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Physiological and ultrastructural effects of acute ozone fumigation in the lichen *Xanthoria parietina*: the role of parietin and hydration state

Andrea Vannini[1] · Luca Paoli[1] · Sara Ceccarelli[1] · Sergio Sorbo[2] · Adriana Basile[3] · Vincenzo Carginale[4] · Cristina Nali[5] · Giacomo Lorenzini[5] · Mario Pica[6] · Stefano Loppi[1]

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Abstract The physiological and ultrastructural effects induced by acute exposure to ozone (O_3) were investigated in the lichen *Xanthoria parietina*. Our working hypothesis was that parietin content and hydration of the thalli may play a role in the modulation of the effects of O_3 exposure. Four batches of *X. parietina* samples, dry and wet, with (P+) and without (P−) parietin, were fumigated for 1 h with 3 ppm O_3. The effects of O_3 were assessed immediately after the fumigation and after one week of recovery under controlled conditions. O_3 fumigation caused physiological and ultrastructural impairment both to the photobiont and the mycobiont, irrespective if samples were fumigated wet or dry, and P+ or P−. However, one week after fumigation, a recovery was observed in P+ samples for the photobiont and in dry samples for the mycobiont. We suggest that the hydration state may play a major role in determining the severity of the damage, while the presence of parietin may promote the recovery. Our results provide physiological and ultrastructural basis to explain the ecological insensitivity of lichens to high environmental levels of ozone occurring during dry Mediterranean summers.

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✉ Stefano Loppi
stefano.loppi@unisi.it

[1] Department of Life Sciences, University of Siena, Siena, Italy
[2] CeSMA, University of Napoli, Naples, Italy
[3] Department of Biology, University of Napoli, Naples, Italy
[4] National Research Council (CNR), Naples, Italy
[5] Department of Agriculture, Food and Environment (DAFE), University of Pisa, Pisa, Italy
[6] Bioredox, Rome, Italy

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Introduction

Ozone (O_3) is a strongly oxidizing air pollutant originating from photochemical solar radiation driven reactions between anthropogenic or biogenic gases, and is known to be harmful to all forms of life (WHO 2008). Lichens seem an exception to this general rule of thumb, and several field studies failed to find any correlation between this air pollutant and the biodiversity of epiphytic lichens (Gombert et al. 2004, 2006; Nali et al. 2007), suggesting that lichens are not suitable bioindicators of the effects of O_3, mostly because high O_3 atmospheric concentrations occur in summer, when lichens are mostly dehydrated and hence in a reduced metabolic state (Lorenzini et al. 2003). Also laboratory investigations confirmed the resistance of these organisms to environmental levels of O_3. Riddell et al. (2010, 2012) did not find physiological effects on the photosynthesis of several lichen species fumigated with 10–120 ppb of O_3 for a few days. Pellegrini et al. (2014) exposed *Flavoparmelia caperata* to a relatively high O_3 concentration (250 ppb, 5 h per day for 2 weeks) and did not report negative effects on the photosynthetic apparatus. Similarly, Bertuzzi et al. (2013) fumigated the lichens *X. parietina*, *F. caperata,* and *Parmotrema perlatum* in open top chambers with a daily O_3 concentration of 40 ppb for several weeks and did not find any apparent damage to the photosynthetic system. In all these works, lichens were exposed to realistic O_3 concentrations, comparable or slightly higher than the background values reported for the Northern hemisphere (Virganzan 2004; Laurila and Lättilä 1994; WHO 2008). These environmental concentrations are probably too low to cause significant adverse physiological effects on

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lichens. However, several studies were run fumigating several lichen species with much higher (>1 ppm) O_3 concentrations. Also, in these cases, no effects on the photosynthetic system were detected (Brown and Smirnoff 1978; Nash and Sigal 1979), supporting the idea that lichens are not susceptible to O_3.

Lichens synthesize peculiar secondary metabolites, which protect the photosynthetic apparatus against UV radiations, have a strong antioxidant power (Hill and Woolhouse 1966; Hidalgo et al. 1994) and are probably responsible for the protection against the oxidant activity of O_3. Parietin is the main secondary compound of *X. parietina* and, being located in the upper cortex, protects the photobiont against strong light irradiance (Solhaug et al. 2002). Parietin concentration undergoes a seasonal variation directly correlated with solar irradiance (Gauslaa and Ustvedt 2003). Parietin has attracted much interest over time, especially in medicine, leading to the appraisal of its antibacterial and anticancer activity (Bačkorová et al. 2012; Basile et al. 2015). Considering that lichens may produce several antioxidant compounds, a hypothetical role of parietin as antioxidant against O_3 is worthy of being investigated.

