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# Ozone tolerance in lichens: A possible explanation from biochemical to physiological level using *Flavoparmelia caperata* as test organism

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SUMMARY

Lichens are among the best biomonitors of airborne pollutants, but surprisingly they reveal high tolerance to ozone ($O_3$). It was recently suggested that this might be due to the high levels of natural defences against oxidative stress, related to their poikilohydric life strategy. The objective of this work is to give a thorough description of the biochemical and physiological mechanisms that are at the basis of the $O_3$-tolerance of lichens. Chlorophyll *a* fluorescence ($Chl_aF$) emission, histochemical ROS localization in the lichen thallus, and biochemical markers [enzymes and antioxidants involved in the ascorbate/glutathione (AsA/GSH) cycle; hydrogen peroxide ($H_2O_2$) and superoxide anion ($O_2^{\bullet -}$)] were used to characterize the response of the epiphytic lichen *Flavoparmelia caperata* (L.) Hale exposed to $O_3$ (250 ppb, 5 h $d^{-1}$, 2 weeks) at different watering regimes and air relative humidity (RH) in a fumigation chamber. After two-week exposure $Chl_aF$ was affected by the watering regime but not by $O_3$. The watering regime influenced also the superoxide dismutase activity and the production of ROS. By contrast $O_3$ strongly influenced the AsA/GSH biochemical pathway, decreasing the reduced ascorbate (AsA) content and increasing the enzymatic activity of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) independently from the watering regime and the relative humidity applied. This study highlights that *F. caperata* can face the $O_3$-induced oxidative stress thanks to high levels of constitutive enzymatic and non-enzymatic defences against ROS formed naturally during the dehydration–rehydration cycles to which lichens are frequently exposed.



## Introduction

Lichens, a symbiotic association between a fungus (the mycobiont) and one or more photosynthetic partners (the photobionts), are among the best biomonitors of airborne pollutants, so that a norm for their use has just been edited by the European Committee for Standardization (CEN, EN 16413:2014). Lichens are particularly sensitive to sulphur dioxide ($SO_2$) (Nash, 2008), hydrogen sulphide (Bertuzzi and Tretiach, 2013) and nitrogen oxides (Tretiach et al., 2007), but few data are available concerning their response to tropospheric ozone ($O_3$). This is surprising since $O_3$ has detrimental effects on many organisms at physiological, biochemical and molecular level (Heat, 2008; Goumenaki et al., 2010), and the concentrations of this pollutant are progressively increasing in vast areas of the world (Gillespie et al., 2011).

Field studies show that high concentrations of $O_3$ do not impact the lichen flora (*e.g.* Ruoss and Vonaburg, 1995; Lorenzini et al., 2003), at least if other organic pollutants, in particular peroxyacetylnitrates, are absent (Zambrano and Nash, 2000). With the exception of Scheidegger and Schroeter (1995), all the studies carried out under controlled conditions showed that $O_3$ has only limited (Tarhanen et al., 1997) or no significant consequences on the physiology of lichens (Calatayud et al., 2000; Riddell et al.,

*Abbreviations:* APX, ascorbate peroxidase; AsA, reduced ascorbate; AsA/GSH, ascorbate/glutathione cycle; $Chl_aF$, chlorophyll *a* fluorescence; DCF, dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DTNB, 5′,5′-dithiobis-2-nitrobenzoic acid; $F_v/F_m$, maximum quantum efficiency of photosystem II; GR, glutathione reductase; GSH, reduced glutathione; GSH + GSSG, total glutathione; GSSG, oxidized glutathione; $H_2O_2$, hydrogen peroxide; NBT, NitroBlueTetrazolium; NPQ, non-photochemical quenching; $O_2^{\bullet -}$, superoxide anion; $O_3$, ozone; qP, photochemical quenching; RH, air relative humidity; ROS, reactive oxygen species; SOD, superoxide dismutase; XTT, 3′-(1-[phenylamino-carbonyl]-3,4-tetrazolium)bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate.

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2010, 2012; Bertuzzi et al., 2013). This is not unexpected since the maximum daily concentration of $O_3$ commonly occurs in the central hours of hot, sunny days, when lichens are dry and, therefore, metabolically inactive. In contrast to vascular plants, in fact, the water content of lichens varies according to that of the environment (Nash, 2008). When dry, they can tolerate very harsh environmental conditions, such as high temperatures, UV, and X rays, up to space vacuum (Tretiach et al., 2012a), because their cytoplasm vitrifies, and their metabolism is suspended (Kranner et al., 2008). It could be questioned whether lichens are actually $O_3$-avoidant or $O_3$-tolerant (Tretiach et al., 2012b). More recently, Bertuzzi et al. (2013) proposed that lichens are $O_3$-tolerant, possibly because they have high levels of natural defences against different forms of oxidative stress, but particularly against those derived from the fluctuation in the cell water content, that is an intrinsic feature of poikilohydry. In homoiohydrous vascular plants, the mechanisms involved in the defence against oxidative stress derived from $O_3$ exposure have been studied extensively (Dizengremel et al., 2008; Pellegrini et al., 2013), from the ascorbate-glutathione cycle (Nali et al., 2004) to many other enzymatic (guaiacol peroxidase, glutathione S-transferases, glutathione peroxidase) and non-enzymatic systems (*e.g.* proline, flavonoids and lipoic acid) (Gill and Tuteja, 2010). By contrast, in lichens, the working principles of these mechanisms are largely unknown, above all in relation to the $O_3$-derived oxidative stress.

In this study, physiological [chlorophyll *a* fluorescence ($Chl_aF$) emission], histochemical (ROS localization in the lichen thallus) and biochemical parameters (reduced ascorbate (AsA), dehydroascorbate (DHA), reduced glutathione (GSH) and total glutathione (GSSG) as well as the correlated antioxidant enzymes) were used to characterize thalli of a common epiphytic lichen fumigated with $O_3$ at different water and air relative humidity (RH) regimes. The objective is to give a thorough description of the biochemical and physiological mechanisms that are at the basis of the $O_3$-tolerance of lichens, answering to the following open questions: (i) Do water-activated thalli react to $O_3$ similarly to air-dried, metabolically inactive thalli? (ii) Is air humidity an environmental factor influencing the responses to $O_3$? (iii) Is ROS production enhanced in $O_3$-exposed thalli, and is the cellular localization the same than in air-dried thalli? (iv) Which metabolites/enzymes of the ascorbate/glutathione (AsA/GSH) cycle are more affected by high $O_3$ levels, and why?

