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Ozone and desiccation tolerance in chlorolichens are intimately connected: a case study based on two species with different ecology

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Abstract Tropospheric ozone (O_3) causes severe damage to many vascular plants but not to lichens. It was recently suggested that this may be due to their high levels of natural defences against the oxidative bursts associated to their fluctuating water content. In this study, the combined effects of watering regime (with or without a daily spray of distilled water), air relative humidity (20 ± 5 vs. 80 ± 5% RH) and O_3 (250 vs. 0 ppb, 5 h day^{-1} for 2 weeks) were monitored in two chlorolichens with different ecology, *Parmotrema perlatum* and *Xanthoria parietina.* Modulated chlorophyll *a* fluorescence (Chl_aF), superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) production, antioxidant content and enzyme activity of the ascorbate/glutathione cycle were measured after exposure and, for Chl_aF, after 1 and 2 days of recovery. The species differed in the antioxidant profile (ascorbate was higher in *X. parietina*, glutathione in *P. perlatum*), and in the activity of ROS-scavenging enzymes, more intense in the hygrophilous *P. perlatum* than in the meso-xerophilous *X. parietina.* O_3 slightly modified Chl_aF parameters related to the controlled dissipation, with reduction of F_m, F_v/F_m (both species) and ETR (in *P. perlatum*), and increase in NPQ and qN (in *X. parietina*). It also influenced, particularly in *P. perlatum*, the content of H_2O_2, glutathione (GSH) and oxidized glutathione (GSSG) (but not that of $O_2^{\bullet-}$ and AsA + DHA) and the activity of superoxide dismutase, ascorbate peroxidase and dehydroascorbate reductase. These parameters, however, were more heavily affected by water availability. The hypothesis that lichens are O_3-tolerant thanks to the constitutive antioxidant systems, intimately related to their poikilohydric life-style, is thus confirmed.

Keywords Antioxidants · Halliwell-Asada cycle · Chlorophyll *a* fluorescence · Oxidative stress · Poikilohydric organisms

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Introduction

Tropospheric ozone (O_3) is an important component of global change (Feng et al. 2015): it is mostly produced by a complex series of photochemical reactions from volatile organic compounds and nitrogen oxides generated by human and natural sources (Logan 1985). The increasing emissions of precursor substances coupled with high temperatures and UV irradiances result in a progressive elevation of the ground-level O_3 concentration (Wu et al. 2008). Ozone is considered as a major air pollutant with negative effects not only on human health but also on plants, affecting growth and primary production of crops (Fuhrer 2009; Betzelberger et al. 2012) and native vegetation (Gottardini et al. 2014), with impairment of leaf physiology and biochemistry (Matyssek et al. 2010; Pellegrini et al. 2011; Demidchik 2015).

The action mechanisms of O_3 are mediated by the formation of reactive oxygen species (ROS). In plants, these are formed as soon as O_3 penetrates the leaf through open stomata (Laisk et al. 1989). ROS are dangerous molecules, but they are

also normal by-products physiologically generated by cell metabolism. For this reason, vascular plants have evolved efficient enzymatic and non-enzymatic antioxidant systems responsible for maintaining the baseline levels of ROS. Oxidative stress occurs when the production of ROS exceeds the cell scavenging potential, and the rate of repair processes fails to keep pace with the proceeding damage (Vainonen and Kangasjärvi 2015). One major biochemical marker of plant sensitivity to O_3 is an apoplastic ROS burst after O_3 exposure, which is absent or reduced in O_3-resistant individuals (Wohlgemuth et al. 2002).

In contrast to the large majority of vascular plants (e.g. van Goethem et al. 2013; Döring et al. 2014), lichens - symbiotic associations between a fungus (the mycobiont) and one or more photosynthetic partners (the photobionts) - are considered O_3-tolerant (Calatayud et al. 2000; Lorenzini et al. 2003; Riddell et al. 2010, 2012). This was originally explained as due to the apoplastic compounds produced by the mycobiont (Valencia-Islas et al. 2007), but more recently an alternative hypothesis was advanced (Tretiach et al. 2012a; Bertuzzi et al. 2013): the tolerance would be a side effect of the constitutive natural defences intimately related to poikilohydry. Notoriously, the water content of lichens changes readily in dependence of the water potential of the surrounding environment (Nardini et al. 2013). These organisms face more or less long periods of time in a deep desiccation state and others in different degrees of hydration (Lange and Green 2008), with dehydration/rehydration events that may occur more times per day (Jonsson et al. 2008). In both the key phases, when water is lost and when it is gained, there is an intense ROS formation that is mostly due to the imbalanced electron transport of respiratory and/or photosynthetic chains, to non-enzymatic and auto-oxidation reactions (Kranner 2002; Weissmann et al. 2005a, b) and to the prolonged exposure to an oxidative atmosphere in an inactive state (Candotto Carniel et al. 2015). This stochastic oxidative load is faced by the metabolites and the enzymes linked to the ascorbate/glutathione cycle, more or less efficiently in dependence to the ecology of the species (Mayaba and Beckett 2001; Kranner et al. 2008). In general, it was observed that, under stress, tolerant lichens modulate the status and activity of their antioxidant systems in response to increased levels of ROS (del Hoyo et al. 2011). According to Kranner et al. (2006), failure of the antioxidant systems during stressful conditions could trigger ageing and eventual death of portions of the thallus. This suggests a potent antioxidant machinery as one of the underlying mechanisms of desiccation tolerance (del Hoyo et al. 2011), possibly of constitutive nature (Candotto Carniel et al. 2016). According to Tretiach et al. (2012a) and Bertuzzi et al. (2013), the exposure of lichens to O_3 should actually imply just a slight aggravation of the overall oxidative load associated to the fluctuating hydration status, with scarce effects in terms of vitality loss. This hypothesis was recently tested by Pellegrini et al. (2014), who studied the effects of O_3 on the mesophytic *Flavoparmelia caperata* (L.) Hale, a lichen regarded as moderately tolerant to airborne pollutants (Nimis and Martellos 2008). It was shown that the functionality of the photosystems of the photobiont was never impaired, with minimal fluctuations in reduced ascorbate (AsA) and an increase in the enzymatic activity of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). The authors also suggested that thalli equilibrated with air at different water vapour contents can differ in their redox status.

The aim of this study is to deepen the role of the ROS scavenging systems in the O_3 tolerance of lichens in dependence to water availability and species ecology. The experimental design of Pellegrini et al. (2014) has thus been applied to two lichens with green algae ("chlorolichens") selected for their different preferences for humid vs. dry habitats. Hygrophilous vs. xerophilous lichens, with their different inherent desiccation tolerance, actually differ in the amount of scavenging enzymes (e.g. superoxide dismutase, SOD), antioxidant substances (e.g. glutathione) (Silberstein et al. 1996), and speed of recovering the intracellular reduction power and enzymatic activity upon rehydration (Kranner 2002; Kranner et al. 2008). Therefore, they represent an interesting dual model for testing the hypothesis that water availability (in terms of air relative humidity and watering regime) may have heavier effects on the pool of metabolites and enzyme activity of the ascorbate/glutathione cycle than the O_3 exposure itself.

