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Chlorophyll *a* fluorescence emission, xanthophyll cycle activity, and net photosynthetic rate responses to ozone in some foliose and fruticose lichen species

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Abstract

The lichens *Parmelia quercina*, *Parmelia sulcata*, *Evernia prunastri*, *Hypogymnia physodes*, and *Anaptychia ciliaris* were exposed to ozone (O_3) in controlled environment cuvettes designed to maintain the lichens at optimal physiological activity during exposure. Measurements of gas exchange, modulated chlorophyll (Chl) fluorescence, and pigment analysis were conducted before and after exposure to 300 $mm^3(O_3)\ m^{-3}$, 4 h per d for 14 d. No changes in the efficiency of photosystem 2 (PS2) photochemistry, the reduction state of Q_A, or the electron flow through PS2, measured by Chl fluorescence, were detected in any of the five lichen species studied. Additionally, neither photosynthetic CO_2 assimilation nor xanthophyll cycle activity or photosynthetic pigment concentration were affected by high O_3 concentrations. Thus the studied lichen species have significant capacities to withstand oxidative stresses induced by high concentration of O_3.

Additional key words: *Anaptychia*; antheraxanthin; *Evernia*; *Hypogymnia*; *Parmelia*; photosystem 2; quantum efficiency; violaxanthin; zeaxanthin.

Introduction

Tropospheric ozone is a major regional air pollutant over wide areas downwind of urban and industrialised areas of the world. The adverse effects of ozone on growth and yield of agricultural crops and native plants have been documented (Lefohn 1992, U.S.E.P.A. 1996, Davison and Barnes 1998). The mechanisms of ozone toxicity to plants have not been completely defined, but ozone or its free radical by-products impair membrane functions, leading to declines in photosynthesis, accelerated foliar senescence, and premature leaf abcission (Farage *et al.* 1991, Pell *et al.* 1992, Moldau *et al.* 1993, Heath 1994, Soldatini *et al.* 1998). In contrast, research into the effects of ozone on lichens has not led to a coherent synthesis regarding mechanisms of toxicity or its potential for adverse effects on these organisms. Significant reductions in lichen species diversity and symptoms of thallus injury were observed in the field in areas where ozone produces visible injury symptoms and reduces growth of vascular plants (Nash and Sigal 1979, Sigal and Johnston 1986, Barreno *et al.* 1999). However, a low sensitivity of lichens to ozone in the laboratory has been reported (Rosentreter and Ahmadjian 1977, Brown and Smirnoff 1978, Ross and Nash 1983, Bates *et al.* 1996). Various effects, sometimes conflicting, were reported at physiological and structural levels (Egger *et al.* 1994, Scheidegger and Schroeter 1995, Balaguer *et al.* 1996).

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Abbreviations: Anth, antheraxanthin; Chl, chlorophyll; F_m, maximum fluorescence yield obtained with dark-adapted sample; F_m', maximum fluorescence yield in irradiated samples; F_0, minimum fluorescence yield in dark-adapted state; F_0', level of modulated fluorescence during a brief interruption of actinic irradiation in the presence of far-red irradiation; F_s, Chl fluorescence yield during irradiation; F_v, (F_m-F_0) variable fluorescence in dark-adapted thalli; NPQ, non-photochemical quenching from Stern-Volmer equation; q_p, photochemical fluorescence quenching coefficient; P_N, net photosynthetic rate; PS2, photosystem 2; RuBPCO, ribulose-1,5-biphosphate carboxylase/oxygenase; Vio, violaxanthin; Zea, zeaxanthin; Φ_{exc}, quantum efficiency of excitation energy capture by oxidised reaction centres of PS2; Φ_{PS2}, quantum efficiency of PS2.
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Because lichens are extraordinarily sensitive to environment, these laboratory exposures raise the question of whether the reported negative effects were the result of the lichens being in an unresponsive condition, or whether the adverse effects constituted a "false positive" due to unmeasured extraneous stress on the lichens.

In the present study we detected stress effects of O_3 on lichens by means of Chl fluorescence measurements, CO_2 gas exchange techniques, and pigment analyses. The combined study follows changes and possible damage to the photosynthetic apparatus. Our final aim was to clarify whether O_3 affects the photosynthetic performance of lichens. We used cuvettes and gas delivery systems specifically designed to maintain the lichens under optimal physiological conditions during the exposure (Deltoro *et al.* 1999).