## Materials and methods

### *Target species, collection and pre-treatment of samples*

*Flavoparmelia caperata* (L.) Hale is a widespread, mesophytic chlorolichen common throughout the mild temperate regions of Europe and North America, mostly epiphytic in sites with diffuse light to sun-exposed sites, with good tolerance to protracted desiccation (Tretiach et al., 2012b). The photobiont has been identified as *Trebouxia gelatinosa* Archibald on the basis of ITS sequence data.

Lichen thalli were collected from northerly exposed bark of ash (*Fraxinus ornus* L.) trees in a wood far from known air pollution sources (Classic Karst, NE Italy). The thalli were detached using a sharp blade, put in open Petri dishes and immediately transported to the laboratory, where the material was left to dry out at room temperature in dim light (<10 μmol photons $m^{-2}$ $s^{-1}$), carefully cleaned from debris and bryophytes, put in a desiccator with silica gel for 2 days and then sealed in vacuum bags and stored at −20 °C. Before use, the material was thawed in a desiccator with silica gel for 2 days, then the marginal parts of the thallus (c. 3 cm from the margin) were selected for the experiments, since they have considerably higher chlorophyll *a* fluorescence ($Chl_aF$) emission than the central ones (Tretiach et al., 2007).

For the experiments (1 and 2, see below), about (i) 100 lobes of 60 ± 5 mg each for $Chl_aF$ measurements and histochemical localization of ROS production, and (ii) 8 g of mixed lobes for biochemical assays were randomly selected. The material was closed in Petri dishes and sealed in vacuum bags until use. Prior to exposure, the samples were subjected to a conditioning process lasting 2 days: they were 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, that were put in a growth chamber with charcoal-filtered air at 20 °C. The photosynthetically active radiation flux, checked using a LI-COR-calibrated Micro-Quantum 2060-M Sensor (Walz, Effeltrich, Germany), was set at 20 μmol photons $m^{-2}$ $s^{-1}$, with a light/dark regime of 12/12 h. Light was provided by four quartz metal halide lamps with clear outer bulb (400 W, MASTER HPI-T Plus, Philips, Netherlands) and by four high pressure sodium lamps with clear tubular outer bulb (250 W, SON-T, Philips, Netherlands).

After this conditioning process a first series of $Chl_aF$ measurements (see below) were taken to exclude those samples with low $Chl_aF$ values [maximum quantum efficiency of photosystem II ($F_v/F_m$) < 0.670] and to divide the samples in homogeneous groups (see below).

### *Sample exposure*

The two sets of samples were divided in 4 (A–D) groups (respectively 12 individual lobes and 2 g of mixed lobes) that were exposed for 2 weeks to 250 ppb ozone ($O_3$) (for $O_3$, 1 ppb = 1.96 μg $m^{-3}$, at 20 °C and 101.325 kPa) for 5 h in form of a square wave, at 20 °C, 37 μmol photons $m^{-2}$ $s^{-1}$, with a light/dark regime of 12/12 h, at four combinations of $O_3$ and artificial daily watering, as follows: without $O_3$ with (group A) or without (group B) watering; with $O_3$ with (group C) or without (group D) watering.

During the exposure, groups A, B were maintained in a controlled environment facility ventilated with charcoal filtered air, whereas groups C, D were maintained in a 0.90 m × 0.90 m × 0.65 m Perspex chamber continuously ventilated with the inlet air enriched with $O_3$ generated by electrical discharge, using a Fisher 500 air-cooled apparatus (Zurich, Switzerland) supplied with pure oxygen (two complete air changes $min^{-1}$). The concentration of $O_3$ was continuously monitored with a photometric analyzer (Monitor Labs, mod. 8810, San Diego, CA, USA) connected to a computer. To moisten the A, C samples, a spray of distilled water (c. 0.01 mL $cm^{-2}$) was applied immediately before the input of $O_3$ to activate the metabolism when the concentration of $O_3$ was the highest.

The exposure was carried out at 30% air relative humidity (RH) (experiment 1) and at 70% RH (experiment 2). The RH values were checked automatically in the controlled environment facility (F.lli Bertagnin, Bologna, Italy).

### *$Chl_aF$ measurements*

The samples were immersed for 3 min in distilled water, gently shaken by hand, and dark-adapted for 30 min in a dark box. $Chl_aF$ measurements were taken with a pulse-amplitude-modulated fluorometer PAM-2000 (Walz, Effeltrich, Germany), positioning the measuring fibre optic (length: 100 cm; active diameter: 5.5 mm) at 60°, on the upper surface of terminal parts of the lobe margin. The modulated light, the saturating light and the internal led light were used to determine $F_v/F_m$, non-photochemical quenching (NPQ), photochemical quenching (qP) and qN, as described by Bertuzzi et al. (2013).

Standard $Chl_aF$ measurements were taken on 6 individual lobes of each exposure group before exposure, after exposure and after

1 and 2 days of recovery with the samples subjected to the same conditioning process described above. The water content of each sample was estimated by measuring the impedance with a Protimeter mini-3 (Parametrics, Shannon, Ireland) (Bertuzzi et al., 2013).

*Histochemical localization of ROS production*

The histochemical observations were carried out on 6 individual lobes of each exposure group: 3 lobes were immersed in liquid nitrogen for c. 5 s immediately after the end of the exposure, while the other 3 lobes were left to recover for 2 days, and only then they were immersed in liquid nitrogen. The samples were stored at −80 °C in a freezer for a maximum of 30 days, then were let to warm up at room temperature (c. 20 °C and 50% RH). Rectangular fragments (4 mm × 3 mm) were cut radially from the margin of the lobes with a stainless blade and rehydrated in distilled water for 5 min. These fragments were mounted in a cryostat embedding medium (Killik, Bio-Optica, Milan, Italy) and then cut with a cryotome LEICA CM 1510 S (Leica, Wetzlar, Germany) to obtain 30 μm thick transversal sections. The sections were immersed in 15 mL of 10 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (HPLC grade, Sigma–Aldrich, St. Louis, USA) aqueous solution inside a vacuum chamber in the dark for 90 min. After this treatment the transversal sections were put on glass slides and observed *in vivo* with a Confocal Laser Scanning Microscope Nikon C1-si (Nikon, Tokyo, Japan). Samples were excited with an argon laser at 488 nm with an intensity of 10.5%. Signal from the excited dichlorofluorescein (DCF) was acquired with a 515/30 nm band pass filter. Emission of the auto-fluorescence from chlorophyll was acquired with a 650 nm long pass filter ($\lambda \geq 650$ nm). Acquisitions were elaborated with the Nikon EZ-C1 FreeViewer software (Nikon, Tokyo, Japan) and with the freeware suite ImageJ 1.46r (Wayne Rasband, National Institutes of Health, Washington DC, USA).

