



Research article

The multi-purpose role of hairiness in the lichens of coastal environments: Insights from *Seiophora villosa* (Ach.) Frödén

Elisabetta Bianchi^a, Renato Benesperi^a, Ilaria Colzi^{b,*}, Andrea Coppi^b, Lorenzo Lazzaro^a, Luca Paoli^c, Alessio Papini^d, Sara Pignattelli^b, Corrado Tani^d, Pamela Vignolini^e, Cristina Gonnelli^b

^a Department of Biology, University of Florence, via G. La Pira 4, I-50121, Florence, Italy

^b Department of Biology, University of Florence, via Micheli 1, I-50121, Florence, Italy

^c Department of Biology, University of Pisa, via L. Ghini 13, I-56126, Pisa, Italy

^d Department of Biology, University of Florence, via Micheli 3, I-50121, Florence, Italy

^e Phytolab - Department of Statistics, Informatics, Applications, University of Florence, Via U. Schiff 6, I-50019, Sesto Fiorentino, FI, Italy

ARTICLE INFO

Keywords:

Antioxidant activity
Chlorophyll *a* fluorescence
Juniperus shrublands
Hair
MDA
Salt stress

ABSTRACT

The fruticose epiphytic lichen *Seiophora villosa*, strictly associated with *Juniperus* shrublands in the Mediterranean basin, was used to investigate the role of hairiness on a lichen thallus, as a characteristic morphological trait. We evaluated the effect of hair removal on the physiological parameters of a set of samples, during desiccation and on exposure to different salt concentrations. Hairy thalli were less affected by salt, suggesting that during dehydration, the presence of hair protects the thallus from light irradiance, oxidative stresses and the lipid peroxidation generated by free radicals, and could offer passive, but selective, water control. Our results showed that hair could not only increase thallus surface and promote water absorption when availability is low, but could also repel the salt dissolved in water by activating a passive resistance mechanism, by preventing salt entering.

1. Introduction

The close relationship between the mycobiont and the photobiont/s in lichens allows them to survive in a wide range of habitats, where symbiotic partners would not manage separately. Lichens occur in almost all terrestrial habitats of the world, including in the most inclement environments (Larson, 1987; Green et al., 2012; Zedda and Rambold, 2015). They are physiologically adapted and chemically diverse, and so are able to face abiotic and biotic environmental stresses and survive in extreme environments (Upreti et al., 2015). They can tolerate hot dry environments, arctic climate, salt spray, immersion in water and they colonize many types of substrata (Nash, 2008).

Coastal environments are dynamic ecosystems influenced by prolonged sun exposure, rapid change in water availability, and wind-dependent fluctuations in salinity and temperature (Le Devehat et al., 2014). Stress factors such as those mentioned above induce an overproduction of reactive oxygen species (ROS) and can therefore unbalance the relationship between oxidants and antioxidants in favor of the former, thus increasing the level of oxidative stress (Green et al., 2011). Heat can denature proteins in lichens, so that they must activate

detoxification mechanisms in order to re-establish homeostasis efficiently (Wang et al., 2003). Moreover, the accumulation of sea salt may induce strong dehydration, ion imbalances, and significant loss in net photosynthesis (Matos et al., 2011). A few studies have addressed the impact of salinity stress on lichens, mostly focusing on the cellular location of elements and its effects on net photosynthesis (Smith and Gremmen, 2001). Investigations on *Ramalina canariensis* Steiner, indicated that saline stress may irreversibly impair photosynthesis, thus compromising lichen vitality (Matos et al., 2011). In such saline stress coastal environments, sea spray is one of the main abiotic factors influencing lichen distribution (Fletcher, 1976), with lichens demonstrating considerable variability in salt (NaCl) sensitivity proportionate to their distance from the coast (Nash and Lange, 1988; Malaspina et al., 2015). Winds can carry sea spray inland for considerable distances, thus not limiting marine influence to the immediate shore, but potentially extending it inland for many kilometers, creating small scale variations depending on local topography and shelter (Fletcher, 1976; Nash and Lange, 1988).

Lichens living near the seashore need a morphological and chemical organization to adapt and/or acclimate to extremely variable

* Corresponding author. Department of Biology, via Micheli 1, I-50121, University of Florence, Florence, Italy.

E-mail address: ilaria.colzi@unifi.it (I. Colzi).

<https://doi.org/10.1016/j.plaphy.2019.06.022>

Received 13 March 2019; Received in revised form 14 June 2019; Accepted 15 June 2019

Available online 16 June 2019

0981-9428/ © 2019 Elsevier Masson SAS. All rights reserved.