Materials and methods

Lichens: Five of the most widespread and commonly occurring lichens in Mediterranean sclerophyllous oak-forest, *Parmelia quercina* (Willd.) Vainio, *Parmelia sulcata* Taylor, *Evernia prunastri* (L.) Ach., *Hypogymnia physodes* (L.) Nyl., and *Anaptychia ciliaris* (L.) Massal, were collected from Sierra del Toro (Castellón, Spain) remote from major local industrial and urban sources of pollution. This site has been used in the past as an un-polluted control (Calatayud *et al.* 1996) for experiments comparing responses of lichens collected from more polluted regions. On return to the laboratory, lichens were transferred to a climatic chamber and maintained at 200 μmol m^{-2} s^{-1}, 70 % relative humidity, 18/14 °C, 12/12 h photoperiod for 4 d before fumigation. The lichens were sprayed with distilled water once every morning to simulate the daily re-moistening cycle.

Fumigation system: The fumigation system was a modified version of that described in Deltoro *et al.* (1999) for exposing lichens to SO_2. The system consisted of four stainless steel teflon-coated cuvettes of an internal volume of 500 cm^3, fitted with transparent lids. The cuvettes were flushed with ambient air pumped through a wash bottle filled with distilled water at room temperature, to maintain a high humidity. An activated charcoal filter was placed immediately downstream from the air pump to remove background levels of pollutants. The humidity was regulated to a dew point of 15 °C (±0.1 °C), using a cold trap (*KF 18/2*, *Walz*, Germany) and a dew point measuring system (*TS-2*, *Walz*, Germany). After preconditioning, the air stream was split into two separate lines, control and ozonated, each one with two cuvettes. In the control line, one of the cuvettes was used for temperature and relative humidity monitoring whilst the other held the control plants during experiments. In the ozonated line, O_3 is generated by passing the air flow through a laboratory ozonator (UV-source, *SIR*, Spain). The O_3 concentration was monitored before entering the cuvettes with an O_3 analyser (*1008-RS*, *DASIBI Env. Corp.*, Glendale, CA, USA). The flow rate was maintained at 16.7 cm^3 s^{-1} with calibrated mass flow controllers (*5850 TR*-series, *Brooks Instruments*, The Netherlands). Temperature control inside the cuvettes was achieved by submersion of the cuvettes in a large, thermostatically controlled water bath set at 15±0.1 °C.

Fumigation experiments: For the fumigation, 0.5 g air dry material per cuvette was selected. Prior to the experiments, thalli were sprayed with distilled water and kept in the climatic chamber for 30 min. Subsequently, the thalli were shaken to remove externally adhering water and inserted in the cuvettes. Each cuvette contained six lichen thalli of the same species. Because one control cuvette was used for environmental monitoring, 6 control thalli and 12 O_3-exposed thalli were available for measurements. Experiments were conducted with 300 mm^3 m^{-3} ozone for 4 h daily during 14 consecutive days. Experimental conditions were 15±0.5 °C, 97±1 % relative humidity, and irradiance of 500 μmol m^{-2} s^{-1}. Upon conclusion of experiments, the thalli were returned to the climatic chamber. Although high by ambient standard, this exposure regime was selected to elicit a potentially significant O_3 response in the studied species.

Measurement of *in vivo* Chl *a* fluorescence: Chl fluorescence and gas exchange measurements were performed prior to and after 14-d exposure for each sample set (fumigated and control thalli); in this way, each sample set had its own control (measurements prior to the experiments). This allowed comparisons within and between sample sets so that the effects of ozone and/or experimental manipulation could be confidently differentiated. The Chl fluorescence values are means of 12 samples for ozonated thalli and 6 samples for control thalli. Chl *a* fluorescence at room temperature was measured with a portable pulse-modulated fluorometer (*PAM-2000*, *Walz*). Before fluorescence measurements, samples were kept in the dark for 15 min. The minimum (dark) fluorescence yield F_0 was obtained upon excitation of thalli with a weak measuring beam (14 μmol m^{-2} s^{-1}) from a light-emitting diode. The maximum fluorescence yield (F_m) was determined with a 600 ms saturating pulse of "white light" (4000 μmol m^{-2} s^{-1}). Variable fluorescence (F_v) was calculated as $F_m - F_0$. Following 2 min for dark re-adaptation, actinic "white light" [260 μmol(photon) m^{-2} s^{-1}] was switched on and saturating pulses were