Our working hypothesis was that parietin content and the hydration state of the thalli may play a role in the modulation of the effects of exposure to O_3. To test this hypothesis, samples of *X. parietina* were fumigated with a high O_3 concentration, under both dry and wet conditions, and with or without parietin. The effects of O_3 were assessed at physiological and ultrastructural level, immediately after the fumigation as well as after 1 week of recovery under controlled conditions.

Material and methods

Samples of the lichen *Xanthoria parietina* (L.) Th. Fr., a species commonly used in biomonitoring studies (Loppi et al. 2006), were collected in a rural area of Tuscany (Italy) far removed from local sources of air pollution (43°14′07″ N, 11°20′26″ E, Ville di Corsano, Siena). Samples were harvested from the branches of *Ulmus* trees, at ca. 1.5 m from ground. Within 1 h from collection, the material was placed in a climatic chamber at 15 °C, RH 60%, photoperiod of 12 h at 40 μmol m^{-2} s^{-1} photons PAR. In the laboratory, samples were cleaned with nylon forceps under a stereoscopic microscope, to remove extraneous material.

Prior to the treatments, parietin was removed from 50% of the samples, following the recommendations of Gauslaa and McEvoy (2005): dry lichen thalli were immersed six times for 5 min in 50 mL 100% acetone buffered with $CaCO_3$ and were then air-dried for 30 min at room temperature, to allow complete acetone evaporation.

Dry samples with (P+) or without (P−) parietin were left in the dark overnight in a climatic chamber at 16 °C and RH 55%; in these conditions, lichen samples were characterized by a residual water content <10%. Humid samples (P+ and P−) were sprayed with deionized water and left in the dark overnight in a climatic chamber at 16 °C and RH 90%; wet samples were characterized by a water content up to 240% (calculated as % ratio between the weight of wet and dry samples).

Lichen samples were fumigated for 1 h at an O_3 concentration of 3 ppm (3000 ppb), using an ozone generator (GPC2000, Ozonosoluzioni, Italy). Control samples were treated for 1 h with ozone-free air. Each experiment was replicated three times. To evaluate the possible recovery, after the treatment, lichen samples were placed inside Petri dishes and stored at ambient conditions for 1 week.

Only the peripheral parts of the thalli, i.e., those most active metabolically (generally up to 5 mm from lobe tips), were selected for the analysis. All the experiments, including the collection of the samples, were carried out during January 2016.

Physiological parameters

The following parameters were measured to assess the physiological status of the samples: content of chlorophylls *a* and *b*, chlorophyll integrity, photosynthetic efficiency, content of ergosterol, and content of reactive oxygen species (ROS).

Content of chlorophylls *a* and *b*

About 20 mg of lichen material were dissolved in 1 mL of ethanol and filtered at 0.45 μm. 50 μL of the resulting supernatant were promptly analyzed by HPLC (Waters LC I Plus) using an Ascentis Supelco column as separator (5 μm C18, 250 × 4.6 mm). The concentration of chlorophylls *a* and *b* was determined according to Suzuki et al. (1993) using water/methanol/acetone as mobile phase with a flow rate of 1 cm^3 min^{-1}. Runs were monitored at 440 nm. Quantification was performed with a calibration curve from Sigma-Aldrich (USA). Three replicates were measured each treatment.

Chlorophyll integrity

Photosynthetic pigments were extracted in DMSO as described by Pisani et al. (2007). Absorbance of the extracts was measured using a UV–visible spectrophotometer (Agilent 8453). Chlorophyll integrity was expressed by the ratio between the absorbance at 435 and 415 nm (OD_{435}/OD_{415}). A decrease of this ratio from the value of 1.4 reflects chlorophyll *a* degradation to phaeophytin *a* (Garty et al. 2000). Three replicates were measured for each treatment.