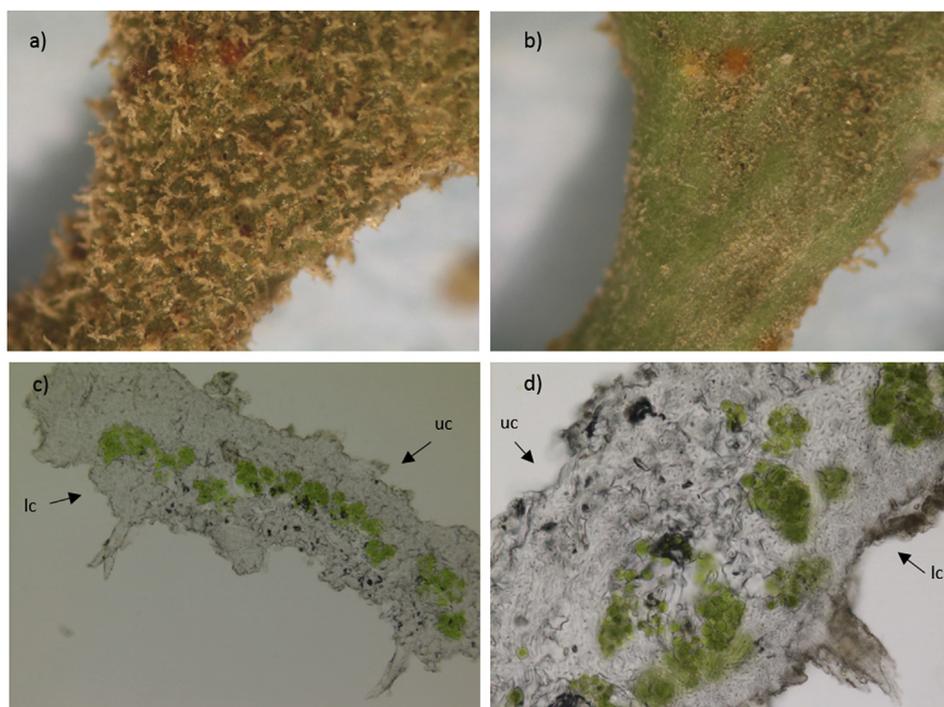


Fig. 1. *Seirophora villosa* thalli. (a), hairy thalli, H; (b), hairless thalli HL; (c–d), cross fresh sections (10 μm and 20 μm , thick) the arrows specify the upper (uc) and the lower (lc) cortex.

disturbances (Delmail et al., 2013). For instance, *Ramalina menziesii*, a typical Californian species of the aerohaline zone, is characterized by a morphological adaptation consisting of a reticulate structure that increases its surface, thereby enhancing the water-holding capacity of its thallus that protects it from fast dehydration when its host trees are ventilated by the ocean (Rundel, 1974). To avoid the harmful effects of the overexcitation of the photosynthetic apparatus, many species colonizing sunny environments possess a complete set of photoprotection mechanisms (Fernández-Marín et al., 2010), as the widespread coastal lichen *Xanthoria aureola*, which has developed a protection system that includes the synthesis of photoprotective pigments, bringing a yellow pigmentation caused by parietin derivatives in the cortex (Smith et al., 2009). Some lichens have a super-hydrophobic upper surface that chemically repels water drops, as is the case with *Cladonia chlorophaea* (Hamlett et al., 2011). As a consequence of these various adaptation strategies, the ability to survive in stressful environments may vary among lichen species (Delmail et al., 2013).

In the Mediterranean ecosystems, coastal juniper habitats are known to host numerous epiphytic lichens, among them the Union for Conservation of Nature (IUCN) red-listed macrolichen *Seirophora villosa* (Ach.) Frödén (Nascimbene et al., 2013) which is strictly associated with dune environments, such as coastal *Juniperus* shrublands (a priority habitat for conservation according to Habitats Directive 92/43/EEC, habitat code 2250*) (Benespero et al., 2013). Recently, it has been demonstrated that *S. villosa* thalli are susceptible to sudden increases in light exposure, especially in the case of small specimens, which after photoinhibition exhibited a reduced ability to recover (Bianchi et al., 2019). Actually, light regime and water availability are generally the main ecological factors modified by habitat fragmentation that can be detrimental to the colonization and survival of *S. villosa* populations, especially in dispersal and establishment phases (Bianchi et al., 2019). *Seirophora villosa* is a fruticose lichen characterized by the presence of compressed canaliculated laciniae covered by thin hairiness and the absence of secondary surface metabolites (Söchting and Frödén, 2002). The role of hair on the thallus has been only partially investigated. Since lichens do not have active mechanisms to regulate water content, hair is presumed to increase the absorption surface and break the

superficial tension of drops, making their absorption easier (Ros and Werner, 1997; Esseen et al., 2015).

Our working hypothesis is that morphological and physiological traits of lichens are linked to specific functions, such as hair in *S. villosa*. In fact, hair could regulate water penetration in the thallus, hence protecting the thallus from salt particles carried by sea spray. Therefore, in this study we investigated the role of the hair layer as a characteristic morphological trait of *S. villosa*, comparing the susceptibility of physiological parameters according to the presence or absence of hair i) during dehydration and ii) on exposure to different NaCl concentrations.

2. Materials and methods

2.1. Lichen material and experimental conditions

Thalli of the epiphytic macrolichen *S. villosa* were collected in Marina di Castagneto Carducci, (Livorno, Tuscany, Italy 43°9'42"12 N, 10°36'42"84 E) in April 2017 and then transferred to the laboratory. Residual tree bark pieces were carefully removed and the thalli slowly moistened by repeated spraying with distilled water. Samples were kept at 16 °C in dim light (200 photons $\mu\text{mol m}^{-2}\text{s}^{-1}$) until treatment. For each treatment, five replicates were used.