applied at 60 s intervals for determination of the maximum fluorescence yield during actinic irradiation (F'_m), the modulated fluorescence during a brief interruption of actinic irradiation in the presence of far-red radiation (F_0'), and the Chl fluorescence yield during actinic irradiation (F_s). Calculation of quenching due to non-photochemical dissipation of absorbed radiant energy (NPQ) was determined at each saturating pulse, according to the equation NPQ = $(F_m - F_m')/F_m'$ (Bilger and Björkman 1991). The coefficient for photochemical quenching, q_p, was calculated as $(F_m' - F_s)/(F_m' - F_0')$ (Schreiber *et al.* 1986). The quantum efficiency of PS2 photochemistry, Φ_{PS2}, closely associated with the quantum yield of non-cyclic electron transport, was estimated from $(F_m' - F_s)/F_m'$ (Genty *et al.* 1989).

Gas exchange measurements: Six control and six O_3-exposed lichen thalli were removed from the cuvettes after the 4-h exposure. Net photosynthetic rate (P_N) was measured using clean compressed air, at an irradiance of 260 μmol m^{-2} s^{-1} and 20 °C. Prior to measurements, the species was adapted to the irradiance of the experiment to ensure photosynthetic induction. Measurements started with maximally hydrated plants and proceeded until CO_2 uptake ceased due to water deficit. The highest values obtained were considered for each sample. P_N was measured using an *LCA-4* (*ADC*, Hoddesdon, UK) infrared gas analyser operated in the differential mode. P_N response was calculated on a dry mass basis after drying at 105 °C for 24 h.

Chl and carotenoid estimation: The thalli used for pigment analysis were the same used for Chl fluorescence and P_N measurements. Pigment analysis was conducted on three hydrated control and fumigated thalli at the end of the fumigation. The samples were irradiated (260 μmol m^{-2} s^{-1}, 4 h), quick-frozen with liquid nitrogen, and ground with pure acetone and sodium ascorbate (Abadía and Abadía 1993). Pigments were separated and quantified by non-aqueous reverse-phase HPLC as described by De las Rivas *et al.* (1989). Pigment concentrations were expressed on a Chl *a* basis. The displacement of the xanthophyll pigments towards violaxanthin (Vio) or zeaxanthin (Zea) and antheraxanthin (Anth) is described by the de-epoxidation state of the xanthophyll pool or DPS = (Anth + Zea)/(Vio + Anth + Zea).

Statistical analysis: Significance of differences between control and treated samples before and after O_3 exposures was determined by analysis of variance followed by the least significance differences (LSD) test.

Results

P_N: After the 14-d treatment, P_N in control samples declined on average of 10 % for *P. quercina*, *P. sulcata*, and *H. physodes*, and did not decrease in *A. ciliaris*. P_N decreased by 43 % in control *E. prunastri* (Table 1). Thus, except for *E. prunastri*, the cuvette system was successful in maintaining relatively stable P_N in these thalli. Similarly, in lichen thalli exposed to O_3 for 14 d, P_N declined on average of 10.4 % in *P. quercina*, *P. sulcata*, and *H. physodes*, and 1.6 % in *A. ciliaris*, similar to decreases in control samples. Thalli of *E. prunastri* showed a 35 % reduction in P_N after 14 d of O_3 exposure, less than that of controls. Thus, exposure to O_3 had an apparent effect on P_N in these lichens.

Chl *a* fluorescence: At the end of fumigation (14 d) lichens did not show any visible injury symptoms. Table 2 shows the values of the ratio F_v/F_m, the actual quantum yield (Φ_{PS2}), and the photochemical (q_P) and non-photochemical quenching coefficients (NPQ) for control and ozonated lichen thalli at the end of a 15 min fluorescence induction kinetics. No significant changes for any parameter were detected between controls and fumigated lichens in any of the species studied.

Table 1. Means and standard deviations ($n = 6$) of CO_2 exchange [mol(CO_2) kg^{-1} s^{-1}] for control and ozone exposed samples [300 $mm^3(O_3)$ m^{-3} per 4 h and 14 d] of the studied lichen species. Analysis of variance (ANOVA) followed by the least significance difference (LSD) test, calculated at 95 % confidence level, was performed. Values followed by the same letter indicate no significant differences.