Materials and methods

Collection and pre-treatment of lichen samples

The species selected for this study are *Parmotrema perlatum* (Huds.) M. Choisy and *Xanthoria parietina* (L.) Th. Fr., two lichens with green algae of the genus *Trebouxia* as photobionts (Table 1). The former is rather hygrophilous, avoids extreme solar irradiation and is highly sensitive to air pollution, while the latter is meso-xerophilous, can sustain very high direct solar irradiation and is moderately tolerant to SO_2 and NO_x pollution (Nimis 2016). Several healthy-looking adult thalli were collected from deciduous trees in areas of NE Italy far from known direct air pollution sources. In the laboratory, the thalli were cleaned from debris and bryophytes, put in a desiccator with silica gel for 2 days, sealed in vacuum bags and stored at −20 °C. Before use, the material was thawed in a desiccator with silica gel for 2 days, and then the central parts of the thalli (ca. 3 cm from the margin) were removed, since they have considerably lower modulated chlorophyll *a* fluorescence (Chl_aF) emission than the peripheral, lobate parts (herein, "lobes") (Tretiach et al. 2007). For the exposures, about (i) 80 individual lobes of 60 ± 5 mg each for Chl_aF measurements and (ii) 16 g of mixed

Table 1 Investigated lichens, with respective photobionts, sampling sites, altitude (Alt., m above sea level), collection dates, substrata, species-specific photosynthetic photon flux density at which the quantum yield of CO_2 assimilation is the highest ($PPFD_{Ik}$, μmol photons m^{-2} s^{-1}) (Piccotto and Tretiach 2010) and categories of acidophytism (pH), hygrophytism (H), eutrophication (N) and pollution tolerance (Poll.) according to Nimis and Martellos (2008)

Species	Photobionts[ab]	Sampling site	Alt. (m)	Date	Substratum	$PPFD_{Ik}$	pH	H	N	Poll.
Parmotrema perlatum (Huds.) M. Choisy	*Trebouxia crenulata* Archibald *T. decolorans* Ahmadjian *T.* sp.	Italy, Classical Karst, Trieste prov., 45° 44′ N 13° 44′ E	170–200	23-June-2010	Northerly exposed bark of *Quercus petraea* (Matt.) Liebl.	108	2	2–3	1–2	2–3
Xanthoria parietina (L.) Th. Fr.	*T. irregularis* Hildreth & Ahmadjian *T. arboricola* de Puymaly *T. decolorans* Ahmadjian	Italy, Friulian Plain, Udine prov., 45° 43′ N 13° 03′ E	5–10	29-June-2010	Northerly exposed bark of *Acer* sp.	131	2–4	3–4	3–5	1–3

[a] Ahmadjian (1993)

[b] Candotto Carniel et al. (2015)

lobes for biochemical assays were randomly selected. Prior to exposure, the material was subjected to a 2-day-long conditioning process: it was immersed in distilled water for 3 min twice a day and maintained on rigid plastic nets within plastic boxes containing water at the bottom, covered (but not sealed) with transparent plastic wrap and put in a growth chamber with charcoal-filtered air at 20 °C. The photosynthetic photon flux density (PPFD), checked using a LI-COR-calibrated Micro-Quantum 2060-M Sensor (Walz, Effeltrich, Germany), was set at 18 μmol photons m^{-2} s^{-1} (*P. perlatum*) and 22 μmol photons m^{-2} s^{-1} (*X. parietina*), corresponding to one sixth of the respective PPFD at which the quantum yield of carbon dioxide (CO_2) assimilation is the highest ($PPFD_{Ik}$, Table 1). Light was provided by four quartz metal halide lamps with clear outer bulb (400 W, MASTER HPI-T Plus, Philips, Eindhoven, the Netherlands) and by four high-pressure sodium lamps with clear tubular outer bulb (250 W, SON-T, Philips, Eindhoven, the Netherlands). After this conditioning process, a first series of Chl_aF measurements (see below) were taken to (i) exclude those samples with maximum quantum efficiency of photosystem II (F_v/F_m) below 0.600, as a proxy of non-perfect health conditions, and (ii) divide the samples in homogeneous groups (see below).

Isolation and culturing of the lichen photobiont

An axenic strain of the photobiont of *P. perlatum* was isolated according to Yamamoto et al. (2002). The isolated photobiont was identified as a still undescribed species of *Trebouxia* De Puymaly on the basis of Internal Transcribed Spacer (ITS) sequence data (data available upon request). It was inoculated into sterile plastic tubes filled with 5 mL of slanted solid TM (1.5% agar) (Ahmadjian 1973), and kept in a growth chamber at 18 ± 1 °C and 18 ± 2 μmol photons m^{-2} s^{-1} with a light/dark regime of 14/10 h until an abundant biomass was obtained (Candotto Carniel et al. 2015). The cultures were inoculated with 100 μL of a cell water suspension (density 3.5×10^6 cells mL^{-1}) on hand-cut sterile filter paper discs (Whatman, 60 ± 5 g m^{-2}, diam. 25 mm), laid on solid TM (1.5% agar) inside Petri dishes, four discs per dish. The cultures were let to grow for 30 days before exposure at the same environmental conditions mentioned above.

Sample exposure

The lichen materials were divided in groups of six individual lobes and 2 g of mixed lobes, respectively. The samples were exposed for 2 weeks at 36 μmol photons m^{-2} s^{-1} (*P. perlatum*) and 44 μmol photons m^{-2} s^{-1} (*X. parietina*), with a light/dark regime of 12/12 h, 20 °C, 20 ± 5% relative humidity (RH) or, alternatively, 80 ± 5% RH, at four combinations of O_3 and artificial daily watering: without O_3 ("control"), with or without watering (herein, "wet" and "dry", respectively) and with O_3

("O_3-exposed"), wet or dry. The control samples were put in a ventilated growth chamber with filtered air; the O_3-exposed ones were put in a ventilated 0.90 × 0.90 × 0.65 cm Perspex chamber with the inlet air (two complete air changes min^{-1}) enriched with 250 ppb O_3 for 5 h in form of a square wave generated by electrical discharge, using a Fisher 500 air-cooled apparatus (Zurich, Switzerland) supplied with pure oxygen. The watering consisted in a spray of distilled water of ca. 0.01 mL cm^{-2} applied to the selected samples immediately before the input of O_3. Light was provided by four quartz metal halide lamps with clear outer bulb (400 W, MASTER HPI-T Plus, Philips, the Netherlands) and by four high-pressure sodium lamps with clear tubular outer bulb (250 W, SON-T, Philips, the Netherlands). Air humidity was checked automatically in the controlled environment facilities (F.lli Bertagnin, Bologna, Italy). All the individual lobes selected for the Chl$_a$F measurements, after the post-exposure measurements (see *infra*), were left to recover for 2 days, at the same conditions of the conditioning process described above.

The exposure of the algal discs was carried out in open glass Petri dishes at the same chamber conditions described for *P. perlatum*, at 20 or 80% RH without or with O_3 and without watering. During the 2-day-long recovery, algal discs were maintained in closed Petri dishes on solid 3NBBM (1.5% agar), having hydrated them with 0.3 mL of sterile water, at the same conditions of the conditioning process.

Chl$_a$F measurements

On lichens, Chl$_a$F measurements were taken before and after exposure and after 1 and 2 days of recovery; they were repeated for each treatment on the same individual lobes (n = 6), after rehydration (3 min in distilled water) and dark adaptation (30 min) of the material.

On *Trebouxia*, Chl$_a$F measurements were taken before exposure, after exposure and after 2 days of recovery on different sets of six randomly selected discs, since it is impossible to reuse the algal disc after the measurement due to unavoidable contaminations by bacteria and fungi. With this material, before measurement, 0.3 mL of distilled water was added to each disc, which was then dark-adapted for 30 min.

Chl$_a$F measurements were taken with a pulse amplitude-modulated fluorometer PAM-2000 (Walz, Effeltrich, Germany), positioning the measuring fibre at 60° on the upper surface of the lobes and cultured discs. The modulated light was turned on to obtain the minimal Chl$_a$F level (F_0). A saturating light pulse of ca. 8000 μmol photons m^{-2} s^{-1} for 0.8 s was emitted to obtain the transient maximum Chl$_a$F level (F_m) and thus to calculate the variable Chl$_a$F level (F_v, i.e. $F_m - F_0$) and the maximum quantum efficiency of PS II photochemistry (F_v/F_m). An external actinic light provided by a light unit FL-460 (Walz, Effeltrich, D) with a halogen lamp was turned on to record the Kautsky effect at an intensity consistent with the specific $PPFD_{Ik}$ value of each species (Table 1). Once the emission peak was achieved (F'_m), saturating light pulses were applied at 60-s intervals during actinic illumination to determine photochemical (qP) and non-photochemical (qN and NPQ) quenching (see, e.g. Baker 2008; Roháček 2002; Bussotti et al. 2011). NPQ was calculated as $(F_m - F'_m) / F'_m$.

The water content of each sample was estimated by measuring the impedance with a Protimeter Mini-3 (Parametrics, Shannon, Ireland) (Bertuzzi et al. 2013).