2.2. Microscopy analyses

To elucidate hair morphological characteristics we took the opportunity to refer to the thallus structure, investigating the anatomy of a set of *S. villosa* selected samples, through observations by optical microscope and by scanning electron microscope (SEM).

To obtain fresh sections: samples were divided into rough sections of about 4 mm using a very sharp razor, and were then frozen at $-20\text{ }^{\circ}\text{C}$. Once the required temperature was reached, sections were reduced a freezing microtome (CRYO-CUT), up to a size of 30–40 μm . Observations were done by an optical microscope (Leitz DMRB, Leica).

For the observations to the scanning electron microscope (SEM), $1 \times 1\text{ cm}$ fragments of *S. villosa* thalli were fixed to stabilize the

structure by preventing degeneration of the material. The samples were placed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 6.8 for about 48 h at 4 °C and once the maximum dehydration of the samples was achieved by Critical Point Drying process they were coated with a thin gold layer (10 nm thick). Observations were done by an EVO MA 15 microscope.

2.3. Salt and dehydration treatment

Different types of thallus were used in the salt treatment. Samples were randomly divided into three batches: i) thalli with hair (hereafter referred as H) (Fig. 1a); ii) thalli from which hair had been removed prior to NaCl treatments (HL) (Fig. 1b); and iii) thalli where hair had been removed after NaCl treatments (HL_{after}).

Hair was removed from the thallus surface under a stereomicroscope, using a razor and taking care not to damage the surface (Fig. 1a and b). Five different thalli were used for each of the three following experimental conditions: H₂O (control), 0.5 M NaCl and 1 M NaCl (solutions in deionized water). The upper surface of the thalli was repeatedly sprayed with each solution, until fully hydrated. The thalli were then left to dehydrate (160 min) in a climatic chamber at 60% RH, 16 °C and 200 photons $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity. In parallel with dehydration, chlorophyll *a* fluorescence emission was recorded as described in par. 2.4. At the end of the dehydration, samples were stored at about -18 °C, the recommended way to preserve dry lichens (Honegger, 2003; Paoli et al., 2013) for the later chemical and physiological measurements carried out in this study (par. 2.5; 2.6; 2.7; 2.8).

2.4. Chlorophyll *a* fluorescence

During dehydration, measurements of the rate of water loss and fluorescence were carried out every 40 min from the beginning of salt treatment. To estimate the water storage traits for this experiment, we determined the wet mass (WM_{max}) once sprayed each thallus until full hydration. Then, we removed excess surface water by gently shaking the thalli three times and weighed the wet mass (WM_{shaking}). Five random thalli were dried at 70 °C for 24 h to calculate a correction factor to estimate the oven-dry mass (DM). DM was calculated by multiplying air-dry masses of each thallus with the mean correction factor (DM/air-dry mass-ratio). Percent water content, $WC = (WM_{\text{shaking}} - DM) * 100 / DM$, was calculate after shaking.

Chlorophyll *a* fluorescence emission was recorded with a portable fluorimeter (Plant Efficiency Analyzer – Handy PEA, Hansatech Ltd, Norfolk, UK), on two different laciniae per thallus, until the thalli were completely dry and, consequently, a fluorescence signal was no longer obtained. Fluorescence was measured on dark-adapted samples (10 min), which were exposed to light for 1 s with a saturating excitation pulse ($3000 \mu\text{mol s}^{-1} \text{m}^{-2}$) of red light (650 nm).

2.5. Chemical analysis

At the end of the dehydration after salt treatment, sodium concentrations were determined in all three types of *S. villosa* thalli. To obtain the internal fraction, half of the samples were shaken in 5 mL of nickel chloride (NiCl_2 20 mM) for 20 min to remove the adsorbed Na from the cell wall (Matos et al., 2011). The other half were shaken in deionized water for 20 min and used to determine the total Na fraction. Aliquots of oven-dried material were weighed (about 100 mg) and mineralized with concentrated nitric acid (HNO_3) (Applichem, 65%) at 200 °C for 20 min in a microwave digestion system (Mars 6, CEM, Matthews, North Carolina, USA). After the process of digestion, solutions were adjusted to a volume of 25 mL with deionized water, and Na concentrations were determined by atomic absorption spectrophotometry (AAnalyst 200, PerkinElmer). Certified reference materials (LGC No 7162) were used to verify the accuracy and precision of the

methods, whose values were < 10% and < 5% RDS, respectively.

2.6. MDA and H_2O_2 concentration assay

Malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations were determined as indicators of membrane lipid peroxidation. MDA was estimated using thiobarbituric acid reactive substances (TBARS) assay (Taiti et al., 2016). Fresh samples (about 0.5 g) were homogenized with 2.5 mL of 0.1% (w/v) trichloroacetic acid (TCA). 1.5 mL of the homogenate was put in Eppendorf tubes and centrifuged at 12,000 g for 20 min. The supernatant (0.5 mL) was collected and added to 1.5 mL of 0.6% thiobarbituric acid in 10% TCA and put in glass tubes. Tubes were put in the oven at 95 °C for 30 min, cooled in an ice bath, and then solutions were centrifuged again at 12,000 g for 10 min. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific absorption at 600 nm. Concentration of MDA was calculated using the extinction coefficient for the TBA–MDA complex ($155 \text{ mM}^{-1}\text{cm}^{-1}$) and the results expressed as $\mu\text{mol g}^{-1}$ (d.w.). H_2O_2 is one of the reactive oxygen species that accumulates as a consequence of oxidative stress. For H_2O_2 assay, 1 mL of the supernatant was added to 1 mL of 10 mM potassium phosphate buffer (pH 6.8) and 2 mL of 1 M KI. The reaction mixture was incubated for 1 h in darkness. H_2O_2 concentrations were calculated based on the supernatant absorbance at 390 nm and on a standard curve with known concentrations (Alexieva et al., 2001).