Photosynthetic efficiency

The photosynthetic efficiency of the samples was assessed in terms of chlorophyll *a* fluorescence emission, by the classical physiological indicator F_V/F_M, representing the potential quantum yield of primary photochemistry, where $F_V = (F_M - F_0)$ is the variable fluorescence and F_0 and F_M are minimum and maximum chlorophyll *a* fluorescence. Samples were lightened 1 s with a saturating 3000 μmol m^{-2} s^{-1} light pulse of red light and fluorescence emission was recorded for 1 s. Samples were measured with a Plant Efficiency Analyzer (Handy PEA, Hansatech, Norfolk, UK). Up to 10 replicates were recorded for each treatment.

Ergosterol content

Each lichen sample (50 mg) was put into test tubes and 1 cm^3 of absolute ethanol was added. Samples were homogenized with Ultraturrax for 1 min, centrifuged at 20,000 g for 5 min and filtered at 0.45 μm; samples were kept at 4 °C until use. Ergosterol absorption was measured with HPLC (Agilent 1100 Series). Ergosterol was separated with an Agilent C18 column (25 × 4.6 mm) using methanol as mobile phase at the flow rate of 1 cm^3 min^{-1} and then read at 280 nm (Dahlman et al. 2002). Three replicates were measured for each treatment.

ROS content

A fluorescent technique using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) has been used for quantitative measurement of ROS production in lichen samples. DCFH-DA is deesterified intracellularly and turns to nonfluorescent 2′,7′-dichlorofluorescin (DCFH). DCFH is then oxidized by ROS to highly fluorescent 2′,7′-dichlorofluorescein (DCF) (LeBel et al. 1990). Briefly, samples were immediately frozen in liquid nitrogen and ground thoroughly with prechilled mortar and pestle. The resulting powder (150 mg) was then resuspended in TrisHCl 40 mM pH 7.4, sonicated and centrifuged at 12,000 g for 30 min. The supernatant (500 μL) was collected and protein content determined. An aliquot (0.01 cm^3) of each sample was incubated with 5 μM DCFH-DA for 30 min at 37 °C followed by recording of the final fluorescence value, which was detected at excitation (488 nm) and emission (525 nm) wavelength. DCF formation was quantified from a standard curve (0.05–1.0 μM).

Transmission electron microscopy

Before preparation for TEM analysis, lichen samples were hydrated for 24 h on filter paper wet with deionized water. Specimens underwent fixation with glutaraldehyde 3% for 2 h at room temperature, post-fixation with osmium tetroxide 1% for 1.5 h at room temperature, dehydration with ethanol to propylene oxide and embedment in Spurr's epoxy medium. Ultrathin sections (50 nm), obtained by ultramicrotomy, were collected on copper grids and stained with uranyl acetate and lead citrate. A FEI EM 208S transmission electron microscopy (TEM) (FEI, Eindhoven, NL), with an accelerating voltage of 80 kV, was used for observations. The image analysis of the cellular ultrastructural features within a median section of algal and fungal cells was made on electron micrographs by the software program AnalySIS (FEI, Eindhoven, NL). Ten ultrathin sections were examined for each treatment.

Statistical analysis

For the interpretation, data were normalized calculating the percentage ratio between values after O_3 exposure to that of control samples. A two-way ANOVA was run to check for statistically significant differences ($P < 0.05$) between treatments, using the Tukey HSD test for post-hoc comparisons. Prior to the analysis, data were subjected to angular arcsin transformation, as requested for percentage values. Data not matching a normal distribution (Kolmogorov–Smirnov test, 95% confidence interval) were processed using the Box-Cox transformation.

Results

Physiological parameters

With the sole exception of chlorophyll *b* in wet samples, O_3 fumigation caused a marked decrease in all the investigated physiological parameters of *X. parietina*, both in the humid and dry state, as well as with or without parietin, but with a different sensitivity according to the parameters (Table 1). In general, dry samples were more severely affected than wet ones. After the treatment, dry samples, irrespective if P+ or P−, showed a significantly lower content of chlorophylls *a* and *b*, paralleled by a higher chlorophyll degradation. In wet samples, irrespective whether P+ or P−, the alteration of chlorophylls *a* and *b* and the degradation of chlorophylls were less marked. Photosynthetic efficiency and the content of ergosterol were affected by O_3 regardless of the hydration state and parietin content. ROS content was significantly higher in wet samples, especially in those P−. No interaction emerged between hydration and parietin.