Biochemical markers

Three samples of 250 mg (air-dry weight) were analysed for each biochemical marker, with the exception of hydrogen peroxide (30 mg per sample). Parallel samples kept at 80 °C for 48 h were used to determine the water content of air-dry materials and calculate the corresponding dry weight (d.w.).

ROS determination

The superoxide anion radical ($O_2^{\bullet-}$) determination was based on the reduction of a tetrazolium dye sodium, 3′-(1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) by O_2 to a soluble XTT formazan, according to Able et al. (1998). Samples were frozen in liquid nitrogen, ground and then added to 1.0 mL of 50 mM Tris-HCl buffer (pH 7.5). After centrifugation, the supernatant was incubated with 50 mM Tris-HCl buffer (pH 7.5) and 0.5 mM XTT at room temperature for 15 min. The reduction of XTT formazan was quantified at 470 nm. For more details, see Supplementary Material (S1).

Hydrogen peroxide (H_2O_2) production was measured fluorimetrically using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA), according to Shin et al. (2005). The assay is based on the reaction of 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent, a highly sensitive and stable probe for H_2O_2) with H_2O_2 in a 1:1 stoichiometry which produces the red-fluorescent oxidation product resorufin. Samples were frozen in liquid nitrogen, ground and then extracted with 0.8 mL of 20 mM potassium phosphate buffer (pH 6.5). After centrifugation, the supernatant was incubated with 50 μM Amplex Red reagent and 0.1 U mL^{-1} horseradish peroxidase at 25 °C for 30 min in the dark. The resorufin fluorescence (E_x/E_m = 530/590 nm) was quantified with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA).

Activity of antioxidant enzymes

Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was assayed according to Nali et al. (2005) by measuring the oxidation of AsA at 290 nm at 25 °C for 1 min. The reaction

mixture contained 50 mM potassium phosphate buffer (pH 6.6), 1 mM AsA, 0.4 mM Na_2EDTA and 50 μL of enzyme extract. Activity was expressed as units per milligram of protein (one unit of APX was defined as the amount of enzyme required to oxidize 1 μmol of AsA min^{-1}).

Dehydroascorbate reductase (DHAR) (EC 1.8.5.1) activity was assayed according to Kawakami et al. (2000) by measuring the production of AsA by dehydroascorbate (DHA) reduction at 265 nm at 25 °C for 1 min. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM GSH, 20 mM DHA and 30 μL of enzyme extract. Activity was expressed as units per milligram of protein (one unit of DHAR was defined as the amount of enzyme required to produce 1 μmol of AsA min^{-1}).

Glutathione reductase (GR) (EC 1.6.4.2) activity was assayed according to Gillham and Dodge (1986) by monitoring the oxidation of NADPH by oxidized glutathione (GSSG) at 30 °C for 3 min through the decrease in absorbance at 340 nm. The reaction mixture contained 400 mM potassium phosphate buffer (pH 7.5), 6.4 mM $MgCl_2$, 5.0 mM GSSG, 0.35 mM NADPH, 0.4 mM Na_2EDTA and 50 μL of enzyme extract. Activity was expressed as units per milligram of protein (one unit of GR was defined as the amount of enzyme required to oxidize 1 nmol of NADPH min^{-1}).

Total SOD (EC 1.15.1.1) activity was assayed in terms of its ability to inhibit the photochemical reduction of Nitro Blue Tetrazolium (NBT) according to Zhang and Kirkham (1994). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM NBT, 2 μM riboflavin and 20–50 μL of enzyme extract. Samples were incubated for 10 min under a fluorescent lamp (150 W, Osram R80, Milan, Italy), and absorbance at 560 nm was read against unilluminated samples. Activity was expressed as units per milligram of protein (one unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction).

For all assays, proteins were determined according to Bradford (1976), using bovine serum albumin as standard. For more details, see Supplementary Material (S2).

Non-enzymatic antioxidant compounds

Reduced ascorbate (AsA) and dehydroascorbate (DHA) content was measured spectrophotometrically according to Wang et al. (1991). Samples were frozen in liquid nitrogen, ground and then added to 6 mL of cold 5% (*w*/*v*) trichloroacetic acid. This assay is based on the reduction of ferric ions to ferrous ions with ascorbic acid in acid solution followed by the formation of the red chelate between ferrous ions and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) that absorbs at 534 nm. Supernatants were also used for the determination of the total glutathione (GSH) and GSSG content according to Sgherri and Navari-Izzo (1995). This assay is based on an enzymatic recycling procedure in which glutathione is sequentially oxidized by 5′,5′-dithiobis-2-nitrobenzoic acid and reduced by NADPH in the presence of GR. GSSG was determined after removal of GSH from the sample extract by derivatization with 2-vinilpyridine. Changes in absorbance of the reaction mixtures were measured at 412 nm and 25 °C. For more details, see Supplementary Material (S3).

Statistics

Calculations were performed with Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA, USA), R version 2.15.1 (R Foundation for Statistical Computing), and Statistica 6 (StatSoft Inc., Tulsa, OK). Non-parametric tests were applied for testing significant differences related to experimental treatments, because the data distribution of some parameters significantly deviated from normality, as tested by the Shapiro-Wilk's test. The following tests were applied: (i) Wilcoxon for paired samples, for comparison of Chl_aF data in the same group of lichen samples between two successive times; (ii) Mann-Whitney *U*, also known as Wilcoxon for non-paired samples, for comparison of Chl_aF post-exposure and post-recovery data from different groups of samples of axenically cultured *Trebouxia* sp. and (iii) Kolmogorov-Smirnov test (biochemical data). In order to disentangle the effects of the different factors considered in the exposures, generalized linear models were separately fitted for each response parameter, considering first Chl_aF levels and derived parameters, and then ROS, enzyme activity and antioxidants levels as the dependent variables. In the models, species, watering regime, air humidity and O_3 were considered as fixed effects, each with two levels; mean pre-exposure values based on all the samples were used in the GLM analysis of Chl_aF data.

Results

After microscopical observations, no visible symptoms were observed in both fumigated and non-fumigated materials, regardless of water regimes and RHs.

Chl_aF measurements

The patterns of F_v/F_m variation were quite similar in the two species, although the median values slightly differed already in the pre-exposure measurements (*P. perlatum* 0.665 ± 0.030, $n = 46$; *X. parietina* 0.700 ± 0.023, $n = 48$) as a result of the different habitats and the climatic conditions in the days immediately before sampling.

The GLM analysis (Table 2) revealed significant first-order effects on F_v/F_m, with a stronger effect for those factors related to the thallus water status, i.e. the watering regime ($F = 47.609$, $p = 1.1 \times 10^{-9}$) and RH (F = 18.94,

Table 2 Values of the *F* statistics from the general linear mixed model (GLM) testing for main and interactive effects of species (*Parmotrema perlatum/Xanthoria parietina*: P vs. X), air relative humidity (20/80% RH: 20 vs. 80), watering (no/yes: 0 vs. 1) and O_3 treatment (no/yes: 0 vs. 1) on the chlorophyll *a* fluorescence-derived parameters (Par.s, top) and levels (bottom) measured in lichen samples after a 2-week exposure

	Species		RH		WR		O_3		Sp. × RH	Sp. × WR	Sp. × O_3	RH × WR	RH × O_3	WR × O_3
	P vs. X		20 vs. 80		0 vs. 1		0 vs. 1							
Derived Par.s														
F_v/F_m	*7.05*	>	**18.94**	>	**47.60**	>	*7.70*	>	**34.11**	–	–	–	–	–
NPQ	**24.74**	>	**31.17**	>	–		4.02	>	**41.25**	–	–	–	–	5.21
qP	–		**27.60**	>	*8.36*	>	–		–	–	–	–	–	–
qN	5.02	>	**25.30**	>	–		4.03	>	**25.87**	–	–	–	–	–
ETR	–		4.62	>	–		*7.56*	>	–	5.10	–	–	–	–
Levels														
F_0	**18.01**	>	4.34	>	–		5.26	>	**41.86**	–	–	–	–	–
F_m	4.84	>	**22.38**	>	**38.71**	>	**15.51**	>	–	–	–	–	–	–
F_0'	–		**16.36**	>	6.01	>	–		–	*9.24*	–	–	–	–