*Biochemical assays*

*ROS determination*

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). Samples were pulverized under liquid nitrogen in a mortar, homogenized with 0.8 mL of 20 mM potassium-phosphate buffer (pH 6.5), and centrifuged at 12,000 × *g* for 5 min at 4 °C. The reaction mixture contained 20 mM potassium-phosphate buffer (pH 6.5), 50 μM of 10-acetyl-3,7-dihydrophenoxazine, 0.1 U $mL^{-1}$ horseradish peroxidase and 50 μL of the supernatant. Samples were incubated at 25 °C for 30 min in the dark and the resorufin fluorescence ($E_x/E_m$ = 530/590 nm) was quantified with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, USA), after subtracting the background fluorescence due to the buffer solution and to the assay reagents. Each result was plotted against a $H_2O_2$-standard curve (from 0 to 5 μM).

The superoxide 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 $O_2^{\bullet -}$ to a soluble XTT formazan, according to the method of Able et al. (1998). Samples were pulverized under liquid nitrogen in a mortar, homogenized with 1 mL of 50 mM Tris–HCl buffer (pH 7.5), and centrifuged at 12,000 × *g* for 15 min at 4 °C. The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 0.5 mM XTT and 100 μL of the supernatant in a final volume of 1 mL. Samples were incubated at room temperature for 15 min and the reduction of XTT formazan was quantified with a spectrophotometer (6505 UV-Vis, Jenway, UK) at 470 nm, after subtracting the background absorbance due to the buffer solution and to the assay reagents. The quantity of $O_2^{\bullet -}$ produced was determined using the molar extinction coefficient $2.16 \times 10^4$ $M^{-1}$ $cm^{-1}$.

*Non enzymatic antioxidant compounds*

Samples were pulverized under liquid nitrogen in a mortar, homogenized with 6 mL of cold 5% (w/v) trichloroacetic acid, and centrifuged at 14,000 × *g* for 15 min at 4 °C. Reduced ascorbate (AsA) and dehydroascorbate (DHA) content were measured spectrophotometrically according to Wang et al. (1991). This assay is based on the reduction of ferric ion to ferrous ion with ascorbic acid in acid solution followed by formation of the red chelate between ferrous ion and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) that absorbs at 534 nm. AsA + DHA was determined through a reduction of DHA to AsA by 3.89 mM dithiothreitol and DHA levels were estimated on the basis of the difference between AsA + DHA and AsA values. A standard calibration curve covering 0–1 mM of AsA or DHA range was used. Supernatants were also used for total and total glutathione (GSSG) determinations by the DTNB-GSSG reductase recycling procedure (see Sgherri and Navari-Izzo, 1995). GSSG was determined after removal of reduced glutathione (GSH) from the sample extract by derivatization with 4-vinilpyridine. Changes in absorbance of the reaction mixtures were measured at 412 nm and 25 °C. The amount of GSH was calculated by subtracting the GSSG amount, as GSH equivalents, from the total glutathione (GSH + GSSG) amount. A standard calibration curve where GSH-equivalents (0–10 mM) were plotted against the slope of change in absorbance at 560 nm.

*Activity of antioxidant enzymes*

Total SOD (EC 1.15.1.1) activity was assayed in terms of its ability to inhibit the photochemical reduction of NitroBlueTetrazolium (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 the enzyme extract. Samples were incubated for 10 min under fluorescent lamp (150 W, Osram R80, Milan, Italy) and absorbance at 560 nm was read against unilluminated samples. One unit of SOD activity was defined as the amount of the enzyme required to cause 50% inhibition of the rate of NBT reduction. The ascorbate peroxidase (APX) (EC 1.11.1.11) activity was assayed according to Francini et al. (2006) by measuring the oxidation of AsA at 290 nm at 25 °C for 1 min. The reaction mixture contained 50 mM potassium phosphate (pH 6.6), 1 mM AsA, 0.4 mM $Na_2EDTA$ and 50 μL of enzymatic extract in 1 mL final volume. Dehydroascorbate reductase (DHAR) (EC 1.8.5.1) activity was assayed according to Kawakami et al. (2000) by measuring the production of AsA by DHA reduction at 265 nm at 25 °C for 1 min. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 20 mM GSH, 2.0 mM DHA and 30 μL of enzymatic extract in 1 mL final volume. One unit of DHAR was defined as the amount of enzyme required to oxidize 1 μmol of AsA $min^{-1}$. The glutathione reductase (GR) (EC 1.6.4.2) activity was assayed according to Gillham and Dodge (1986) by monitoring the oxidation of NADPH by GSSG at 30 °C for 3 min through the decrease in absorbance at 340 nm. The assay mixture contained 400 mM potassium phosphate (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 enzymatic extract in 1 mL final volume. One unit of GR was defined as the amount of enzyme required to oxidize 1 nmol of NADPH $min^{-1}$. For all assays, proteins were determined according to Bradford (1976), using bovine serum albumin as standard.

*Statistics*

The following tests were applied: (i) Mann–Whitney *U* test, also known as Wilcoxon non-paired test ($Chl_aF$ data) for comparison of