2.7. Antioxidant activity

The free radical scavenging activity of samples was measured with the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), according to Hatano et al. (1988): the extract (0.5 mL) at five different concentrations were added to 0.5 mL of a DPPH ethanolic solution. The mixture was shaken vigorously and after 30 min of incubation at room temperature, the absorbance was read at 517 nm in a UV/Vis spectrophotometer (Lambda 35, PerkinElmer), against a blank. Percentage of inhibition free radical DPPH was calculated in the following way: $\text{DPPH scavenging effect (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$, where A_{blank} is the absorbance at the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The inhibition concentration at 50% inhibition (EC50) was the parameter used to compare the radical scavenging activity. A lower EC50 corresponds to a higher antioxidant activity of the lichen extract.

2.8. Measurement of chlorophylls and carotenoids

Total chlorophyll and carotenoid concentrations were determined in *S. villosa* samples. Cold 100% methanol was added to frozen ground lichen material. Samples were left being shaken in darkness at 4 °C for 30 min to extract pigments. After that, they were centrifuged at 1000 g for 10 min. The supernatant was collected and used to read the absorbance at 665, 652 and 470 nm using an UV/Vis spectrophotometer (Lambda 35, PerkinElmer). Chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*) and carotenoid concentrations were determined according to Wellburn (1994).

2.9. Data analysis

To verify whether loss of water content during dehydration was affected by the presence or absence of hair and salt treatments, a Linear Mixed Model (LMM) was fitted in a Repeated Measurement ANOVA design, using thallus identity as a random effect factor to account for the temporal correlation of observations. Water content and F_V/F_M were used as response variables and hairiness, NaCl concentrations and time as explanatory variables in a full factorial design. LMMs served to also verify whether chlorophyll *a* fluorescence (F_V/F_M) was affected by the presence or absence of hair and salt treatments. As the relationship