The symbol > indicates the level of each factor having the highest value of that specific variable (acronyms as in the main text). Significant values are printed in bold ($p < 0.001$), italic ($0.001 \leq p < 0.01$) or normal ($0.01 \leq p < 0.05$) character

$p = 3.9 \times 10^{-5}$). Actually, the F_v/F_m decrease was the highest in the dry samples, at both RHs (Figs. 1 and 2). The factor "species" was the least important ($F = 7.05$, $p = 9.5 \times 10^{-3}$), whereas the effect of O_3 was slightly more pronounced in

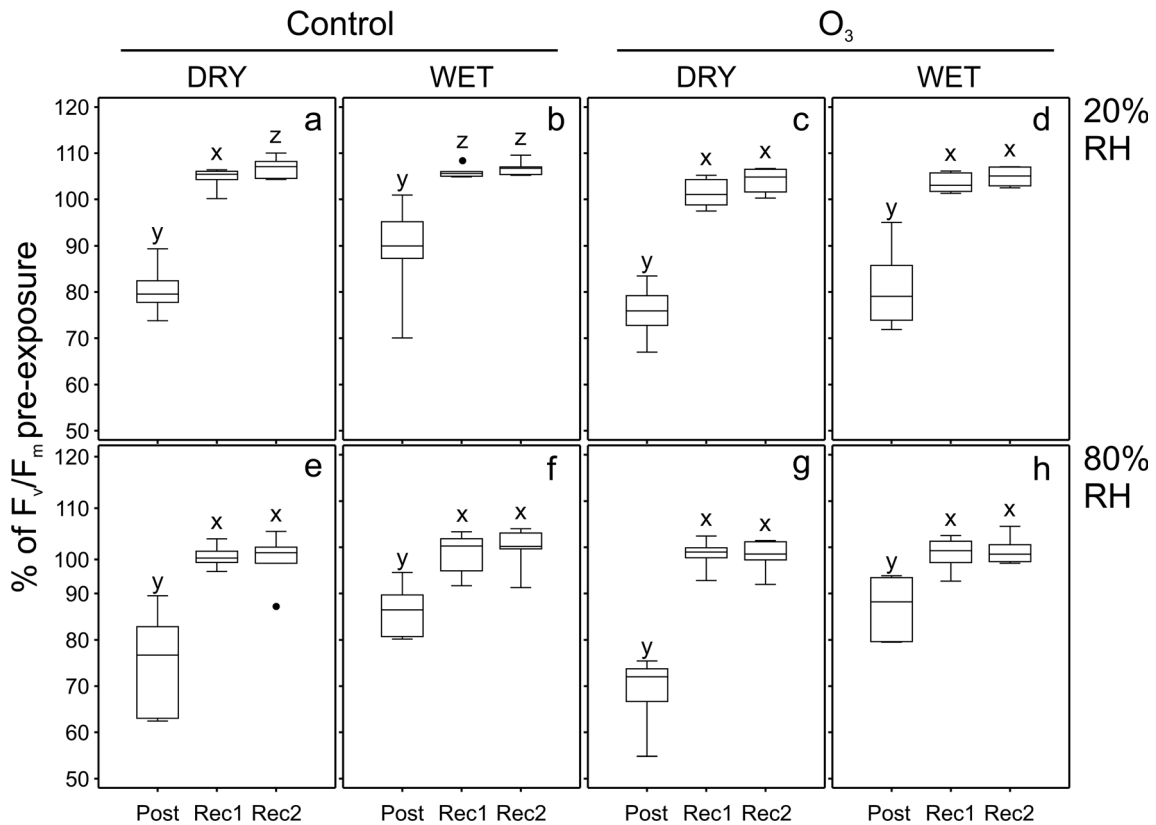

Fig. 1 Percent variation of F_v/F_m after a 2-week exposure (Post) and after a 1-day (Rec1) and 2-day (Rec2) recovery with respect to pre-exposure values measured in samples of the lichen *Parmotrema perlatum* exposed at 20% (**a**–**d**) and 80% (**e**–**h**) RH to filtered air (control) (**a**, **b**, **e**, **f**) and to O_3 (250 ppb, 5 h day^{-1}) (**c**, **d**, **g**, **h**), under DRY (**a**, **c**, **e**, **g**) and WET (**b**, **d**, **f**, **h**) condition. For each boxplot median, 25°–75° percentiles (*boxes*), non-outlier minimum and maximum (*whiskers*) and outlier (*dots*) are reported; the *same letters* near the *boxes* indicate no statistically significant difference at $p \leq 0.05$ by Wilcoxon paired test (comparison between two successive times; x = no difference with pre-exposure values); $n = 6$

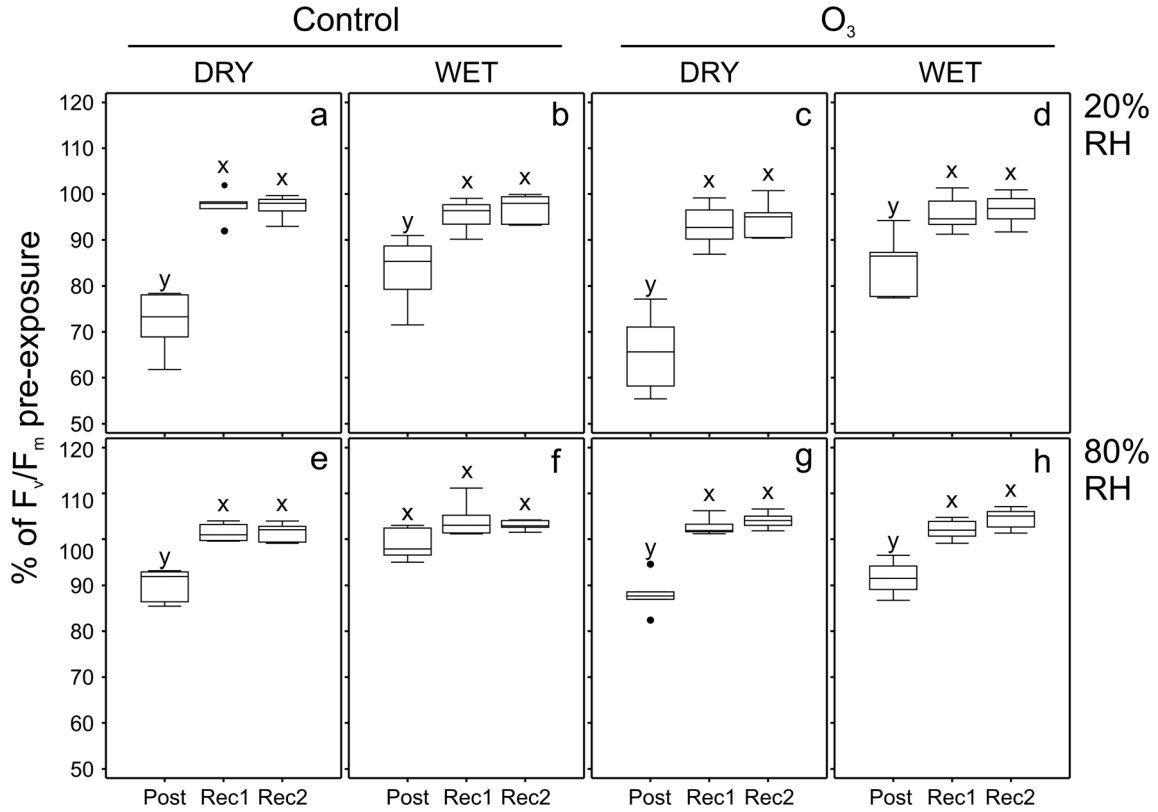

Fig. 2 Percent variation of F_v/F_m after a 2-week exposure (Post) and after a 1-day (Rec1) and 2-day (Rec2) recovery with respect to pre-exposure values measured in samples of the lichen *Xanthoria parietina* exposed at 20% (**a**–**d**) and 80% (**e**–**h**) RH to filtered air (control) (**a**, **b**, **e**, **f**) and to O_3 (250 ppb, 5 h day^{-1}) (**c**, **d**, **g**, **h**), under DRY (**a**, **c**, **e**, **g**) and WET (**b**, **d**, **f**, **h**) condition. For each boxplot median, 25°–75° percentiles (*boxes*), non-outlier minimum and maximum (*whiskers*) and outlier (*dots*) are reported; the *same letters* near the *boxes* indicate no statistically significant difference at $p \leq 0.05$ by Wilcoxon paired test (comparison between two successive times; x = no difference with pre-exposure values); $n = 6$

P. perlatum (SI 4). Also, RH had a different effect on the species, because F_v/F_m did not change significantly between 20 and 80% RH in *P. perlatum*, whereas it was lower at 20% than at 80% RH in *X. parietina* (SI 4). Interestingly, the sole statistically significant interactive effect was that of "species × air humidity" (Table 2), in good agreement with the different degree of hygrophytism of the two species (Table 1).