One week after the O_3 fumigation, the physiological situation was quite different (Table 2), with some parameters that returned to the original state of the controls. This was the case of $OD_{435/415}$ in P+ samples, photosynthetic efficiency in wet and P+ samples, and of ergosterol in dry samples. Notably, all values of chlorophyll *b* were significantly lower than the control. The presence or the absence of parietin was the major

Table 1 Physiological parameters of the lichen *Xanthoria parietina* after exposure to 3 ppm O_3 for 1 h. Results are expressed as % respect to control samples

P	H	Chl *a*	Chl *b*	$OD_{435/415}$	F_V/F_M	Ergosterol	ROS
+	−	0.8 ± 0.1^a	36 ± 5^a	68 ± 5^a	31 ± 23	59 ± 10	44 ± 12^a
+	+	26 ± 16^b	*84 ± 27^b*	77 ± 5^b	14 ± 13	47 ± 16	240 ± 68^b
−	−	0.9 ± 0.1^a	31 ± 22^a	67 ± 5^a	29 ± 23	47 ± 4	124 ± 37^c
−	+	60 ± 29^b	*85 ± 22^b*	78 ± 4^b	25 ± 22	42 ± 8	619 ± 138^d
	H	*	*	*	–	–	–
	P	–	–	–	–	–	–
	H:*P*	–	–	–	–	–	–

In each column, different letters indicate statistically significant ($P < 0.05$) differences. Values in italics are not different from control values. ANOVA results $*P < 0.05$

H hydration, *P* parietin, *H*:*P* interactive hydration:parietin effects

driver of photobiont parameters, while hydration state was the most important factor for the mycobiont. It is noteworthy that in P− samples both chlorophylls *a* and *b* have almost disappeared. ROS content was again lower than in controls in wet and P+ samples. The photosynthetic efficiency was the only parameter responding to the combined effect of hydration and parietin content.

Ultrastructure

In ozone-untreated samples (Fig. 1a–d), the algal cells showed a typical ultrastructure, with a thick cell wall surrounding the protoplast, most of which was occupied by a large chloroplast with wavy thylakoids and a central pyrenoid with pyrenoglobules. Mitochondria had a typical appearance, with numerous clear cristae in an electron dense matrix (Fig. 1b, d). The fungal cells showed membranes and vacuoles in an electron clear cytoplasm.

Ozone fumigation caused a severe damage to the cell ultrastructure in both wet and dry samples (Fig. 1e–l). The strongest effects occurred in samples without parietin (Fig. 1g–h, k–l). Dry P+ samples were those less damaged by ozone (Fig. 1i) and still showed the development of some reactive ultrastructure, such as multivesicular bodies (Fig. 1 j).

One week after ozone fumigation, the less damaged samples were those dry P+ (Fig. 2g–j), which showed a partial recovery of the wild ultrastructure. The algal cell had the typical wild ultrastructural features, but distinctive abnormal features occurred. The cytoplasm developed vacuoles containing membrane bodies; abnormal thylakoid gatherings appeared inside the pyrenoid. The mitochondria still exhibited clear cristae inside an electron dense matrix, like untreated samples. In all the other samples, the algal protoplasts appeared condensed, electron densed, plasmolyzed and vacuolated. The typical ultrastructural features were missing (Fig. 2c–f, k–l).

Discussion

Despite their well-known sensitivity to a wide array of air pollutants, lichens are not regarded as good indicators of the biological effects of O_3 (Lorenzini et al. 2003). However, our study has shown adverse physiological effects both to the photobiont and the mycobiont of *X. parietina* fumigated for 1 h at 3 ppm O_3, i.e., at a concentration ca. 16 times higher than the maximal O_3 concentration detected in central Europe during the hottest summer hours (ca. 189 ppb; EEA 2015).

Table 2 Physiological parameters of the lichen *Xanthoria parietina* after 1 week from exposure to 3 ppm O_3 for 1 h. Results are expressed as % respect to control samples

P	H	Chl *a*	Chl *b*	$OD_{435/415}$	F_V/F_M	Ergosterol	ROS
+	−	13 ± 8^a	53 ± 27^a	*91 ± 10^a*	32 ± 30^a	*95 ± 10^a*	68 ± 21^a
+	+	17 ± 14^a	22 ± 17^a	*99 ± 2^a*	*106 ± 6^b*	18 ± 7^b	188 ± 58^b
−	−	1.1 ± 0.1^b	2.1 ± 0.4^b	67 ± 2^b	10 ± 8^a	*82 ± 39^a*	187 ± 57^b
−	+	0.9 ± 0.1^b	1.7 ± 0.2^b	77 ± 6^b	20 ± 16^a	12 ± 2^b	200 ± 65^b
	H	–	–	–	–	*	–
	P	*	*	*	*	–	–
	H:*P*	–	–	–	*	–	–