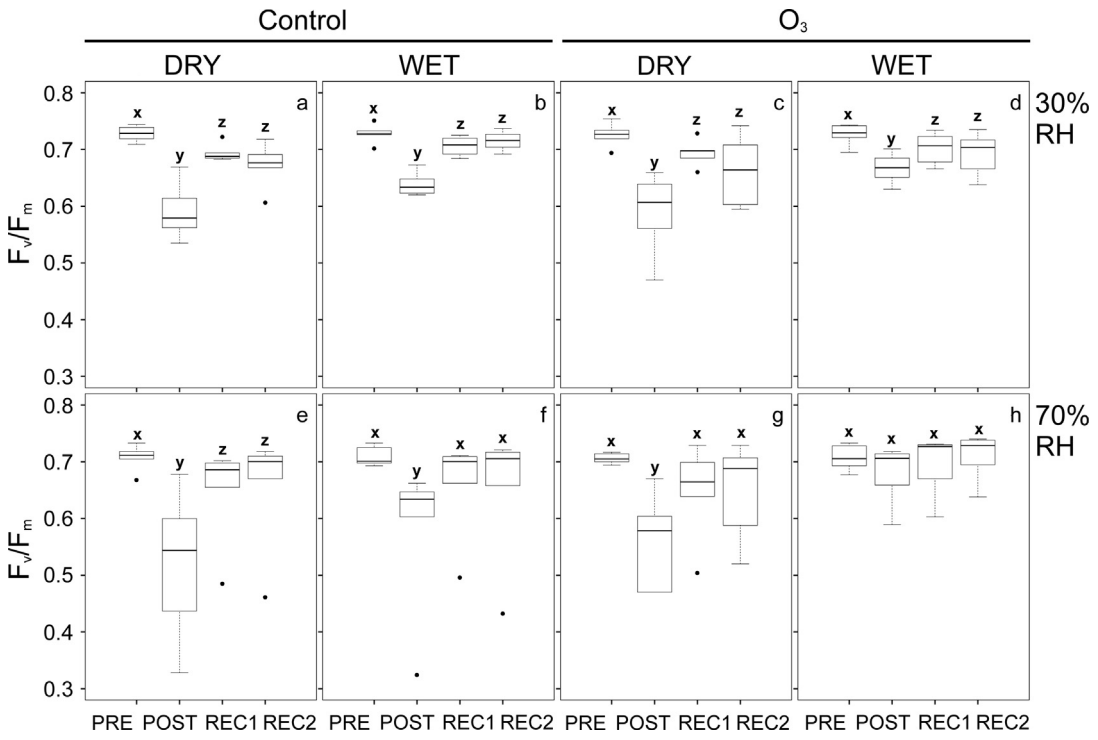


**Fig. 1.** $F_v/F_m$ measured before exposure (PRE), after 2-week exposure (POST) and after 1- (REC1) and 2-day (REC2) recovery in samples of *Flavoparmelia caperata* exposed at 30% (a–d) and 70% (e–h) RH to filtered air (control) (a, b, e, and f) and to $O_3$ (250 ppb, 5 h $d^{-1}$) (c, d, g, and h), under DRY (a, c, e, and g) and WET (b, d, f, and h) treatment. 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 $P = 0.05$ by Wilcoxon paired test (comparison between two successive times). $n = 6$.

groups of samples subjected to different treatments; (ii) Wilcoxon paired test ($Chl_aF$ data) for comparison of the same group of samples between two successive times; (iii) Kolmogorov–Smirnov test (biochemical data for comparison between controls and $O_3$-treated samples). Non-parametric tests were applied because the data distribution curves of some parameters show a noticeable deviation from a Gaussian distribution curve, as tested by the Shapiro–Wilk's test. All calculations were performed with Microsoft Office Excel 2010 (Microsoft Corporation, WA, USA) and R version 2.15.1 (R Foundation for Statistical Computing).

## Results

### *$Chl_aF$ measurements*

No visible symptoms were observed in both fumigated and non-fumigated materials, regardless of water regimes and RHs. There was no statistically significant difference between $O_3$-exposed samples and controls, except for those subjected to the wet regime at 30% RH (Fig. 1, Table 1). Surprisingly, in this case the $O_3$-exposed samples showed the smallest maximum quantum efficiency of photosystem II ($F_v/F_m$) reduction with respect to the pre-exposure values, c. −10% *vs.* −20 to −30% of all the other groups. The dry regime, compared with the wet one, induced a significant reduction of $F_v/F_m$, fully retrieved after the two-day long recovery. By contrast, the two RH regimes did not influence the sample response, also for the relatively large data spread recorded in all the groups subjected to 70% RH.

The exposure conditions were associated with a general increase in non-photochemical quenching (NPQ) after recovery (from +80% to +300% in the samples exposed to 30% RH; see Fig. 2 and Table 2). Other parameters of $Chl_aF$ extinction (*e.g.* qP and qN) followed the same pattern of NPQ, and therefore they are not shown here.

### *ROS quantification and localization*

At both RH regimes, superoxide anion ($O_2^{\bullet -}$) was always higher in the dry samples than in the wet ones, without distinction between $O_3$-exposed samples ($6.0 \pm 0.72$ *vs.* $2.9 \pm 0.43$, $P \leq 0.001$) and controls ($5.9 \pm 0.37$ *vs.* $2.8 \pm 0.63$, $P \leq 0.001$). $O_3$ did not induce any significant change of the $O_2^{\bullet -}$ content in both dry and wet samples (Fig. 3a).

$O_3$ led to an accumulation of hydrogen peroxide ($H_2O_2$) only in the dry samples maintained at 30% RH (+44%, with respect to the control, see Fig. 3b). By contrast, at 70% RH $H_2O_2$ did not significantly change after $O_3$ exposure at both watering regimes (dry *vs.* wet; *data not shown*), although it was higher in the dry than in the wet samples exposed to filtered air (+53%). This pattern is largely congruent with the results of the *in situ* histochemical localization of ROS. ROS production was always heterogeneously distributed in the cross thallus sections, being particularly intense in some spot areas, and absent in others (Fig. 4). ROS production in the mycobiont occurred in the paraplectenchymatous cells of both upper and lower cortex, and in the appressoria, *i.e.* the specialized hyphal cells that are in physical contact with single photobiont cells. No reaction was observed instead in the hyphae of the medulla. The localization was mostly restricted to the cytoplasm of both photo- and mycobiont cells; only when particularly intense, ROS were detected also around the pyrenoid of the large multi-lobed *Trebouxia* chloroplast.

In the photobiont ROS production was not modified either by the watering regime (groups A, B; see Fig. 4) or by $O_3$ (groups C and D), whereas in the mycobiont ROS production was slightly affected by the watering regime, being observed more frequently in both groups of dry samples (B and D) than in the other two (A and C).

After the two-day long recovery, ROS production was generally barely detectable in all the samples (*data not shown*).

**Table 1**
*P*-values (Mann–Whitney *U* test) of the differences among the $F_v/F_m$ values of the a–h groups of samples subjected to different treatments, after exposure at 30% (a–d) and 70% (e–h) RH to filtered air (a, b, e, and f) and to $O_3$ (250 ppb, 5 h/d; c, d, g, and h), under DRY (a, c, e, and g) and WET (b, d, f, and h) treatment. $P \leq 0.05$ underlined. $n = 6$.

| | 30% RH | | | | 70% RH | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | $O_3$ | | Control | | $O_3$ | |
| | DRY<br>a | WET<br>b | DRY<br>c | WET<br>d | DRY<br>e | WET<br>f | DRY<br>g | WET<br>h |
| a | | 0.04113 | 0.8182 | 0.01515 | 0.5887 | 0.3939 | 0.8182 | 0.01515 |
| b | | | 0.1994 | 0.04113 | 0.06494 | 0.6991 | 0.04113 | 0.09307 |
| c | | | | 0.02597 | 0.4848 | 0.4848 | 0.5211 | 0.03704 |
| d | | | | | 0.01515 | 0.04113 | 0.01515 | 0.1797 |
| e | | | | | | 0.3095 | 1 | 0.02597 |
| f | | | | | | | 0.3095 | 0.06494 |
| g | | | | | | | | 0.02597 |