Among the fluorescence levels and the other derived parameters, F_m was positively affected by watering and negatively by O_3 (Table 2).

Two days at optimal conditions allowed the total recovery of the pre-exposure F_v/F_m values (Figs. 1 and 2), when not a slight increase (see e.g. *P. perlatum* control, dry and wet, at 20% RH), with a slight difference between the species, possibly related to the different environmental conditions at the sampling sites, since *P. perlatum* could increase F_v/F_m more than the relatively stable *X. parietina*.

The behaviour of the axenically grown photobiont of *P. perlatum* was basically the same of the lichenized one (Fig. 3), but the post-exposure decrease of F_v/F_m was less intense. A clear effect of O_3 was observed only at 80% RH, with a significant, pronounced decrease (−16%) of F_v/F_m. This difference persisted after recovery.

ROS quantification

The concentration values of the two ROS considered in this study are reported in Table 3. $O_2^{\bullet-}$ and H_2O_2 were differently affected by the experiments. The factor "species" was highly influential for $O_2^{\bullet-}$ ($F = 114.64$, $p = 3.5 \times 10^{-17}$), but not for H_2O_2 (Table 4), since the former parameter was always higher in *P. perlatum*, particularly when the samples were exposed to low air humidity (Table 3).

RH actually affected both ROS but in an opposite way, with $O_2^{\bullet-}$ being significantly higher at 20% than at 80% RH; $O_2^{\bullet-}$ was also subjected to almost all the possible interactive effects (Table 4).

The water regime (WR) did not affect the content of the two ROS whereas O_3 affected ($F = 91.42$, $p = 6.2 \times 10^{-15}$) H_2O_2 concentration in both lichens. O_3-exposed samples, in fact, had higher H_2O_2 concentrations than controls, whereas they remained stable in dependence to the other tested factors.

Activity of antioxidant enzymes

A strong species-specific difference was detected in the activity of ROS-scavenging enzymes (Table 5), since this was

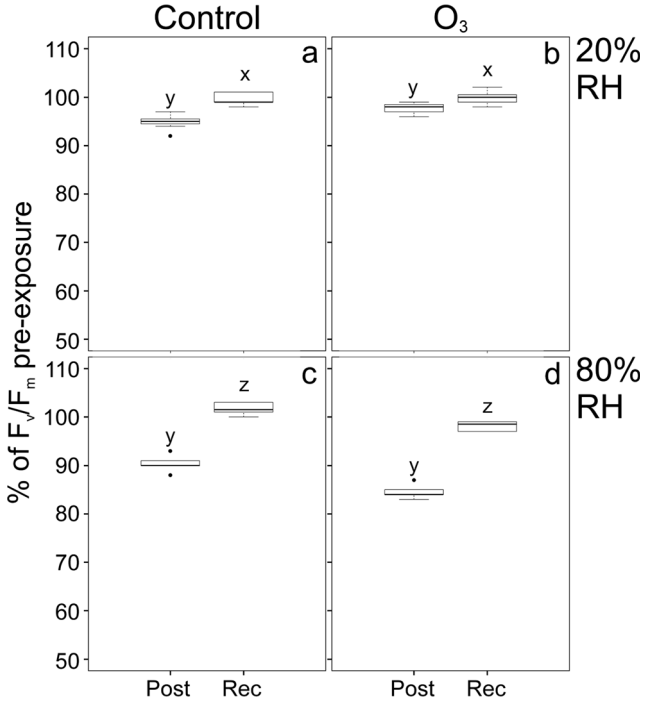

Fig. 3 Percent variation of normalized F_v/F_m values after a 2-week exposure (Post) and after a 2-day (Rec) recovery with respect to pre-exposure values measured in axenically cultured *Trebouxia* sp. exposed at 20% (**a**, **b**) and 80% (**c**, **d**) RH to filtered air (control) (**a**, **c**) and to O_3 (250 ppb, 5 h day^{-1}) (**b**, **d**). For each boxplot median, 25°–75° percentiles (*boxes*), non-outlier minimum and maximum (*whiskers*) and outlier (*dots*) are reported; the *same letters* near the *boxes* indicate no statistically significant difference at $p \leq 0.05$ by Mann-Whitney *U* test (comparison between two successive times; x = no difference with pre-exposure values); $n = 6$

always higher in *P. perlatum*, particularly for DHAR and SOD, up to fivefold in the former, and from twofold to fourfold in the latter (Table 5).

The enzymatic activity was also strongly influenced by water availability during exposure, being generally higher in samples exposed to 80% RH and in the watered ones. By contrast, only DHAR was higher in the 20% RH-exposed samples. O_3 had a more limited, stimulating influence on SOD, APX and DHAR, not on GR (Table 4).

Non-enzymatic antioxidant compounds

The antioxidants, whose concentration values are reported in Table 6, responded differently to the four selected factors, with "species" and "RH" being certainly the most relevant (Table 4). The two species had significant different contents of antioxidant compounds: AsA and DHA were higher in *X. parietina* whereas GSH and GSSG were higher in *P. perlatum* (Table 6).

The effect of RH was particularly significant, since 80% RH compared with 20% RH induced a significant reduction of AsA, GSSG and GSH, and derived parameters AsA + DHA, AsA/DHA and GSH + GSSG. By contrast, the watering regime did not modify the content of these antioxidants (except GSH).

O_3 was highly influential for the couplet GSH-GSSG ($F = 181.30$, $p = 2.3 \times 10^{-22}$ for the latter) and for their total content (but not for their ratio), whereas it was totally uninfluential for the couplet AsA-DHA (Table 4): O_3-exposed samples showed significant differences in GSH content in six of the eight direct comparisons with the respective controls. Interestingly, GSH content was reduced in O_3-exposed *P. perlatum* samples, and increased in *X. parietina* ones, whereas GSSG content (Table 6) increased in seven of the eight O_3-exposed groups of samples with respect to the respective controls.

Table 3 Quantification of $O_2^{\bullet-}$ (nmol $min\ g^{-1}$ DW) and H_2O_2 (nmol g^{-1} DW) in *Parmotrema perlatum* and *Xanthoria parietina* samples under dry and wet conditions for 2 weeks, exposed to filtered air (control) or to ozone (250 ppb, 5 h day^{-1}) at 20 and 80% RH

	20% RH		80% RH	
	$O_2^{\bullet-}$	H_2O_2	$O_2^{\bullet-}$	H_2O_2
		Parmotrema perlatum		
Control dry	0.45 ± 0.05	0.32 ± 0.05	0.39 ± 0.02	0.33 ± 0.03
Control wet	0.65 ± 0.15a	0.32 ± 0.06	0.36 ± 0.04b	0.34 ± 0.04
Ozone dry	0.68 ± 0.08c	0.38 ± 0.03	0.36 ± 0.03b	0.45 ± 0.01bc
Ozone wet	1.00 ± 0.13ac	0.39 ± 0.02	0.37 ± 0.03b	0.40 ± 0.02a
		Xanthoria parietina		
Control dry	0.39 ± 0.08	0.26 ± 0.01d	0.30 ± 0.03d	0.34 ± 0.03b
Control wet	0.35 ± 0.09d	0.30 ± 0.01a	0.23 ± 0.03ad	0.38 ± 0.05b
Ozone dry	0.32 ± 0.02d	0.46 ± 0.01cd	0.28 ± 0.04	0.39 ± 0.03bd
Ozone wet	0.22 ± 0.03acd	0.42 ± 0.03c	0.22 ± 0.04d	0.41 ± 0.01

