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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.

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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

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