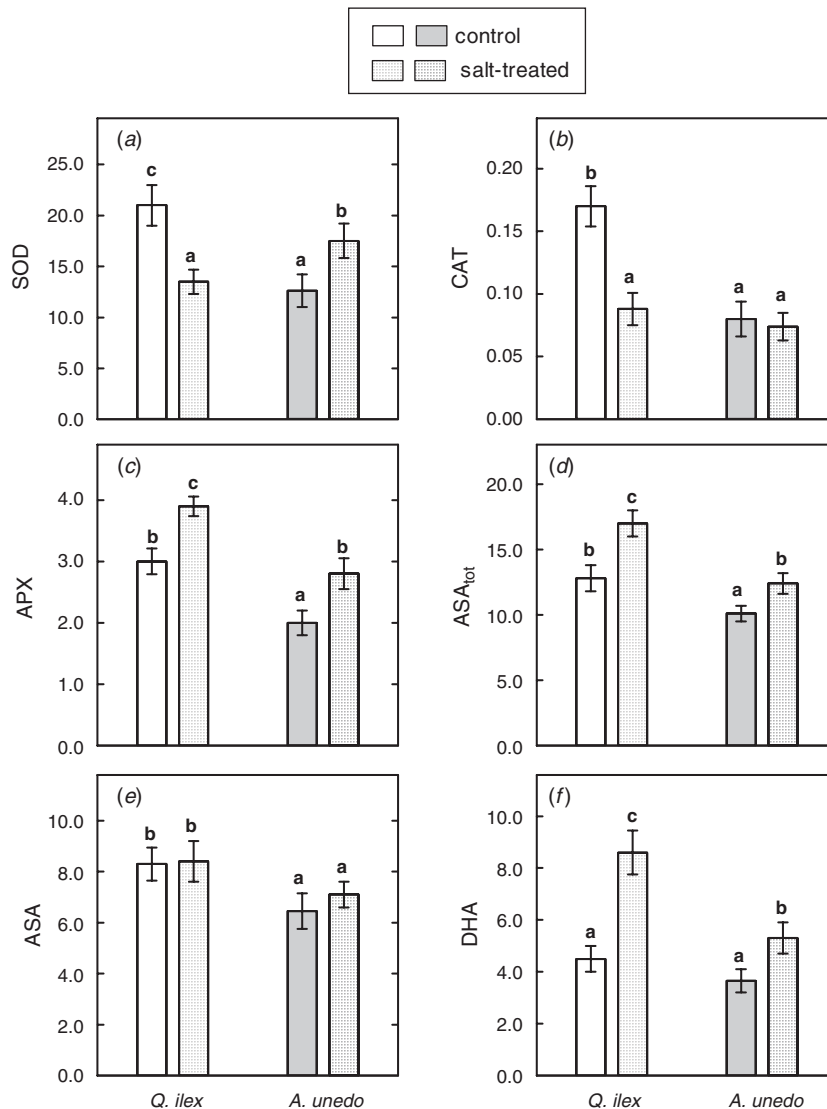


Fig. 4. The activities of superoxide dismutase (SOD, $\text{U mg}^{-1} \text{ protein min}^{-1}$), catalase (CAT, $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$), ascorbate peroxidase (APX, $\mu\text{mol ascorbic acid min}^{-1} \text{ mg}^{-1} \text{ protein}$), the total concentration of ascorbic acid (ASA_{tot} , $\text{mg g}^{-1} \text{ DW}$), the concentrations of reduced (ASA, e) and oxidised ascorbic acid (DHA, f). Data are means $\pm$ s.d. ($n = 4$) and bars not accompanied by the same letter are significantly different at $P < 0.05$, using a least significant difference (l.s.d.) test.

particularly mannitol (Tattini *et al.* 1996, 2002; Gucci *et al.* 1997). Accumulation of soluble carbohydrates in source leaves follows drastic declines in the strength of sink organs (Tattini *et al.* 1996; Munns *et al.* 2000; Munns and Tester 2008), which were not observed in our experiment (e.g. whole-plant DW decreased by only 16 or 11% in *Q. ilex* or *A. unedo*, respectively, data not shown).

Following salt-induced declines in the usage of radiant energy in photosynthetic processes, biochemical adjustments operated to a greater degree in salt-treated *Q. ilex* than in corresponding plants of *A. unedo*. These adjustments primarily included changes in carotenoids, particularly xanthophyll concentration and composition, to effectively sustain thermal dissipation of excess radiant energy through nonphotochemical quenching.