Data are shown as mean ($n = 3$) ± standard deviation. Different lowercase letters (in rows and in columns) are indicative of significant differences among watering regime (a), relative humidity (b), O_3 treatment (c) and species (d) ($P \leq 0.05$; Kolmogorov-Smirnov test)

Table 4 Values of the *F* statistics from the general linear mixed model (GLM) testing for main and interactive effects of species (*Parmotrema perlatum*/*Xanthoria parietina*: P vs. X), air relative humidity (20/80% RH: 20 vs. 80), watering (no/yes: 0 vs. 1) and O_3 treatment (no/yes: 0 vs. 1) on ROS, ROS scavenging enzymes and antioxidants (acronyms as in the main text) measured in lichen samples after a 2-week exposure

	Species		RH		WR		O_3		Sp. × RH	Sp. × WR	Sp. × O_3	RH × WR	RH × O_3	WR × O_3
	P vs. X		20 vs. 80		0 vs. 1		0 vs. 1							
ROS														
$O_2^{\cdot-}$	**114.641**	>	**73.55**	>	–		–		**32.33**	**17.78**	**18.62**	*8.93*	5.47	–
H_2O_2	–		7.10	>	–		**91.42**	>	–	–	–	–	*7.15*	5.45
Enzyme activity														
APX	**90.56**	>	**66.28**	>	*11.51*	>	**17.81**	>	*10.62*	–	*10.78*	–	–	4.24
DHAR	**1096.79**	>	**265.66**	>	**20.04**	>	**12.63**	>	**103.72**	**23.06**	–	4.32	–	–
GR	**14.15**	>	**82.81**	>	**120.91**	>	–		**16.59**	–	4.71	5.21	–	**17.05**
SOD	**311.09**	>	6.94	>	**50.10**	>	**31.76**	>	*8.98*	*17.24*	5.65	*9.01*	–	–
Antioxidants														
AsA	**171.52**	>	**144.42**	>	–		–		**36.40**	4.45	–	–	–	–
DHA	**1030.07**	>	–		–		–		6.67	–	–	–	–	–
AsA+DHA	**927.28**	>	*11.12*	>	–		–		*8.82*	–	–	–	–	–
AsA/DHA	**240.17**	>	**103.75**	>	–		–		–	**17.75**	–	–	–	–
GSH	*7.33*	>	**18.13**	>	**12.21**	>	*11.85*	>	**91.43**	4.37	**168.60**	–	**72.46**	–
GSSG	**234.12**	>	**159.95**	>	–		**181.30**	>	**148.69**	–	–	*9.91*	**23.45**	–
GSH+GSSG	**31.62**	>	**118.82**	>	*10.53*	>	**98.46**	>	**210.01**	6.38	**79.83**	*8.91*	4.67	–
GSH/GSSG	**75.45**	>	–		–		–		**20.04**	–	**61.82**	–	**37.35**	–

The symbol > indicates the level of each factor having the highest value of that specific variable. Significant values are printed in bold ($p < 0.001$), italic ($0.001 \leq p < 0.01$) or normal ($0.01 \leq p < 0.05$) character

Table 5 Ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and superoxide dismutase (SOD) activity (unit mg^{-1} protein) in *Parmotrema perlatum* and *Xanthoria parietina* samples kept under dry and wet conditions for 2 weeks and exposed to filtered air (control) or to O_3 (250 ppb, 5 h day^{-1}) at 20 and 80% RH

	20% RH				80% RH			
	APX	DHAR	GR	SOD	APX	DHAR	GR	SOD
				Parmotrema perlatum				
Control dry	47.8 ± 4.5	163.2 ± 8.2	86.9 ± 7.3	1264 ± 199	70.1 ± 10.7b	73.7 ± 11.4b	96.3 ± 11.1	1815 ± 425
Control wet	77.5 ± 14.4a	182.7 ± 2.5a	100.6 ± 11.0	796 ± 144a	99.6 ± 10.6a	86.8 ± 5.5b	106.4 ± 6.4	1045 ± 45a
Ozone dry	85.6 ± 11.8c	168.6 ± 23.9	74.6 ± 1.9c	1837 ± 493	122.0 ± 20.8c	70.0 ± 5.3b	86.1 ± 10.6	2203 ± 468
Ozone wet	101.9 ± 3.9c	181.7 ± 11.2	104.5 ± 6.0a	1295 ± 237c	87.4 ± 4.7ab	138.8 ± 11.1abc	117.9 ± 7.3a	1416 ± 252ac
				Xanthoria parietina				
Control dry	35.2 ± 0.8d	33.9 ± 6.5d	66.9 ± 6.4d	363 ± 70d	62.6 ± 2.5b	20.1 ± 4.0bd	88.8 ± 5.3b	451 ± 98d
Control wet	25.4 ± 4.6ad	31.4 ± 6.2d	83.0 ± 2.6a	458 ± 16d	87.1 ± 2.6ab	20.8 ± 1.1bd	103.9 ± 3.3ab	160 ± 24abd
Ozone dry	31.3 ± 4.3d	48.4 ± 6.6cd	71.8 ± 3.0	520 ± 93d	62.0 ± 3.8bd	23.4 ± 1.9bd	83.6 ± 3.3b	804 ± 135bcd
Ozone wet	47.3 ± 6.3acd	47.9 ± 3.0cd	82.0 ± 2.5ad	504 ± 51d	82.4 ± 4.7ab	21.7 ± 1.3bd	132.9 ± 7.3abc	348 ± 90acd

Data are shown as mean ($n = 3$) ± standard deviation. Different lowercase letters (in rows and in columns) are indicative of significant differences between watering regime (a), relative humidity (b), O_3 treatment (c) and species (d) ($P < 0.05$; Kolmogorov-Smirnov test). One unit of APX and DHAR was defined as the amount of enzyme required to oxidize or reduce 1 μmol of AsA min^{-1}, respectively. One unit of GR was defined as the amount of enzyme required to oxidize 1 nmol of NADPH min^{-1}. One unit of SOD activity was defined as the amount of the enzyme required to cause 50% inhibition of the rate of NBT reduction

Table 6 Content of ascorbate (AsA), dehydroascorbate (DHA) (μmol g^{-1} DW) and reduced (GSH) and oxidized glutathione (GSSG) (μmol g^{-1} DW) in *Parmotrema perlatum* and *Xanthoria parietina* samples maintained under dry and wet conditions for 2 weeks, exposed to filtered air (control) or to O_3 (250 ppb, 5 h day^{-1}) at 20 and 80% RH

	20% RH				80% RH			
	AsA	DHA	GSH	GSSG	AsA	DHA	GSH	GSSG
				Parmotrema perlatum				
Control dry	2.8 ± 0.4	10.1 ± 0.4	1.92 ± 0.37	1.90 ± 0.17	2.1 ± 0.2b	10.8 ± 0.1	0.38 ± 0.07b	1.06 ± 0.04b
Control wet	2.4 ± 0.1	11.4 ± 0.6a	1.85 ± 0.19	1.99 ± 0.16	2.1 ± 0.1	11.3 ± 0.6	0.70 ± 0.34b	1.27 ± 0.02ab
Ozone dry	2.4 ± 0.1	10.4 ± 0.5	0.74 ± 0.07c	3.34 ± 0.19c	2.1 ± 0.1b	10.5 ± 0.5	0.42 ± 0.26	1.02 ± 0.09b
Ozone wet	2.3 ± 0.4	10.6 ± 0.6	0.89 ± 0.09c	2.86 ± 0.12ac	2.1 ± 0.3	10.6 ± 0.7	0.38 ± 0.13b	1.45 ± 0.05abc
				Xanthoria parietina				
Control dry	3.4 ± 0.1	22.3 ± 1.8d	0.50 ± 0.12d	0.68 ± 0.08d	2.7 ± 0.2bd	20.4 ± 2.3d	0.51 ± 0.04	0.63 ± 0.06d
Control wet	3.7 ± 0.1ad	20.7 ± 0.2d	0.91 ± 0.08ad	0.63 ± 0.02d	2.5 ± 0.2b	19.6 ± 2.2d	0.33 ± 0.08ab	0.59 ± 0.07d
Ozone dry	3.7 ± 0.3d	22.2 ± 0.9d	0.99 ± 0.02cd	1.44 ± 0.04cd	2.3 ± 0.1bcd	19.6 ± 0.5abd	1.62 ± 0.52bcd	1.30 ± 0.03bcd
Ozone wet	3.9 ± 0.4d	21.0 ± 2.1d	1.26 ± 0.08acd	1.47 ± 0.06cd	2.7 ± 0.3b	21.0 ± 1.5d	2.54 ± 0.11abcd	1.61 ± 0.15ac

Data are shown as mean ($n = 3$) ± standard deviation. Different lowercase letters (in row and in column) are indicative of significant differences between watering regime (a), relative humidity (b), ozone treatment (c) and species (d) ($P \leq 0.05$; Kolmogorov-Smirnov test)

Interactions between factors were frequently highly significant for the couplet GSH-GSSG, only sporadically for the couplet AsA-DHA.