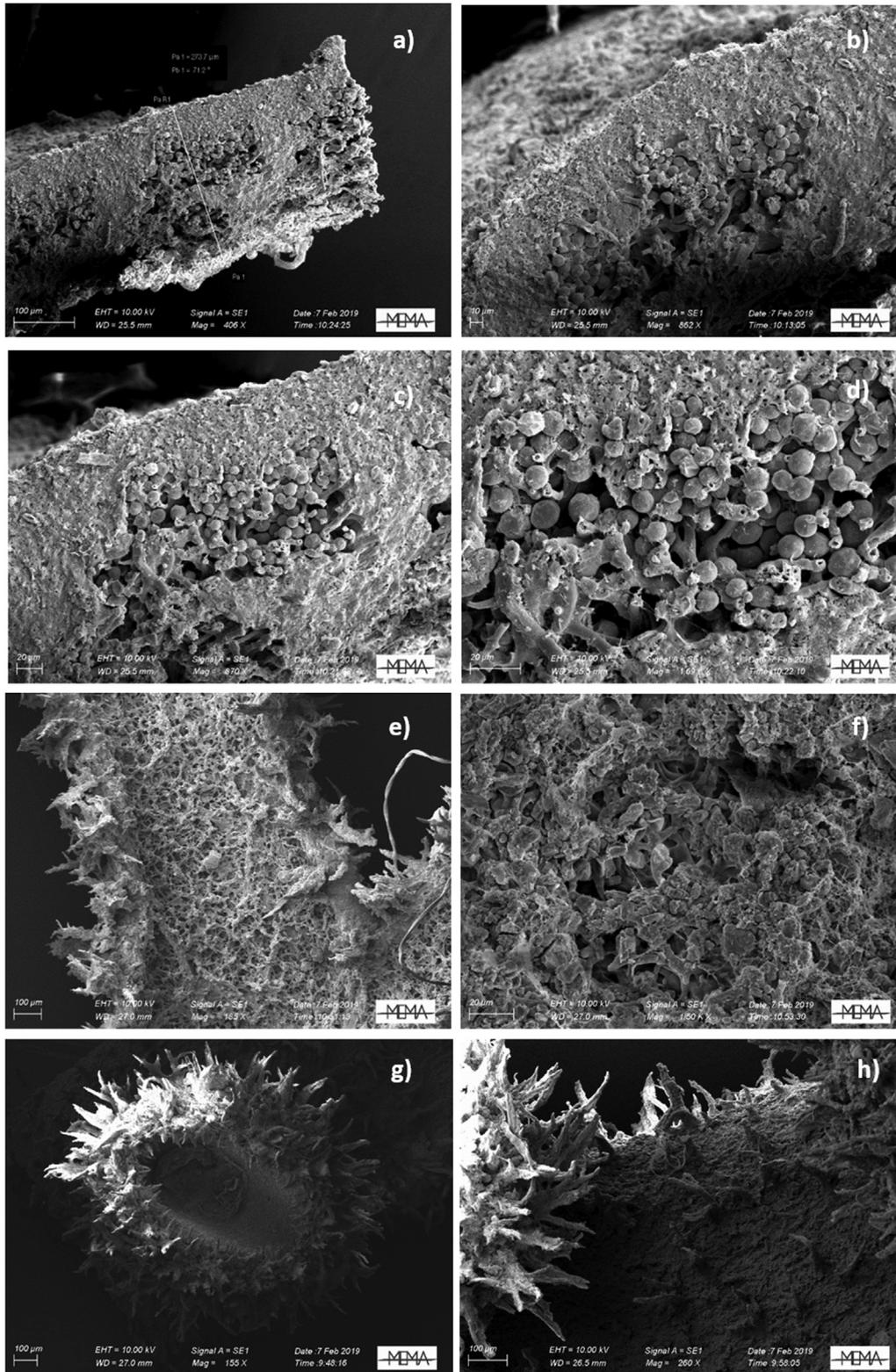


Fig. 2. *Seirophora villosa* anatomical structure. (a), the heteromeric structure of the thallus (100 µm); (b–c), upper cortex consisting of intertwined hyphae and underlying medullary hyphae (10 µm and 20 µm); (d) detail of the algal partner lies; (e–f), reticulate lower cortex with portions of the medulla exposed (100 µm and 20 µm); (g–h) consistent multiserial complex hair around the apothecium and on the entire upper cortex (100 µm).

between the variation in water content or chlorophyll *a* fluorescence and time appeared quite far from a simple linear regression, in all the analyses we used time as an ordinal variable. We evaluated the significance of the fixed effects and of associated interaction factors using an ANOVA type III table, with Kenward-Roger degrees of freedom (df)

approximation. LMM computations were performed using the lmer function of the *lme4* package version 1.1–12 for fitting the models and *car* package to obtain probability (p) values. Means and standard errors (SE) of all studied parameters were checked by one-way and two-way ANOVA and Tukey post hoc test was used for a posteriori comparison of

individual means (with at least $p < 0.05$ as the significance level). The analysis was conducted using the statistical program R environment (R version 3.3.1). All plots were drawn using *SigmaPlot* 8.0.

3. Results

3.1. Microscopy analyses

The microscopical observations highlighted the heteromeric structure of the thallus, characterized by a loosely intertwined upper cortex, a scleroplectenchymatic medulla (Fig. 1c and d; Fig. 2a, b, c), and a reticulate lower cortex that leaves portions of the medulla exposed (Fig. 2e and f). The algal partner lies below the upper cortex (Fig. 2d), well distinct from the hyphal region. The upper hyphal layer is considerably thickened by the formation of plectenchyma, making a compact layer free of gaps (Fig. 2c). Noteworthy, the agglutinated conformation of the hyphae and the arrangement of the photosynthetic partner is a typical scleroplectenchymatic structure of the lichen thallus (Fig. 2a, b, c, d).

SEM analyses highlighted the presence of consistent multiseriate complex hairs exclusively on the entire upper cortex of *S. villosa* thalli, consisting of strongly conglutinated hyphae, approximately 137.5 ± 10.7 (SE) μm (Fig. 2g and h).

3.2. Rate of water loss and photosynthetic activity during dehydration

After spraying with water, H (hairy thalli) and HL (hairless thalli) reached water contents of about 168% and 180% dry weight respectively (Fig. 3a and b). Water loss kinetics changed significantly over

time for H and HL ($p < 0.001$) (Table 1): complete dehydration was achieved in both cases after 160 min, decreasing faster in H than in HL ($p < 0.001$). Following NaCl (0.5 and 1 M) treatments, H did not show significant differences with respect to the control (H_2O), while HL reached a lower water content more rapidly than the control samples ($p < 0.001$).

Along with dehydration, the parameter F_V/F_M (used as a proxy for the photosynthetic activity) decreased significantly both in H and HL ($p < 0.001$) (Table 1), with F_V/F_M values in H generally higher in comparison with HL, both at 0.5 M and 1 M NaCl ($p < 0.001$) (Fig. 3c and d). Initial F_V/F_M values were comparable for H and HL, but changed in different ways depending on salt treatment and the presence of hair: at 1 M NaCl, F_V/F_M values in H decreased more rapidly compared to the control, reaching low values (0.173 ± 0.038 H; 0.400 ± 0.049 control) in 120 min from experiment outset (Fig. 3c). No difference in H thalli was observed between 0.5 M and control samples. Regarding HL, significant differences with control emerged both at 0.5 M and 1 M NaCl, with samples reaching low values (0.164 ± 0.054 , 0.060 ± 0.024 respectively) in 120 min from the beginning of the experiment (Fig. 3d).