Species	Control		Exposed	
	0 d	14 d	0 d	14 d
Parmelia quercina	1.00a	0.88ab	0.82b	0.71b
Hypogymnia physodes	1.00a	0.91ab	0.82b	0.80b
Evernia prunastri	1.89a	1.07b	1.49a	0.97b
Parmelia sulcata	0.83a	0.76a	0.81a	0.67a
Anaptychia ciliaris	0.95a	0.82a	0.82a	0.81a

Pigment composition: No differences in Chl and carotenoid composition and in capacity for de-epoxidising Vio to Anth and Zea were detected in *P. quercina*, *H. physodes*, and *E. prunastri* (Table 3) as a result of ozonation.

Discussion

Under our experimental conditions, ozone did not induce significant stress on PS2 as measured by Chl fluorescence in any of the five lichen species studied. Additionally, neither photosynthetic CO_2 assimilation nor xanthophyll cycle activity or photosynthetic pigment concentration were affected by high concentrations of ozone. Measurements of fluorescence evaluate only a restricted portion of the thallus and therefore the absence of ozone effect on fluorescence parameters might be due to spatial inhomogeneities in the degree of ozone injury. Nonetheless, pigment analysis and gas exchange values represent measurements for the whole thallus and thus support the conclusion that O_3 did not injure the studied lichens.

Table 2. The maximum quantum yield (F_v/F_m), the quantum efficiency of PS2 photochemistry (Φ_{PS2}), and the photochemical (q_P) and non-photochemical (NPQ) quenching coefficients determined prior to and after a 14-d exposure for five species of lichens to 300 mm^3 m^{-3} ozone for 4 h. Values are means of 12 replications for ozone-exposed and of 6 for control thalli. Values followed by the same letter indicate no significant difference in each parameter for the same species of lichen.

Species		Control		Exposed	
		0 d	14 d	0 d	14 d
P. quercina	F_v/F_m	0.739a	0.724a	0.743a	0.681a
	Φ_{PS2}	0.346a	0.361a	0.378a	0.336a
	q_P	0.648a	0.713a	0.646a	0.698a
	NPQ	0.938a	1.147a	0.875a	1.121a
P. sulcata	F_v/F_m	0.735a	0.742a	0.743a	0.728a
	Φ_{PS2}	0.332a	0.340a	0.315a	0.288b
	q_P	0.673ab	0.701b	0.611a	0.612a
	NPQ	1.150a	1.384a	1.077a	1.472a
H. physodes	F_v/F_m	0.639a	0.652a	0.698a	0.624a
	Φ_{PS2}	0.270a	0.266a	0.308a	0.222a
	q_P	0.599a	0.641ab	0.717b	0.683ab
	NPQ	0.847a	1.006a	1.006a	1.396a
E. prunastri	F_v/F_m	0.689a	0.638a	0.696a	0.624a
	Φ_{PS2}	0.260a	0.248a	0.293a	0.222a
	q_P	0.528a	0.631a	0.569a	0.615a
	NPQ	0.805a	0.681a	1.914b	1.392b
A. ciliaris	F_v/F_m	0.743a	0.726a	0.752a	0.729a
	Φ_{PS2}	0.360a	0.303a	0.369a	0.332a
	q_P	0.691a	0.605a	0.691a	0.638a
	NPQ	1.192a	1.081a	1.039a	1.007a

Stress effects on PS2 are often accompanied by a decline in F_v/F_m (Osmond 1994) which reflects the maximal efficiency of excitation capture of a dark-adapted plant (Demmig and Björkman 1987) and is correlated with the number of functional PS2 reaction centres (Öquist and Chow 1992). F_v/F_m was not significantly affected by O_3 exposure in the studied lichens, suggesting that the maximum capacity of the primary reaction of photosynthesis was not impaired by ozone. A lack of effect of ozone exposure on F_v/F_m and Φ_{PS2} in *P. sulcata* has been also observed (Balaguer *et al.* 1996). Similarly, no change in F_v/F_m was recorded in wheat leaves or spruce needles exposed to O_3 (Farage *et al.* 1991, Godde and Buchhold 1992).