In each column, different letters indicate statistically significant ($P < 0.05$) differences. Values in italics are not different from control values. ANOVA results $*P < 0.05$

H hydration, *P* parietin, *H*:*P* interactive hydration:parietin effects

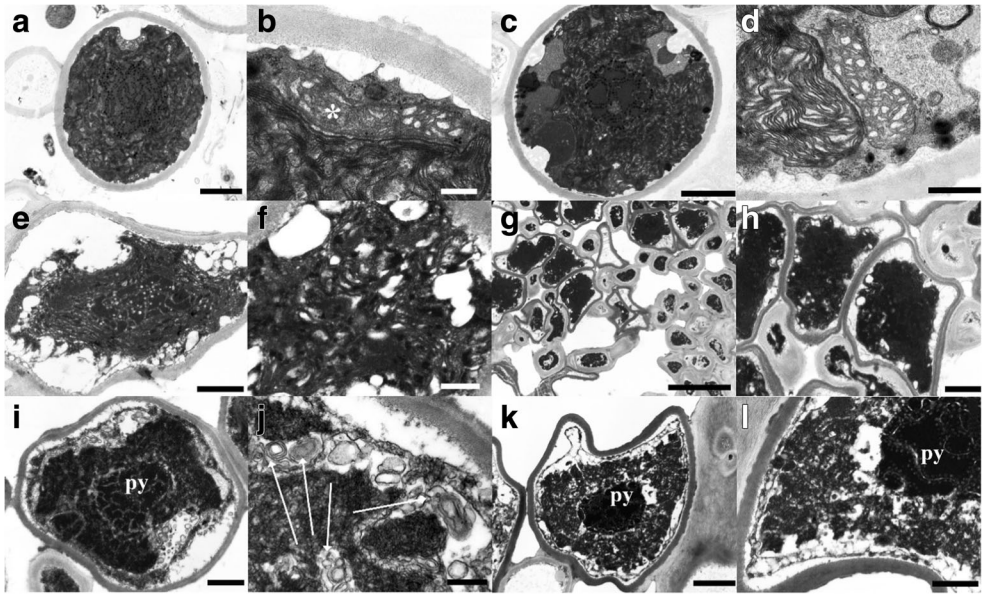

Fig. 1 TEM micrographs of *Xanthoria parietina* samples from untreated (**a**–**d**), and ozone-treated samples (**e**–**l**). **a**–**b** Ozone-untreated P+ samples. **a** A cell wall-surrounded green algal cell with most of the protoplasm occupied by the chloroplast. **b** A mitochondrion (see *asterisk*) with evident cristae and electron dense matrix, next to the wavy thylakoids of a chloroplast. (**c**–**d**) Ozone-untreated P− samples. **c** A cell wall-surrounded green algal cell with a large, central chloroplast. **d** Next to the wavy thylakoids of a chloroplast, a section of a mitochondrion shows well visible cristae and electron dense matrix. **e**–**f** Wet, ozone-treated P+ samples. **e** The algal cell appears heavily plasmolyzed and shows a shrunk, vacuolated protoplast. **f** Details of the heavy vacuolization affecting the electron dense protoplast in an alga. **g**–**h** Wet, ozone-treated P− samples. **g** The medulla of the lichen shows cells with electron dense, plasmolyzed protoplasts. **h** Algal cells with shrunk, electron dense protoplasts, where no typical ultrastructure organization is recognizable. Peripheral clear vacuoles are visible. **i**–**j** Dry, ozone-treated P+ samples. **i** In the algal cell, a central pyrenoid (py) within chloroplast remnants and a highly vacuolated cytoplasm are barely recognizable. **j** The cytoplasm shows multivesicular bodies (*arrows*) and vesicles. **k**–**l** Dry, ozone-treated P− samples. The algal cell shows a central, electron dense pyrenoid (*py*) surrounded by chloroplast remnants. The heavily vacuolated cytoplasm has an almost "empty" appearance. Scale bars: 5 μ (**g**); 2 μ (**a**, **c**, **k**); 1 μ (**e**, **h**, **i**, **l**); 500 nm (**d**, **f**); 300 nm (**b**, **j**)