Discussion

In our hypothesis, the exposure of lichens to O_3 should actually imply just a slight intensification of the overall oxidative load associated to the last phases of dehydration, and first phases of rehydration, with scarce effects in terms of vitality loss (Tretiach et al. 2012a). Therefore, in this study two lichens with different ecology, as well as the cultured photobiont of one of them, were exposed to high levels of O_3 at contrasting air humidity values and watering regimes.

A matter of species

Contrary to the current opinion of most authors, Kranner (2002) demonstrated that hygrophilous and relatively sciaphilous lichens possess comparable or even larger pools of antioxidants than lichens of dry, sun-exposed environments. The two species used in our experiments were selected in order to compare lichens with different ecology, the hygrophilous *P. perlatum* and the meso-xerophilous *X. parietina*. We could demonstrate that (a) the two lichens have a different protection strategy against oxidative stress, and (b) each of them is characterized by its own peculiar ROS (especially $O_2^{\bullet-}$) metabolism. *Xanthoria parietina* has a larger pool of AsA/DHA that directly and efficiently participates in free radical detoxification, with a less remarkable generation of $O_2^{\bullet-}$. Since the ascorbate and glutathione pools may respond to perturbation in a compensatory manner, *X. parietina* has a higher concentration of GSH in comparison to *P. perlatum*, which conversely has a higher content of the oxidized form, GSSG. Interestingly, the values of GSH and GSSG measured in *X. parietina* daily watered and kept in filtered air at 80% RH were similar to those reported by Silberstein et al. (1996) for material collected in unpolluted sites of Israel.

Parmotrema perlatum revealed a bigger pool of ROS scavenging enzymes as assessed by the higher enzymatic activity for SOD, DHAR and APX, and this agrees with the fact that *P. perlatum* was certainly more responsive than *X. parietina* in terms of both ROS formation and modification of antioxidant levels. It would be interesting to check whether the two species differ in the capability of recovery the intracellular reduction power and enzymatic activity upon rehydration. In fact, Kranner (2002) and Kranner et al. (2008) substantiated a significant difference in this sense between hygrophilous and xerophilous lichens, as first observed by Mayaba and Beckett (2001).

Finally, it is worth noting that in both lichens, GSSG of wet controls at 80% RH equalled that of GSH. In vascular plants under optimal conditions, the GSH pool (and that of AsA as well) is highly reduced and the accumulation of GSSG is often correlated to increased stress (Foyer and Noctor 2011). In lichens, however, GSSG is useful, because it is used to form PSSG, thereby protecting free –SH residues in proteins from irreversible auto-oxidation during the tricky phase of cell dehydration (Kranner and Grill 1996).

Watering regime and air relative humidity

Among the three factors considered in this study as potentially influencing the examined parameters, the change of the cell water status strongly affected the content of selected ROS and antioxidants, and the activity of specific ROS scavenging enzymes (Table 6), i.e. it influenced the most important

components of the intracellular redox state (Cruz de Carvalho et al. 2012) of the two lichens. Interestingly, $O_2^{\bullet-}$ (but not H_2O_2) generation was clearly influenced by air humidity, probably because the hydration of the cytoplasm derived from air water vapour plays an important role in the activation of the antioxidant system. At equilibrium with 80% RH, most *Trebouxia*-containing lichens are metabolically active (Lange and Kilian 1985; Lange et al. 1986), whereas at 20% RH metabolic reactions are unlikely to occur (Candotto Carniel, unpubl. results). The direct demonstration of this phenomenon is that at 20% RH, AsA and GSH levels were always higher than at 80% RH. The vitrification of the cytoplasm evidently reduces the consumption of these antioxidants, which are free to react with the respective ROS species at 80% RH.

Contrary to air humidity, the watering regime did not significantly influence the content of the metabolites linked to the AsA-GSH cycle, but at the same time it markedly influenced the activity of the enzymes involved in the redox homeostasis, although not certainly through the over-expression of the corresponding genes, as demonstrated in a recent transcriptomic study on *Trebouxia gelatinosa* (Candotto Carniel et al. 2016). APX, DHAR and GR activity of watered samples was higher in comparison to non-watered ones indicating that these general "housekeeping" protectants may contribute to the tolerance against oxidative stress preventing H_2O_2-mediated damage. It should be underlined that in our experimental design, the daily watering to which the wet samples were subjected was intended as a rhythmic interruption of an otherwise unnatural period of dryness. The samples for the biochemical assays, however, were always processed when air-dry. In other words, we did not compare metabolically active vs. metabolically inactive samples, but samples that had been without liquid water for, respectively, 14 days and one single day. When watered, the samples were metabolically active for several hours per day (see Bertuzzi et al. 2013), and theoretically they could increase the stock of antioxidants required to cope with the unpredictable but certainly incipient cycle of dehydration-rehydration. We cannot exclude that small changes actually occurred, but were obscured by the relatively large variability typical of lichen samples.

Interestingly, also in this case the factor "species" showed the highest number of significant combinations with the factor "air humidity" and "watering", with the former certainly prevailing on the latter (18 against 7 combinations, see Tables 2 and 4). This is an indirect confirmation that the two species have different water requirements, which can explain at least in part their different ecology and distribution.

Ozone

O_3 had scarce effects on Chl_aF (see *infra*), but it caused specific effects on (i) H_2O_2 formation and (ii) activity of the antioxidant machinery. However, the limited number of cases in which O_3 influenced the concentration of H_2O_2 confirmed that the equilibrium between production and scavenging rates was not drastically altered by O_3 exposure. The modified APX activity observed in the O_3-exposed samples confirms that this enzyme may function as a regulator of ROS production, although this hypothesis is not corroborated by a corresponding modification of AsA or DHA content in O_3-treated thalli. Perhaps the quantity of AsA was probably sufficient for maintaining the increased APX activity. The availability and the redox state of AsA generally correlates with O_3 sensitivity in many vascular plants (Vainonen and Kangasjärvi 2015), and it is known that species and/or cultivars with high AsA concentrations are O_3-tolerant (Conklin and Barth 2004). Similar findings were reported by Pellegrini et al. (2014) for the lichen *F. caperata*, suggesting that the AsA content is a relevant trait involved in the oxidative stress tolerance also in lichens.

The oxidative perturbation of the GSH pools has been well documented in vascular plants exposed to O_3 (Vainonen and Kangasjärvi 2015). Most data suggest that enhanced ROS evolution has less impact on the AsA/DHA ratio than on the GSH/GSSG one (Foyer and Noctor 2011). In our target species, however, O_3 influenced differently the GSH-GSSG cycle. In *P. perlatum*, it caused a decrease in the GSH content with the concomitant formation of GSSG, whereas in *X. parietina* it caused a GSH increase, more pronounced at 80% RH than at 20% RH. This difference might be due to the different species ecology. Moreover, it is not surprising that the levels of GSSG are equal to or greater than the levels of GSH: similar amounts of GSSG and GSH were found by Vráblíková et al. (2005), while Kranner et al. (2005) suggested that the GSH/GSSG ratio tends to decrease in samples under stress. A moderate "chamber effect", already identified e.g. by Bertuzzi et al. (2013) in lichens exposed in open top chambers, could be responsible for the slight decrease of this ratio as well as to a low value of the AsA/DHA ratio.