3.3. Na accumulation

Total Na concentration increased progressively in H, HL and HL_{after} as the concentration in the treatment solution increased, with the same trend observed for intracellular concentrations ($p < 0.001$) (Fig. 4). At treatment end, total Na concentration was significantly higher than the intracellular Na concentration ($p < 0.001$). There was also no significant difference in total Na concentrations between H and HL_{after},

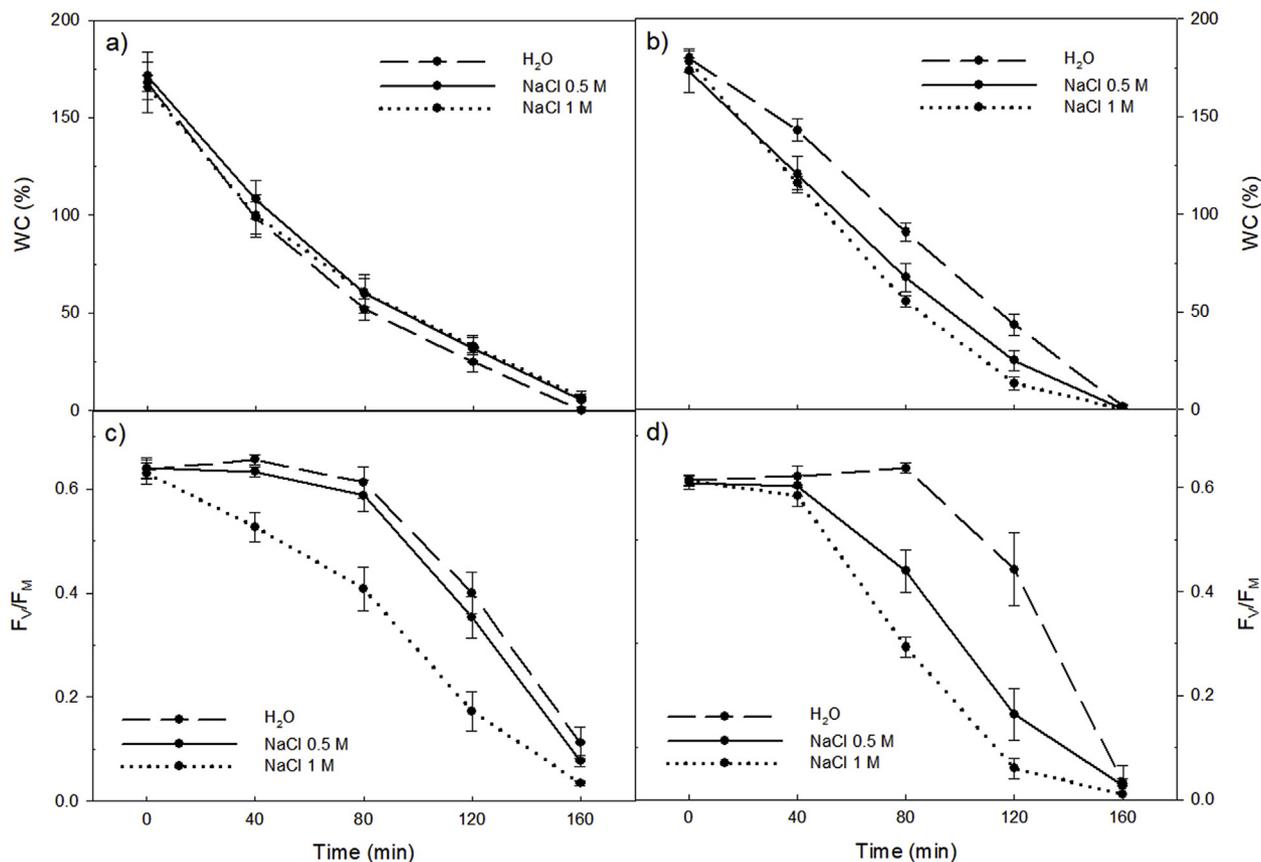


Fig. 3. Rate of water loss and reduction of F_V/F_M during a complete drying cycle in *S. villosa* thalli after having been sprayed with different NaCl concentrations (dark adaptation = 10 min). The plots depict a decline in water content in hairy thalli (a) and in hair-less thalli (b) during a complete drying cycle, variation of photosystem II (F_V/F_M) efficiency in hairy thalli (c) and in hair-less thalli (d) over time. The interaction terms are statistically significant, according to a mixed model ANOVA with p value < 0.001 . The dashed line = control; the solid line = NaCl 0.5 M; the dotted line = NaCl 1 M.

Table 1

ANOVA Type III table with Kenward-Roger approximation for degrees of freedom (df) for repeated measures analysis of variance (rANOVA) for the relationship between water content and efficiency of photosystem II (F_v/F_m) over time and the presence or absence of hair. During the experiment thalli weights were measured every 40 min. Significance codes: p value < 0.001 ****; p value < 0.01 ***; p value < 0.05 **.