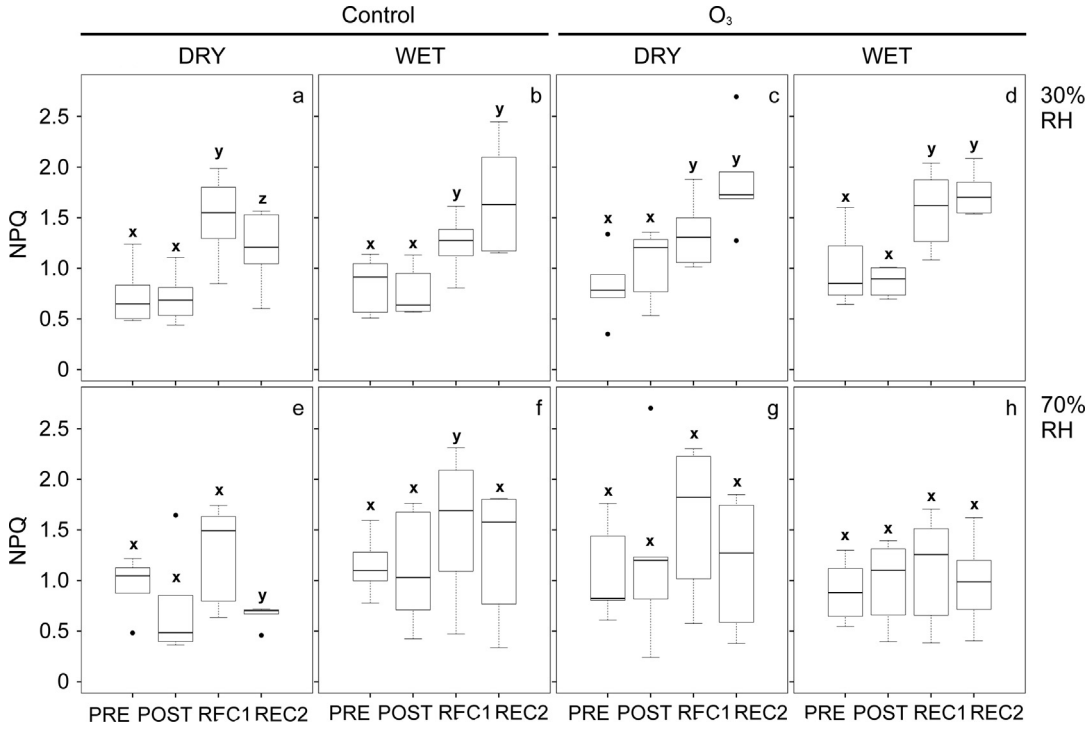


**Fig. 2.** NPQ measured before exposure (PRE), after 2-week exposure (POST) and after 1- (REC1) and 2-day (REC2) recovery in samples of *Flavoparmelia caperata* exposed at 30% (a–d) and 70% (e–h) RH to filtered air (control) (a, b, e, and f) and to $O_3$ (250 ppb, 5 h $d^{-1}$) (c, d, g, and h), under DRY (a, c, e, and g) and WET (b, d, f, and h) treatment. Symbols as in Fig. 1. $n = 6$.

**Table 2**
*P*-values (Mann–Whitney *U* test) of the differences among NPQ values of the groups of samples subjected to different treatments, after the 2-day long recovery following the exposure in fumigation chamber at 30% (a–d) and 70% RH (e–h) to filtered air (a, b, e, and f) and to $O_3$ (250 ppb, 5 h/d; c, d, g, and h), under DRY (a, c, e, and g) and WET (b, d, f, and h) treatment. $P \leq 0.05$ underlined. $n = 6$.

| | 30% RH | | | | 70% RH | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | $O_3$ | | Control | | $O_3$ | |
| | DRY<br>a | WET<br>b | DRY<br>c | WET<br>d | DRY<br>e | WET<br>f | DRY<br>g | WET<br>h |
| a | | 0.1797 | 0.01515 | 0.008658 | 0.5887 | 0.3095 | 0.9372 | 0.4848 |
| b | | | 0.8182 | 0.9372 | 0.09307 | 0.3939 | 0.1797 | 0.04143 |
| c | | | | 0.8182 | 0.004329 | 0.2403 | 0.132 | 0.004329 |
| d | | | | | 0.008658 | 0.3939 | 0.09307 | 0.01515 |
| e | | | | | | 0.2403 | 0.6991 | 0.9372 |
| f | | | | | | | 0.8182 | 0.3939 |
| g | | | | | | | | 0.5887 |

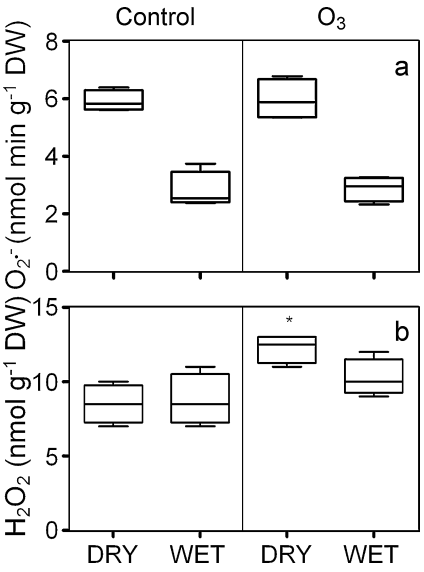


**Fig. 3.** Quantification of $O_2^{\bullet-}$ (a) and $H_2O_2$ (b) in *Flavoparmelia caperata* under DRY and WET treatment, exposed for two weeks to filtered air (control) and to $O_3$ (250 ppb, 5 h $d^{-1}$, two weeks) at 30% RH. For each boxplot median, 25–75° percentiles and non-outlier minimum and maximum are reported. Statistically significant differences between control and ozonated samples are marked (*$0.01 < P \leq 0.05$; Kolmogorov–Smirnov test). $n = 4$.

*Biochemical assays*

*Non enzymatic antioxidant compounds*

$O_3$ induced an evident decline of the concentration and the redox state of ascorbate in dry and wet samples independently of the exposure RH. At 30% RH, ascorbate was lower in $O_3$-treated samples than in the controls, independently of the watering regime (−60 and −54%, respectively in dry and wet, Fig. 5a). Interestingly only at 70% RH reduced ascorbate (AsA) was higher in the wet samples than in the dry ones if exposed to filtered air ($8.3 \pm 0.37$ *vs.* $6.1 \pm 0.72$, $P \leq 0.01$) (*data not shown*).