Actually DES differed little in salt-treated *Q. ilex* and *A. unedo*, despite large differences in NPQ. We note that zeaxanthin may have served a prominent role to protect thylakoid membranes from photo-oxidation (Beckett *et al.* 2012; Ramel *et al.* 2012) due to high VAZ to Chl_{tot} ratios detected in our experiment. This high concentration of zeaxanthin in salt-treated *Q. ilex*, $144 \text{ mmol mol}^{-1} \text{ Chl}_{\text{tot}}$ ($94 \text{ mmol mol}^{-1} \text{ Chl}_{\text{tot}}$ in salt-treated *A. unedo*), is unlikely to be bound to light-harvesting chlorophyll-protein complexes (Havaux *et al.* 2004), and therefore reside in other parts of the thylakoids thus limiting the penetration of ROS inside the thylakoid other than preserving disruption of chloroplast envelope membranes (Havaux *et al.* 2004; Beckett *et al.* 2012). It is worth noting that lutein may have also contributed

to NPQ in *Q. ilex* (Jahns and Holzwarth 2012). De-epoxidation of lutein epoxide to lutein (a cycle operating in *Quercus* spp., García-Plazaola *et al.* 2002), not only of violaxanthin to zeaxanthin, may indeed contribute in pH-dependent NPQ (Müller *et al.* 2001). Therefore, we suggest that despite excess of excitation energy to PSII was greater in *Q. ilex* than in *A. unedo*, more active processes of carotenoid de-epoxidation allowed the former species to preserve PSII from impairment, possibly fulfilling multiple functions.

There is consensus that primary mechanisms adopted by plants to counter an increase in ROS generation are supported by antioxidant enzymes and low-molecular weight antioxidants, such as ascorbic acid (Apel and Hirt 2004). However, the question of how primary antioxidant defences respond to salinity stress (or confer salt tolerance) is still a matter of debate. Constitutively or salt-induced enhancement in antioxidant enzymes has been reported to correlate or not with salt-tolerance (Hernández *et al.* 2003; Noreen and Ashraf 2009). It is noted that in addition to species, intensity of salinity stress, and sunlight irradiance plants face may have been responsible for such conflicting data (Noreen and Ashraf 2009; Ding *et al.* 2010). Severe excess excitation energy deleteriously affects the activities of ROS detoxifying enzymes (Peltzer and Polle 2001; Fini *et al.* 2012), as here observed for both SOD and CAT activities in salt-treated *Q. ilex*. However, we note that CAT activity did not differ whereas APX activity was 40% greater in salt-treated *Q. ilex* than in corresponding *A. unedo* plants. Greater APX activity and ASA_{tot} concentration, which are key determinants of salt tolerance (Shalata and Neumann 2001), likely conferred a greater capacity for reducing H₂O₂ to *Q. ilex* than *A. unedo*. However, the finding that the salt-induced increase in DHA/ASA ratio (+49%) was much greater than the corresponding increases in APX activity (+27%) and ASA_{tot} concentration (+30%) suggests that recycling of DHA to ASA was in some way compromised in salt-treated *Q. ilex*.

Conclusions

Here we have described the strategies adopted by two-occurring Mediterranean species to allocate potentially toxic ions at organism level when subjected to mild salinity. Such strategies seem to be strongly determined by their relative leaf life-spans and abilities to withstand salt-induced leaf dehydration. How these morpho-functional features may confer 'salt tolerance' to the examined species is a complex issue that needs further investigation. The ability to survive severe stress or achieve acceptable yield under mild stress are conflicting descriptors of stress tolerance *sensu lato* (Tardieu and Tuberosa 2010). We have shown here that *Q. ilex* (1) displays greater ability to limit the transport of harmful ions in shoot organs, but concomitantly (2) activates costly biochemical adjustments as a consequence of greater salt-induced depression of photosynthesis, than *A. unedo*. This may have significance for the species ecology, e.g. when plants face to unpredictable severe increases in root zone salt concentration. Salt-tolerance based on gas-exchange/growth performance, which has great horticultural value, may conflict with salt-tolerance based on species survival, which has instead great ecological significance (Munns and Tester 2008; Flowers *et al.* 2010). In evergreens, a species' ability to resume gas

exchange and growth performance following late-summer/early-autumn rainfalls, which is a key component of salt-tolerance, depends strongly on its ability to exclude harmful ions from actively shoot organs during salt stress events.

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