Parameter	Hairy thalli "H"				Hair-less thalli "HL"					
	Water content				Efficiency of photosystem II (F_v/F_m)					
Factor	F	df	Df.res	Pvalue	F	df	Df.res	Pvalue		
(Intercept)	800.432	1	188.82	< 0.001	***	2143.626	1	104.56	< 0.001	***
NaCl	43.302	2	269.00	< 0.001	***	322.3045	5	245.00	0.991	
Time	283.882	1	269.00	< 0.001	***	0.8411	1	245.00	< 0.001	***
Hairiness	432.750	1	269.00	< 0.001	***	0.0090	2	245.00	0.359	
NaCl:Time	13.539	2	269.00	< 0.001	***	1.4101	5	245.00	< 0.001	***
NaCl:Hairiness	17.882	2	269.00	< 0.001	***	8.6286	10	245.00	0.981	
Time:Hairiness	6.918	1	269.00	0.009	**	0.0187	2	245.00	0.221	
NaCl:Time:Hairiness	3.899	2	269.00	0.021	*	1.9268	10	245.00	0.042	*

which accumulated significantly lower amounts of Na than HL both at 0.5 M and 1 M NaCl ($p < 0.05$). A similar pattern was also observed for intracellular Na accumulation, which was significantly higher in H and HL than in HL_{after} both at 0.5 M and 1 M NaCl ($p < 0.05$).

3.4. Chlorophyll and carotenoid concentrations

In control samples, Chla and Chlb concentrations were significantly higher in H than HL after 160 min of dehydration, as reported in Table 2; while higher carotenoid concentrations were measured in HL than in H ($p < 0.05$) (Table 2). In H in the presence of increasing salt treatments, the concentration of Chla, Chlb and total carotenoids did not change compared to control samples. In HL, both Chla and Chlb significantly decreased at the highest salt concentration (1 M NaCl), while carotenoids were significantly affected both at 0.5 M and 1 M NaCl, compared to control values (Table 2).

3.5. MDA and H₂O₂ concentrations and antioxidant activity

In control samples, MDA production and H₂O₂ concentration were higher in HL compared to H ($p < 0.05$) (Table 3). MDA production and H₂O₂ concentration were not affected by NaCl in H and seemed reduced in HL, both at 0.5 M and 1 M NaCl, compared to the controls.

The free radical scavenging activity of the samples, as reflected by DPPH, significantly decreased in HL compared to in H ($p < 0.05$) (Fig. 5). DPPH values were not affected by salt treatments in H and

showed a significant decrease in HL treated with the highest salt concentration.

4. Discussion

The anatomical investigation highlighted a heteromeric structure characterized by a loosely intertwined upper cortex, a scleroplectenchymatic medulla, and a reticulate lower cortex that leaves portions of the medulla exposed. This structure and the morphological characteristics (such as the presence of hair) are considered typical for desert fog-zone lichens, such as other species belonging to the genus *Seiropora* (del Prado and Sancho, 2007). This fruticose growth form allows the thallus to use the dew (del Prado and Sancho, 2007) as well as water vapor. The presence of hair further increases the absorption surface due to the greater surface to volume ratio (Ros and Werner, 1997; Esseen et al., 2015). Noteworthy, our results show that hairy thalli of *S. villosa* store less water than hairless ones. We therefore hypothesize that in our model species, hair plays a principal role in regulating the absorption of water in cases of both scarcity and excess. Hair could not only increase the thallus surface and promote water absorption in case of low availability (Kärnefelt, 1989), but also repels excess water, to prevent oversaturation. Considering that *S. villosa* does not produce hydrophobic metabolites on the hyphal reticulum (Söchting and Frödén, 2002), the presence of hair can play an important role in limiting the uptake of excessive water that could be detrimental, since it increases the resistance to gas diffusion in lichen thalli by constricting

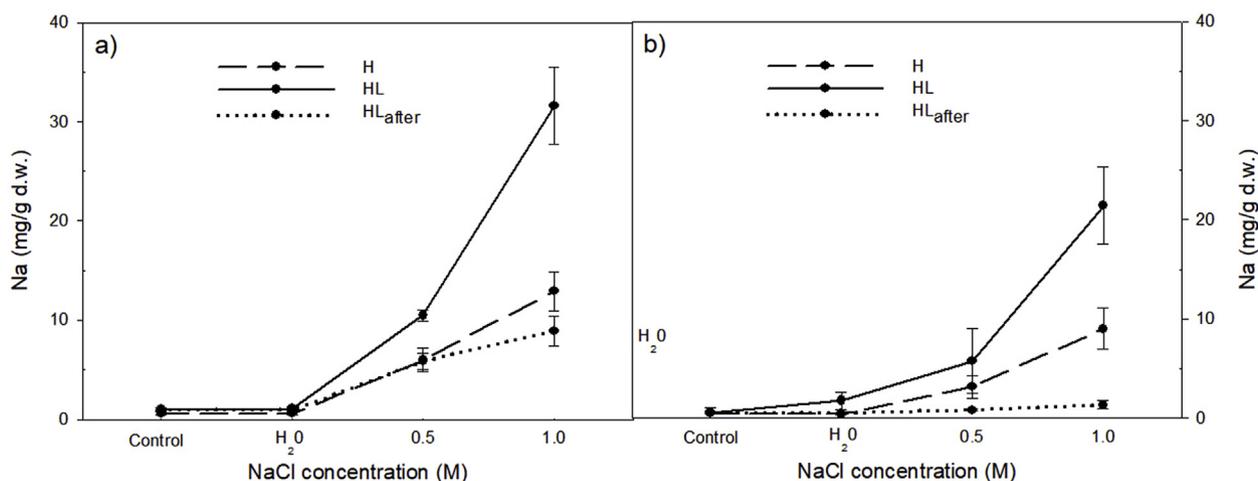


Fig. 4. Sodium concentrations of *S. villosa* thalli sprayed with different NaCl concentrations. The plots depict a) the total Na concentration b) the intracellular Na concentration at the end of the salt treatments. Values are means of 5 replicates \pm standard error (at least, $p < 0.05$). The dashed line = hairy thalli (C); the solid line = hair-less thalli (HL); the dotted line = hair-less thalli after salt treatment (HL_{after}).