At 30% RH, $O_3$ led to a slight decrease of total ascorbate (AsA + DHA) only in wet samples, in comparison to the filtered-air exposed controls (−14%, $P \leq 0.05$, Fig. 5b). By contrast, at 70% RH total ascorbate did not change independently of the treatment applied (*data not shown*).

The redox state of ascorbate showed a similar trend in dry and wet samples at both RHs. In particular at 30% RH, $O_3$ led to a marked decline in the AsA/DHA ratio in comparison to the filtered-air exposed controls (−66 and −57%, respectively, Fig. 5c).

$O_3$ induced an evident decline of the concentration and the redox state of glutathione in dry and wet samples in relation to the exposure RH. At 30% RH, GSH did not show significant change in dry and wet samples exposed to $O_3$ and to filtered air (Fig. 6a). Only at 70% RH, GSH underwent a significant increase when dry and

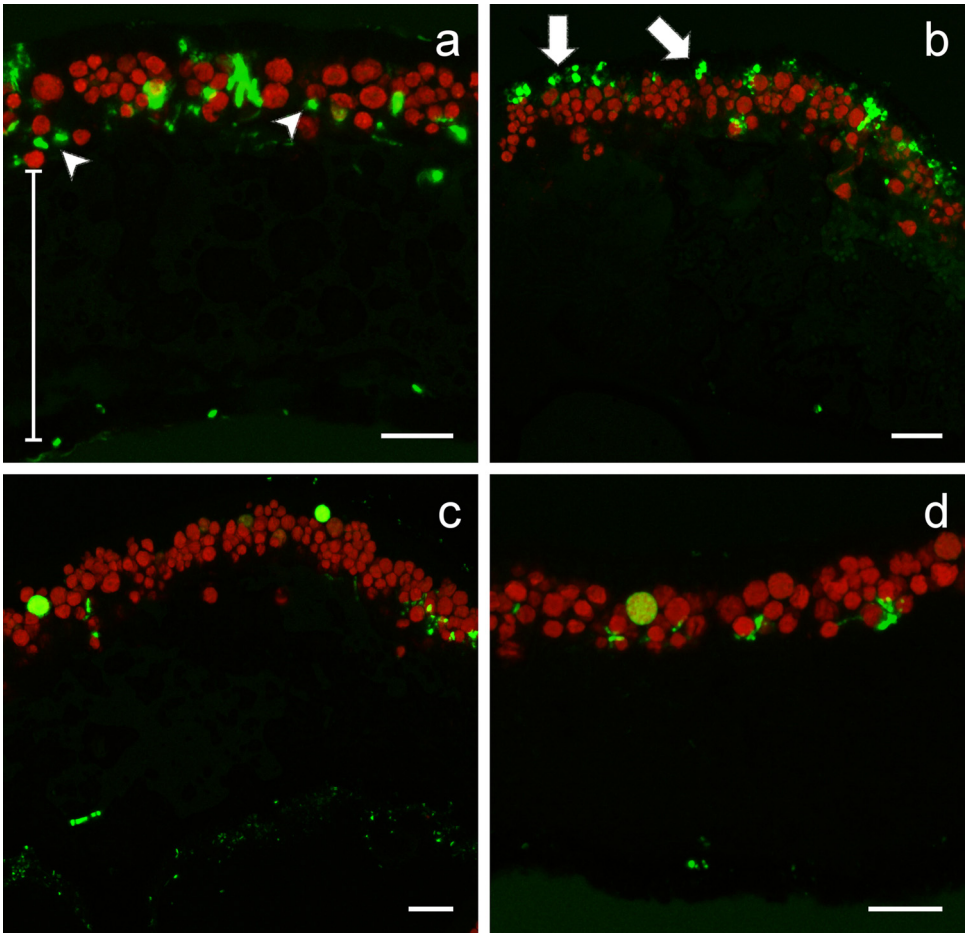


**Fig. 4.** Histochemical localization of ROS production in *Flavoparmelia caperata* under DRY (a and b) and WET (c and d) treatment exposed to filtered-air (a and c) and to $O_3$ (250 ppb, 5 h $d^{-1}$) (b and d) for two weeks at 30% RH. Cross sections of *F. caperata* were stained with DCFH-DA and observed at the confocal laser scanning microscope. Green signal emitted by DCF, red signal by chlorophyll *a* after an excitation with lasers at λ = 488 and 637 nm, respectively. Appressoria are indicated by arrowheads (a), and paraplectenchymatous cells of the upper cortex by arrows (b); the non-reactive medullar region corresponds to the vertical segment in (a). Bar = 30 μm.

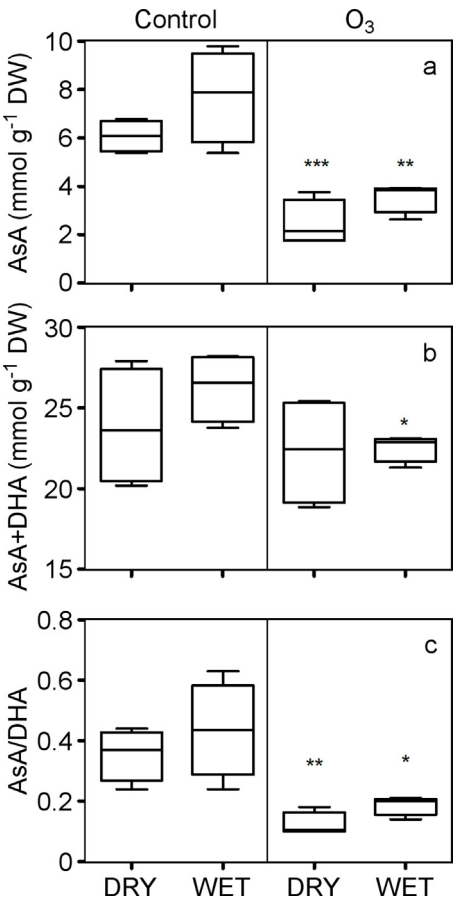


**Fig. 5.** Content of AsA (a), total ascorbate (AsA + DHA) (b), and ratio AsA/DHA (c) in *Flavoparmelia caperata* exposed for two weeks to filtered air (control) and to $O_3$ (250 ppb, 5 h d$^{-1}$) at 30% RH. Symbols as in Fig. 3; statistically significant differences between control and ozonated samples are marked (***$P \leq 0.001$; **$0.001 < P \leq 0.01$; *$0.01 < P \leq 0.05$; Kolmogorov–Smirnov test). $n = 4$.