When samples were fumigated in a dry state (water <10%), irrespective if with or without parietin, chlorophyll degradation occurred and the content of chlorophylls *a* and *b* was greatly reduced. We hence suggest that water may play a significant role in limiting the diffusion of O_3 through the lichen cortex, hence reducing its interaction with the algal cells. Previous studies reported that gas diffusion through the cortex could be limited at a water content of 150–200% (as in the case of our wet samples), due to the formation of a water film that acts as a barrier (Green et al. 1981), and to the swelling of the cells and the cell wall, further limiting air spaces and gas exchange (Snelgar et al. 1981). In *X. parietina*, gas exchange is determined by gas diffusion through the cortex. In fully hydrated conditions, the amount of air spaces (18% of the total volume) may be reduced, hence generating resistance to gas diffusion (Collins and Farrar 1978). This is consistent with the findings of Scheidegger and Schroeter (1995) that fumigated several lichen species with ecologically relevant O_3 concentrations (up to 85 ppb for 80 days) and reported a physiological impairment to the photosynthetic apparatus after the fumigations. The effect was attributable to the hydration state of the thalli, suggesting that when lichens are metabolically active, they are more susceptible. Therefore, when thalli are extremely dry or extremely hydrated, O_3 fumigation may lead to insignificant injury probably because of the limited photosynthetic and respiratory activity.

Lichens are poikilohydric organisms; their water content is strictly related to water availability in the environment and their metabolism, meant as net photosynthetic rate (NPR), is regulated by the water content inside the thallus (Lange and Matthes 1981). Specifically, the NPR may increase with the water content inside the thallus until a species-specific maximal value, generally in the range 30–60% (Lange and Green 2005). When the water content increases above this threshold, NPR decreases proportionally to the increase of water content (Green et al. 1981). Hence, the excess of water limits gas diffusion through the lichen cortex and reduces the metabolic activity of the thallus (Green and Snelgar 1981) causing a depression of CO_2 uptake (Green et al. 1981) as a consequence of the increasing resistance to CO_2 to the sites of carboxylation (Lange and Tenhunen 1981). Consequently, we may argue that also in the case of O_3, the uptake could be reduced when water content in the thallus increases.

In our study, O_3 fumigation decreased chlorophyll *a* fluorescence emission in *X. parietina* by about 50% regardless of thallus hydration and parietin content. Similarly, decrease of chlorophyll *a* fluorescence emissions were observed in five out of seven lichen species fumigated with an O_3