Lichenized vs. isolated *Trebouxia* photobionts

In the first study carried out on isolated vs. lichenized *Trebouxia*, Rosentreter and Ahmadjian (1977) identified only minor effects induced by 0.1 ppm O_3 for 1 week, and therefore they defined the investigated photobiont as O_3-tolerant. In our case, the axenically grown algae of *P. perlatum* showed a pattern similar to the lichenized ones, but with two noteworthy differences: (i) when exposed to O_3 at 80% RH, they suffered a significant—although not severe—reduction of F_v/F_m, and (ii) they were faster than the lichenized ones to recover the original F_v/F_m value.

Several works suggested that lichenization increases the antioxidant and photoprotective mechanisms, particularly in favour of the photobiont (Kranner et al. 2005; Kosugi et al. 2009, 2013), and at the same time in this study we circumstantiated the importance of the ROS scavenging system for

the lichen O_3 tolerance. Therefore, it can reasonably be deduced that the decrease in F_v/F_m observed caused by O_3 can be due to a less efficient antioxidant system of the isolated algae. By contrast, the apparently better performance of the algal cultures in terms of recovery time after the O_3 exposure is to refer to the speed of rehydration, certainly faster in the cultured algae than in the lichenized ones, due to the inertia offered by the predominant mass of the mycobiont.

Modulated chlorophyll *a* fluorescence as a tool to study the effects of ozone

Chl_aF has often been used to study the effects on lichens of several gaseous pollutants, being a signal of the functionality of the photosynthetic process. The most frequently used parameter is the maximum quantum yield of primary photochemistry in dark adapted samples, F_v/F_m, which is temperature-independent and is an ideal tool for checking the vitality of photosynthetic organisms (Jensen 2002). For instance, SO_2 causes a decrease of F_v/F_m before any other signs of injury can be detected (Gries et al. 1995), whereas H_2S causes an even faster reduction of F_v/F_m, due to the rapid block of the oxygen-evolving complex associated to PS II (Bertuzzi and Tretiach 2013). When used to characterize the effects of O_3, F_v/F_m offered less clear-cut results (Scheidegger and Schroeter 1995; Riddell et al. 2010, 2012; Bertuzzi et al. 2013). The latter authors concluded that O_3 has no effect on lichen photosynthesis, since the decrease in F_v/F_m was relatively small, and possibly obscured by the influence of other factors. Actually, F_v/F_m is sensitive not only to phytotoxic molecules (pollutants, biocides, etc.) but also to environmental factors, the most important being light and water status of the thallus (Calatayud et al. 1997; Barták et al. 2015).

In our study, all Chl_aF levels and derived parameters were largely species-specific (Table 2). This agrees with the assumption that lichens from different light-regime habitats differ in their Chl_aF fingerprint (Piccotto and Tretiach 2010), and thus in their response to a prolonged light exposure, although the light regime applied to our species was voluntarily differentiated according to their respective optimum. In general, the low-light-adapted *P. perlatum* had considerably higher fluorescence levels (F_o, F_m) than the sun-loving *X. parietina*, showing an optimisation of light absorption and a decreased photosynthetic quantum conversion, as observed by Piccotto and Tretiach (2010) in several low-light vs. high-light lichens. Since our exposure lasted 2 weeks, this also suggests that photo-acclimation phenomena were certainly slowed down at the artificial conditions of our experiment.

Air humidity and watering had a strong impact on the Chl_aF response, but in a contrasting way. Thus, samples exposed to 80% RH had higher F_v/F_m, NPQ, qN, and ETR values than samples exposed to 20% RH (SI4), suggesting that the photosynthetic as well as the quenching machineries were active. This was recently confirmed by an unpublished study on the chlorolichen *F. caperata*, which demonstrated that the xanthophyll cycle—to which NPQ is strictly related—is fully active when the samples are in equilibrium at 80% RH, whereas it progressively ceases to work between 55 and 35% RH. Conversely, the daily treatment with liquid water, mimicking the daily load eventually provided by pre-dawn dew, basically increased F_m, the transient maximum emission yield of dark-adapted samples during a single saturation light pulse (SI4). This phenomenon was already observed by Tretiach et al. (2012b) in natural populations of the lichen *Flavoparmelia soredians* subjected to an artificial cycle of watering at different time-lapses during the day. Apparently, liquid water reinstates a quencher-free situation, with the deactivation of energy-dissipative mechanisms through heath emission. This dissipation is caused by a long-wavelength quencher embedded in the antenna complex of PS II (Heber et al. 2000), which is related to the characteristic F740-nm fluorescence band and works as an energy-collecting structure or as an energy-dissipating one, depending on the structural changes of the antenna complex determined by the water status (Kosugi et al. 2013; Veerman et al. 2007). A further and more efficient quenching process (providing more than 70% of the total PS II quenching) is shown to involve an efficient transfer of excitation energy from PS II to PS I, which is then efficiently quenched due to the formation of a long-lived P700+ state in the dried state in the light (Slavov et al. 2013).

With these premises, the effects of O_3 on Chl_aF levels and parameters observed in this study were relatively small, but still significant (Table 2 and SI5): F_m and F_v/F_m (both species) and ETR (*P. perlatum* only) were lower in O_3-exposed samples than in the respective controls; on the contrary, NPQ and qN increased in O_3-exposed samples of *X. parietina* with respect to the corresponding controls and to the pre-exposure values, whereas it decreased in all samples of *P. perlatum* after the exposure experiment, but to a lesser extent in the O_3-exposed samples than in the control samples (SI4, 5). These data suggest that the differences between the two species are relatively small in terms of the answer to this pollutant, with *P. perlatum* being slightly less tolerant than *X. parietina*, for partial impairment of the redox chain (see ETR data) and minor flexibility in using non-photochemical quenching (a phenomenon already hypothesized for this species; see Piccotto and Tretiach 2010).

The change occurring in all parameters connected with the controlled dissipation (F_m, F_v/F_m, NPQ etc.) can be considered as the result of a regulation process which acclimates the intensity of electron flux to the needs of reduced net photosynthesis in stressful conditions (Bussotti et al. 2011). The increase of NPQ and qN and the decrease of F_m, F_v/F_m and ETR, observed also in many vascular plants (Carrasco-Rodriguez and del Valle-Tascon 2001; Loreto et al. 2001; Shavnin et al. 1999), are fully congruent with the need of diversion of excitation energy from the photochemical to the

non-photochemical quenching (Krause and Jahns 2004), possibly for damage to the thylakoid membranes. This might imply a possible reduced production of ATP and NADPH, with successive impairment of primary production and growth. In vascular plants, this factor is particularly well-studied (Ciompi et al. 1997; Ojanperä et al. 1998), since it influences the dynamics of foliar demography, which eventually allows a sensitive plant to retain an effective photosynthetic apparatus at all times (Bussotti et al. 2011). In lichens, none of these factors has been investigated so far in connection to O_3 pollution, for the difficulties to carry out growth experiments in controlled conditions over long periods of time.

Conclusions

Our results confirm that lichens are O_3-tolerant basically because this pollutant does not drastically impair the efficient antioxidant machinery that allows lichens to behave as poikilohydric organisms. The effects of O_3 on the two lichens were comparatively negligible, but for the first time we could document the perturbation of selected Chl_aF parameters, and significant changes in the content of H_2O_2, GSH and GSSG, but not that of $O_2^{\cdot-}$ and AsA + DHA. However, most of the examined parameters were more heavily affected by water availability, and particularly by air humidity. A clear species-specific trend was observed, with the hygrophilous *P. perlatum* being more reactive than the mesoxerophilous *X. parietina* to the experimental conditions applied in the experiments. Conversely, minimal differences were observed in the behaviour of axenically grown cultures of the photobiont of *P. perlatum*, in comparison to the lichenized form, when both were exposed to the pollutant. There are no doubts, however, that we need much more work at the biochemical level on the isolated mycobionts and photobionts in order to better understand the interplay of the two organisms and the role of lichenization in the protection mechanisms against oxidative stress.

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Author contribution All authors took part to the experimental design development. EP and SB performed, respectively, the biochemical analysis and the Chl_aF measurements, whereas EP, SB, FCC and GI performed the data analysis. MT, SB, EP, FCC and GI wrote the manuscript. GL, CN and MT are the project supervisors: they contributed actively with their expertise in each step of the work, and co-edited the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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