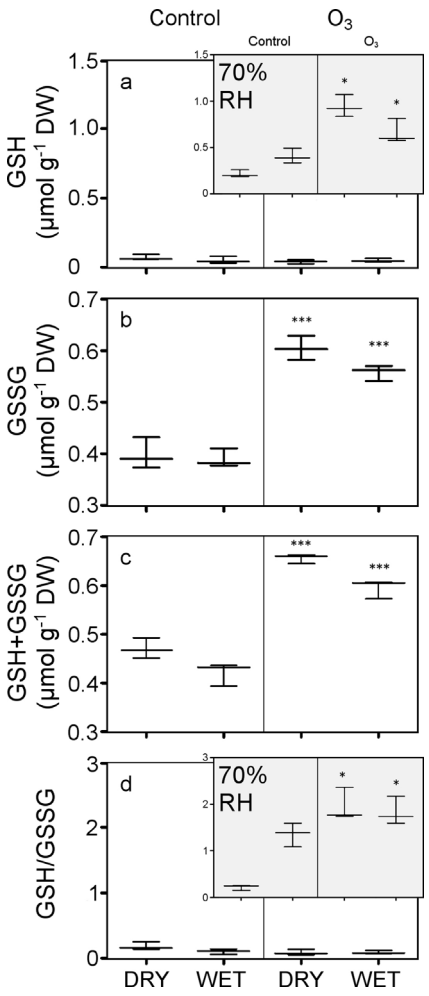


**Fig. 6.** Content of GSH (a), GSSG (b), GSH + GSSG (c), and GSH/GSSG ratio (d) in *Flavoparmelia caperata* exposed for two weeks to filtered air (control) and to $O_3$ (250 ppb, 5 h d$^{-1}$) at 30% RH. Symbols as in Fig. 3; statistically significant differences between control and ozonated samples are marked (***$P \leq 0.001$; Kolmogorov–Smirnov test). The most significant results of the parallel experiment carried out at 70% RH are reported in the grey inserts (a and d). $n = 4$.

wet samples were exposed to $O_3$ (about 4- and 2-fold higher than in the controls, Fig. 6a). At both RHs, $O_3$ led to a marked increase of GSSG independently of the watering regime. At 30% RH, GSSG was higher in $O_3$-treated samples than in the controls (+52 and +43%, respectively, Fig. 6b); at 70% RH, GSSG was higher in dry than in wet samples exposed to filtered air ($0.37 \pm 0.019$ *vs* $0.29 \pm 0.016$, $P \leq 0.01$) (data not shown).

A similar trend was observed for total glutathione (GSH + GSSG) that showed a marked rise at the end of the $O_3$ treatment independently of the watering regime. At 30% RH, it reached a 1.4-fold higher values than in the control (Fig. 6c). In dry $O_3$-treated samples, the total content of this metabolite was higher than in the wet counterparts (+10%).

At 30% RH, the GSH/GSSG ratio did not show significant change related to the water regime or to the $O_3$ exposure (Fig. 6d). On the contrary, at 70% RH $O_3$ induced a marked increase of the redox state of glutathione in comparison to the control in both dry and wet samples (9-fold higher, insert of Fig. 6d).

*Activity of antioxidant enzymes*

At both relative humidities, SOD activity was always higher in wet than in dry samples exposed to $O_3$ or to filtered air (*data not shown*), although $O_3$ did not induce any significant change in the activity of this enzyme at both watering regimes.

At the end of the $O_3$ treatment, an evident increase in ascorbate peroxidase (APX) activity was observed in dry and wet samples in comparison to the control, independently of the RH conditions. In particular, at 30% RH it reached a 2-fold higher value than in the filtered-air controls with both watering regimes (Fig. 7a). In wet $O_3$-treated samples, the activity of this enzyme was higher than in the dry counterparts ($50.4 \pm 6.76$ *vs.* $32.1 \pm 1.70$, $P \leq 0.001$).

At 30% RH, dehydroascorbate reductase (DHAR) activity was always lower in dry than in wet samples exposed to $O_3$ ($138.4 \pm 13.80$ *vs.* $91.2 \pm 2.83$, $P \leq 0.001$). $O_3$ induced a significant rise of the activity of this enzyme at both watering regimes independently of the RH conditions. In particular at 30% RH, DHAR activity reached 1.4- and 2.9-fold higher values than the filtered-air controls (Fig. 7b).

Only at 30% RH, glutathione reductase (GR) activity was higher in wet than in dry samples maintained in filtered air ($11.9 \pm 1.59$ *vs.* $34.9 \pm 1.67$, $P \leq 0.001$). $O_3$ induced a significant increase of the activity of this enzyme at both watering regimes independently of the RH conditions. In particular at 30% RH, GR activity was higher than in the filtered-air control (5- and 2-fold, respectively in dry and wet samples, see Fig. 7c).

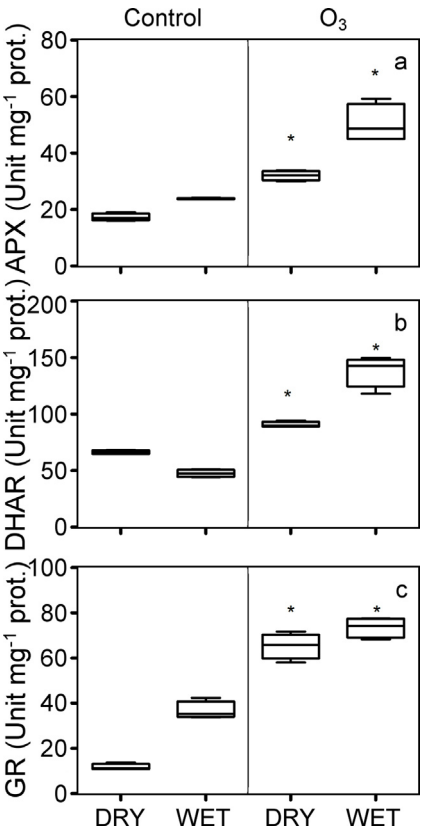


**Fig. 7.** Enzymatic activity of APX (a), DHAR (b) and GR (c) in *Flavoparmelia caperata* exposed for two weeks to filtered air (control) and to $O_3$ (250 ppb, 5 h $d^{-1}$) at 30% RH. Symbols as in Fig. 3; statistically significant differences between control and ozonated samples are marked (*$0.01 < P \leq 0.05$; Kolmogorov–Smirnov test). $n = 4$.