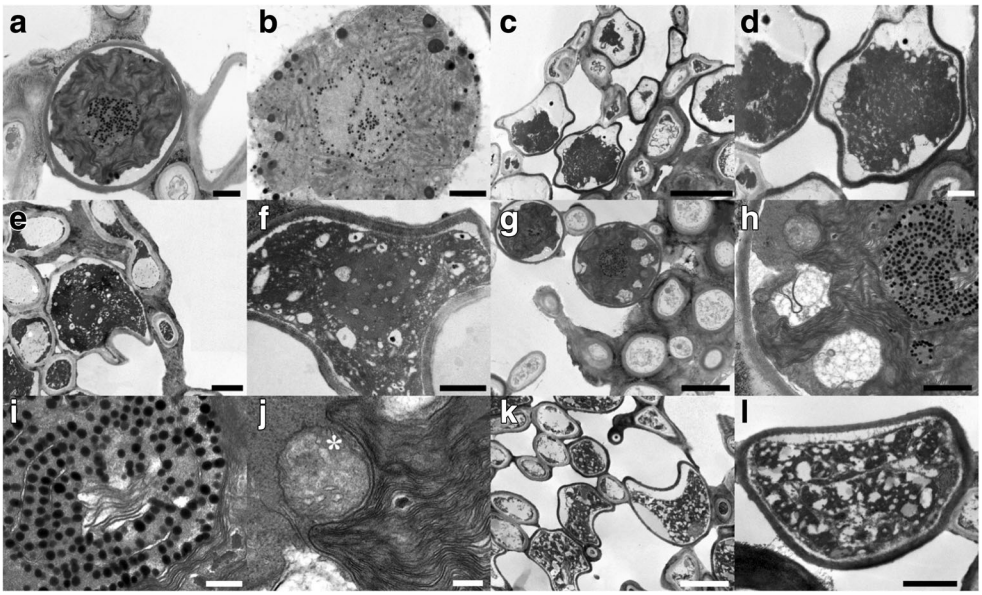

Fig. 2 TEM micrographs of *Xanthoria parietina* samples from recovered untreated (**a**–**b**), and ozone-treated samples (c–l). Ozone-untreated P+ samples. **a** A wild-looking alga surrounded by fungal cells. Ozone-untreated P− samples. **b** The algal cell shows a pyrenoid with unevenly arranged pyrenoglobules and lipid droplets. **c**–**d** Wet, ozone-treated P+ samples. **c** The lichen medulla shows severely plasmolyzed algal and fungal cells. **d** The algal protoplasm appears condensed, plasmolyzed and peripherally vacuolated; the typical ultrastructure is not recognizable. (**e**–**f**) Wet, ozone-treated P−samples. **e** The medulla shows severely altered algal and fungal cells, where the typical organization is not still recognizable. Some cells appear empty. **f** The protoplast of the algal cell appears condensed and vacuolated and the typical ultrastructure is not recognizable. (**g**–**j**) Dry, ozone-treated P+ samples. **g** The medulla shows algal cells with protoplasts featured by a recognizable ultrastructure. **h** The protoplast of the algal cell shows, in addition to a wild-looking pyrenoid and thylakoid system, large cytoplasmic vacuoles containing membrane bodies. **i** Details of a pyrenoid where, in addition to wild-like thylakoids and pyrenoglobules, an abnormal thylakoid gathering is shown. **j** A wild-looking mitochondrion (see *asterisk*) with cristae in an electron dense matrix, next to the wavy thylakoids of a chloroplast. (**k**–**l**) Dry, ozone-treated P−samples. **k** The medulla shows heavily plasmolyzed and vacuolated or even almost empty cells. **l** The algal cell shows a condensed and vacuolated protoplast, where the typical ultrastructure is not yet recognizable. Scale bars: 5 μ (**c**, **g**, **k**); 2 μ (**e**, **l**); 1 μ (**a**, **b**, **d**, **f**, **h**); 300 nm (**i**, **j**)

concentration of 90 ppb for 6 weeks (Scheidegger and Schroeter 1995) and in several plant leaves exposed to O_3, as reported, e.g., for pumpkins (Ciompi et al. 1997), beans (Schreiber et al. 1978), and poplars (Soldatini et al. 1998). Significant reductions of chlorophyll *a* fluorescence were also observed in clones of *Fagus sylvatica* during prolonged exposures (Paoletti et al. 2007).

Also the mycobiont of *X. parietina* was affected by O_3 fumigations. Ergosterol is the main sterol of fungal cell membranes and is commonly used as an indicator of mycobiont vitality, interpreted as metabolically active membranes and cell membrane integrity (Ekblad et al. 1998; Sundberg et al. 1999). Our results showed ergosterol reductions by ca. 50% both in dry and wet samples and regardless of parietin content, confirming the susceptibility of this sterol to O_3 exposures, as already suggested by Takamiya (1929). Recent observations indicated that O_3 is not harmful to the membrane integrity of lichens (Riddell et al. 2010, 2012); however, it may alter membrane permeability in plants, e.g., in clones of *F. sylvatica* at the concentration of 150 ppb (Paoletti et al. 2007), in leaves of *Phaseolus vulgaris* after a fumigation with 0.6 ppm for 1 h (Evans and Ting 1973), in spring barley and winter rape treated with 180 ppb already after 6 h of exposure (Plazek et al. 2000). ROS represent partially reduced or excited forms of atmospheric oxygen that can react with different cellular components and cause their oxidation. In plants, they can be generated in normal metabolic pathways, e.g., as a result of electron transport chains in chloroplast and mitochondria, and in the peroxisome, which mainly produces H_2O_2 as a byproduct of photorespiration. However, adverse conditions, including abiotic and biotic stress, can significantly accelerate the generation of ROS at cellular level leading to different alterations, such as protein carbonylation and DNA damage, activation of kinase cascades, and transcription factors, which ultimately affect cellular essential metabolic activities and viability.