The redox state of the quinone pool reflects the balance between photons absorbed through photochemical reactions and the energy utilised through electron transport and CO_2 fixation (Gray *et al.* 1996). The fact that no effects on P_N were found did not exclude the possibility of changes in q_P, since this parameter also reflects processes such as electron flow to molecular oxygen, cyclic electron flow around PS2, or charge recombination within PS2. In any case the redox state of the plastoquinone pool was not affected by ozone. Additionally, no differences were observed in Φ_{PS2}, a parameter closely correlated with the quantum yield of non-cyclic electron transport (Genty *et al.* 1989). In line with our results, Aarnes *et al.* (1993) found no effect of O_3 on the reduction of Q_A and PS2 electron transport in O_3-exposed duckweed.

Dissipation of excess absorbed energy requires that the level of excess can be accurately sensed in the light-harvesting complex. The level of thylakoid energisation is the light sensor (Mohanty and Yamamoto 1995), and thus dissipative processes are highly dependent on membrane integrity. Our results showed no differences in the ability to dissipate excess energy non-radiatively from ozonated lichens compared to controls. Additionally, no differences in the DPS of the xanthophyll pool at the end of irradiation period were recorded. Since the de-epoxidation of Vio to Anth and Zea is also controlled by the light-driven acidification of the thylakoid lumen and ascorbate availability (Neubauer and Yamamoto 1994, Eskling *et al.* 1997), our results suggest that thylakoid membrane integrity was not impaired by ozone. These results agree with the absence of an ozone effect on membrane structure in *Cladonia rangiformis* fumigated at 2-3.6 cm^3 m^{-3} (Brown and Smirnoff 1978).

The literature on ozone effects on P_N is conflicting. No reduction in P_N was found in *Ramalina menziesii* and *Cladonia rangiformis* during acute O_3 exposure (Brown and Smirnoff 1978, Ross and Nash 1983). Conversely, O_3 reportedly decreased gross photosynthesis in *Parmelia caperata* (Ross and Nash 1983), *P. sulcata* and *Hypogymnia enteromorpha* (Nash and Sigal 1979), and induced long-term non-significant impairment of P_N in several lichen species (Scheidegger and Schroeter 1995). Additionally, Balaguer *et al.* (1997) showed an O_3-induced ribulose-1,5-bisphosphate carboxylase/oxygenase

(RuBPCO) depletion in the algal pyrenoid, which correlated positively with a decline of P_N at saturating irradiance. In our experimental conditions no declines in P_N were observed. However, this does not preclude any effect of ozone on the RuBPCO concentration of the studied species, since a decline in its content does not necessarily limit photosynthesis measured under irradiance-limiting conditions. Since the aim of this work was to analyse the operation and regulation of electron transport and dissipative activity relative to CO_2 fixation in control and O_3-exposed lichens, the same moderate irradiance was used to assess Chl fluorescence and P_N.

To summarise, the present study does not exclude an effect of O_3 in the field, as O_3 can interact with other pollutants. Nonetheless, our results under conditions maintaining optimal physiological activity in lichen thalli indicate that these species have significant capacities to withstand oxidative stresses induced by high concentrations of O_3.

Table 3. The proportion of carotenoid pigments to the chlorophyll *a* concentration [mmol mol^{-1}(Chl *a*)] in control and ozone exposed lichen thalli. DPS = [(Zea+Anth)/(Vio+Anth+Zea)]. Values are means of three samples. Values followed by the same letter indicate no significant differences.

Species	Treatment	Neo	Lut	β-Car	Vio	Anth	Zea	DPS	Chl *a/b*
P. quercina	control	48.7a	224.1a	62.2a	61.8a	9.4a	14.3a	0.28a	4.26a
	exposed	47.6a	227.2a	62.2a	57.6a	9.5a	19.2a	0.33a	4.25a
H. physodes	control	47.9a	216.0a	61.2a	55.6a	13.4b	26.8ab	0.42ab	4.23a
	exposed	50.6a	223.6a	60.1a	54.9a	15.5b	31.2b	0.45ab	4.30a
E. prunastri	control	51.0a	245.0a	62.2a	39.1b	15.6b	46.5b	0.61b	3.66b
	exposed	33.3b	238.6a	61.5a	33.0b	15.1b	54.1b	0.65b	3.58b

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