## Discussion

In this study, the tolerance of the lichen *F. caperata* to $O_3$ has been tested at physiological, cytochemical and biochemical level by exposing portions of healthy thalli in a fumigation chamber under different air humidity and watering regimes. After two-week exposure, no significant effects were observed on $Chl_aF$ that could be traced back to the action of the pollutant. This suggests, in accordance with Riddell et al. (2010, 2012) and Bertuzzi et al. (2013), that the functionality of photosystems is not impaired by $O_3$, or not as much as by the watering regime. Desiccation caused in fact a strong – but fully recoverable – decrement in $F_v/F_m$, in good accordance with the results of Hájek et al. (2001). Desiccation influenced also some biochemical markers of oxidative stress: independently of the relative humidity conditions, *F. caperata* was not able to fully avoid an increase in ROS production. The development of $H_2O_2$ and $O_2^{\bullet-}$ as a consequence of a prolonged desiccation has been reported not only in lichens (Kranner, 2002; Weissmann et al., 2005a) and in their isolated symbionts (Kranner et al., 2005), but also in yeasts (França et al., 2007), and in resurrection vascular plants (Kranner et al., 2002). In our case, the antioxidant mechanisms affected by desiccation differed in relation to the RH to which the samples had been exposed. At 30% RH, desiccation induced a decrease of SOD activity and therefore an increase in oxidative stress. The concomitant decrease in GR activity strengthens the hypothesis that desiccation partly impaired the efficiency of the ROS detoxifying enzymatic systems. Similar findings have been reported for the lichens *Peltigera polydactila* (Kranner et al., 2002, 2003) and *Ramalina lacera* (Weissmann et al., 2005b), although it must be underlined that the decrease of enzymatic activity seems to be at least in part ecology- and species-dependent (Kranner et al., 2003). At 70% RH, on the contrary, the increase in $O_2^{\bullet-}$ and $H_2O_2$ content in the control samples seems to be correlated with the impairing of the AsA/GSH cycle due to the loss in efficiency of the GSH system. Desiccation induced an increase in GSSG that could be responsible for the accumulation of ROS, notwithstanding the decrease of AsA.

As a matter of fact, our experiments show that ROS production derived from desiccation is predominant over that derived from $O_3$ exposure; only in one case, *i.e.* dry samples at 30% RH, it was possible to observe a significantly higher accumulation of ROS due to $O_3$. Interestingly, the histochemical localization of ROS was congruent with the quantitative measurements of $O_2^{\bullet-}$ and, to a lesser extent, of $H_2O_2$. Previous studies detected ROS in both symbionts (Weissmann et al., 2005a; Catalá et al., 2010), but the photobiont of *F. caperata* seems to scavenge ROS better than the mycobiont. Therefore, it can be argued that the accumulation of ROS measured quantitatively (see Fig. 3) derives totally from the latter.

Although the ROS production derived from $O_3$ exposure was obscured by that derived from desiccation, the metabolite contents involved in the AsA/GSH cycle and the activity of the related enzymes showed, by contrast, to be much more sensitive to $O_3$ than to desiccation. At both watering regimes, and independently of the RH conditions, the evident decrease of AsA could be explained by its consumption by APX for detoxification of $H_2O_2$, as indicated by the low AsA/DHA ratio (Asada, 1999), which is considered one of the first signs of oxidative stress (Smirnoff and Wheeler, 2000). The fact that no corresponding DHA accumulation/reduction occurred (notwithstanding the increase of DHAR activity) suggests that this unstable compound broke down and was lost from the total ascorbate pool. Generally, the AsA/DHA ratio significantly decreases when the GSH pool is involved in oxidative stress avoidance but, at 30% RH, GSH synthesis did not increase, notwithstanding the higher GR activity. The unchanged GSH/GSSG ratio suggests that *F. caperata* was not able to counterbalance GSH oxidation and that oxidative alteration occurred. At 70% RH, the regeneration of GSH by GR was operating, and this is notoriously a critical step in the ROS scavenging system. At different watering regimes, $O_3$ enhanced the content of GSH and the GSH + GSSG content (despite the increase in GSSG), suggesting that an induced GSH biosynthesis might constitute a reserve which allows *F. caperata* to tolerate oxidative alterations derived from the $O_3$ treatment.

The differential alteration of the antioxidant mechanisms observed at the two RHs is noteworthy. A possible explanation can be found in the different lichen water contents at equilibrium with the air vapor pressure and/or the different rate of water loss after the artificial watering (A, C samples only). The quantity of water still present in the samples at 70% RH might have been sufficient to permit a minimal metabolic activity (Nash et al., 1990), *i.e.* some protection mechanisms were still functioning. This hypothesis is reinforced by the recent experimental evidence of Fernández-Marín et al. (2013), who demonstrated that in the moss *Syntrichia ruralis* a residual enzymatic activity is actually detectable down to 15% relative water content calculated as (fresh weight-oven dry weight)/oven dry weight.

In vascular plants, the functional cooperation of the AsA/GSH cycle reactions is a key factor in order to understand the effective detoxification mechanism of ROS in presence of moderate stress conditions (Rao et al., 1995). It is generally accepted that ascorbate and glutathione are associated with the cellular redox balance. In the meantime, both can act as signals for the regulation of antioxidant mechanisms and for the preservation of a wide range of metabolic processes. Unfortunately, the knowledge gained in 30 years of research on vascular plants cannot be automatically extended to lichens, also because information from this side is still

too scanty. In particular, the effects of combined abiotic stressors on the AsA/GSH cycle have never been studied in detail. The few works available so far in fact dealt with the content change of a single compound (Caviglia and Modenesi, 1999; Kranner, 2002) and related enzymes (Deltoro et al., 1999; Weissmann et al., 2005a). However, we can argue that similarly to vascular plants, lichens cannot "distinguish" among different forms of stress, but they actually recognize the derived oxidative damage, and therefore they can respond activating the same enzymatic and non-enzymatic antioxidants mechanisms.

In response to the initial key questions, we can conclude that: (i) thalli of *F. caperata* react to $O_3$-induced oxidative stress in the same manner at physiological, cytochemical and biochemical level, indifferently whether their metabolism is fully active or not; (ii) air humidity is actually a modulating factor that can modify and/or alter the antioxidant mechanisms of lichens, in particular the GSH system; (iii) although ROS production mostly derives from the process of desiccation, high $O_3$ levels can further increase it, especially in the mycobiont; (iv) all enzymes related to the AsA/GSH cycle respond to $O_3$ independently to air humidity and watering regimes, their stimulation probably being due to the reduction of AsA that acts as a radical scavenger.

In conclusion, as supposed by Bertuzzi et al. (2013), *F. caperata* is actually $O_3$-tolerant thanks to the high levels of constitutive natural, enzymatic and non-enzymatic, defences against ROS derived from the frequent dehydration–rehydration cycles to which all lichens are naturally subjected. Possible differences linked to ecological and habitat preferences of lichen species will be discussed in a forthcoming paper.

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