One week after the treatment, a partial recovery occurred to the lichen photobiont, especially if fumigated when wet, in P+ samples, as well as to the lichen mycobiont in samples fumigated in the dry state, irrespective if P+ or P−. The recovery of the photobiont in P+ samples was highlighted by a rise in chlorophyll integrity and photosynthetic efficiency. Parietin may thus have a positive role for the recovery of the functionality of the photosynthetic machinery in *X. parietina*. Parietin

protects the photobiont from strong light irradiance and any reduction of the normal amount of parietin may cause an impairment of the protection system for which parietin is responsible. The damage to the photobiont in P− samples could also be caused by the higher rate of ROS production.

One week after, the cell membrane integrity of the mycobiont showed a recovery dependent to the hydration state, with ergosterol concentrations recovered only in dry samples, while the effect of O_3 progressed in wet samples, resulting in further reductions by about 30% of the ergosterol content. The ability of the fungal partner to restore its membrane integrity when fumigated under dry conditions is probably due to the fast disappearance of O_3 in the air spaces of the medulla after the treatment, while the progression of the effects in wet fumigated samples is probably due to the dissolution of O_3 in the water layer in the lichen cortex, leading to an increase of the toxic effects on the mycobiont.

In water, O_3 has a half-life ranging from seconds to hours; however, the interaction with organic matter dissolved in water can lead to oxidations or generate superoxide radicals, increasing the production of OH radicals (Von Gunten 2003). The production of OH radicals may have generated those further effects on the plasma membrane of the mycobiont, further affecting the ergosterol content, as also suggested by the higher ROS values found in wet fumigated samples. However, in general, the ability of the mycobiont to restore its physiological status even if the photobiont was damaged suggests that the fungal partner may compensate to support its membrane integrity, independently of the physiological status of the photobiont. Since the mycobiont constitutes up to 98% of the lichen biomass, this result may provide a physiological basis for the ecological insensitivity of lichens to environmental levels of O_3.

As for the ultrastructural changes, ozone fumigation caused an overall damage to the cell ultrastructure in all samples, irrespective if wet or dry, and with or without parietin. The less damaged samples, both immediately after O_3 treatment and after the recovery time, were those dry P+, characterized by a healthier visual aspect of membranes. This could be related to the content of ROS, which, as a matter of fact, was always the lowest in samples dry and with parietin. Oxidative stress and, above all, ROS are well-known agents responsible for lipid peroxidation (Farmer and Mueller 2013). So, we can suggest that the less damaged appearance of samples dry and P+ could be related to their lower ROS content and the consequent less severe damage to biological membranes. This is also confirmed by the fact that dry and P+ samples were still able to develop reactive ultrastructures, such as vacuoles and multivesicular bodies. In fact, oxidative stress can also account for our finding of multivesicular bodies and vacuolization in fumigated samples. Similar structures were also reported in antimony- or glyphosate-treated *X. parietina* samples (Paoli et al. 2013; Vannini et al. 2016). Plant vacuoles are known to be involved in autophagy phenomena and are reported to be a major site for the degradation of macromolecules (Thompson and Vierstra 2005; Bassham 2009). Autophagy is enhanced upon stress, for recycling and degradation of damaged proteins and organelles (Bassham 2009; Hayward et al. 2009), which could explain the finding of vacuoles containing membrane bodies with an appearance of autophagosomes. That is in agreement with similar finding also reported in other plant species under stress conditions (An et al. 2006; Basile et al. 2012). Furthermore, the same multivesicular bodies found in our samples have already been related to autophagic and endocytic phenomena (Thompson and Vierstra 2005; Todeschini et al. 2011). After 1 week a partial recovery of the ultrastructure was observed in dry P+ samples.

Conclusions

Short-term fumigations with 3 ppm O_3 caused physiological and ultrastructural impairment both to the photobiont and the mycobiont of the lichen *Xanthoria parietina,* irrespective if samples were fumigated wet or dry, and with or without parietin. However, 1 week after the fumigation, a recovery was observed in P+ samples for the photobiont and in dry samples for the mycobiont. We suggest that parietin content may play a major role for the recovery of the thalli after the exposure to very high ozone concentrations, especially when the thalli are in a dry state. The interaction between ROS production and ultrastructural damage requires further investigation. Our results provide physiological and ultrastructural basis to explain the ecological insensitivity of lichens to high environmental levels of ozone occurring during dry Mediterranean summers.

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