Table 2

Mean values (\pm SE, N = 5) of chlorophyll and carotenoid concentration in hairy thalli and hair-less thalli treated with different NaCl concentrations, at the end of desiccation. Lower case letters denote significant differences between treatment means; upper case denotes sample sets means ($p < 0.05$).

Treatment	Hairy thalli "H"			Hair-less thalli "HL"		
	H ₂ O	0.5 M NaCl	1 M NaCl	H ₂ O	0.5 M NaCl	1 M NaCl
Chlorophyll a ($\mu\text{g mg}^{-1}$ d.w.)	0.94 \pm 0.13 a A	1.11 \pm 0.08 a A	1.09 \pm 0.03 a A	0.46 \pm 0.04 a B	0.44 \pm 0.04 a B	0.19 \pm 0.02 b B
Chlorophyll b ($\mu\text{g mg}^{-1}$ d.w.)	0.44 \pm 0.02 a A	0.47 \pm 0.02 a A	0.38 \pm 0.06 a A	0.22 \pm 0.06 a B	0.22 \pm 0.06 a A	0.10 \pm 0.005 b B
Carotenoid ($\mu\text{g mg}^{-1}$ d.w.)	0.06 \pm 0.01 a A	0.06 \pm 0.01 a A	0.06 \pm 0.01 a A	0.14 \pm 0.02 a B	0.03 \pm 0.006 b A	0.05 \pm 0.006 b A

the pore space for gas exchange (Cowan et al., 1992). Moreover, our results showed that immediately after spraying water or salt solutions, hairy thalli reached a lower water content than hairless ones and also dehydrated faster, suggesting that hair could favor a lower water-penetrability into the thalli and at the same time a rapid evaporation due to a greater thallus surface in contact with the air. These results support those reported in the literature for species living in arid environments, which tend to go into cryptobiosis more quickly to escape photoinhibition (Lange et al., 1990; Nardini et al., 2013). In addition, *S. villosa* does not synthesize photoprotective pigments in the cortex against UV radiations, suggesting that the involvement of the mycobiont in protecting the photobiont might not be determined by the presence of secondary chemical compounds in the thallus. In Mediterranean ecosystems, such as *Juniperus* shrublands in the Mediterranean basin, periods of excess thalli hydration and rapid dehydration are linked to their distance from the sea, habitat fragmentation, daily and seasonal fluctuations of microclimatic parameters, and to wet and rainy winters and hot and dry summers (Castro et al., 2004). During dry periods, lichens of dry environments hydrate at night through the uptake of fog, dew or water vapor. The first peak of positive net photosynthesis is seen to occur immediately after sunrise (Lange et al., 2006), with higher air humidity causing a second peak in the late afternoon. This behavior could allow thalli to be fully photosynthetically active even with low water content (Lange et al., 2006).

As expected, our results showed that the length of the photosynthetically active period decreased during dehydration. Hair-less thalli maintained high photosynthetic performances (as suggested by F_v/F_m values) at 90% of their water content, remaining active at up to 40%; hairy thalli maintained high photosynthetic performances even at 50% of their water content and started to deactivate when water content reduced below 20%. Hence, hair could be a strategic morphological trait for this species, not only enhancing a faster dehydration but also a differential evaporation between the hyphal network and the photosynthetic layer. In fact, the photosynthetic activity of a lichen photobiont depends on its water content, and hence, within a certain range, on its cellular turgor status (Bianchi et al., 2018; Petruzzellis et al., 2018). The presence of hair could regulate the evaporation of water initially from the hyphal network, thus enhancing water persistence in the photosynthetic layer. In addition, hair could play a photoprotective effect: during dehydration, the absence of hair affected the concentrations of photosynthetic pigments as well as of oxidative stress markers. None of the parameters investigated changed in hairy thalli. By contrast, chlorophyll *a* and *b* concentrations significantly decreased in hair-

Table 3

Mean values (\pm SE, N = 5) of MDA and H₂O₂ content of lichen samples treated with different NaCl concentrations, at the end of desiccation. Lower case letters denote significant differences between treatment means; upper case denotes sample sets means ($p < 0.05$).

Treatment	Hairy thalli "H"			Hair-less thalli "HL"		
	H ₂ O	0.5 M NaCl	1 M NaCl	H ₂ O	0.5 M NaCl	1 M NaCl
MDA ($\mu\text{mol g}^{-1}$)	0.85 \pm 0.09 a A	0.852 \pm 0.03 ab A	0.72 \pm 0.06 b A	1.23 \pm 0.12 a B	0.94 \pm 0.12 ab A	0.82 \pm 0.07 b B
H ₂ O ₂ ($\mu\text{g g}^{-1}$)	0.41 \pm 0.03 a A	0.41 \pm 0.10 a A	0.25 \pm 0.08 a A	0.57 \pm 0.04 a B	0.24 \pm 0.01 b A	0.27 \pm 0.08 b A

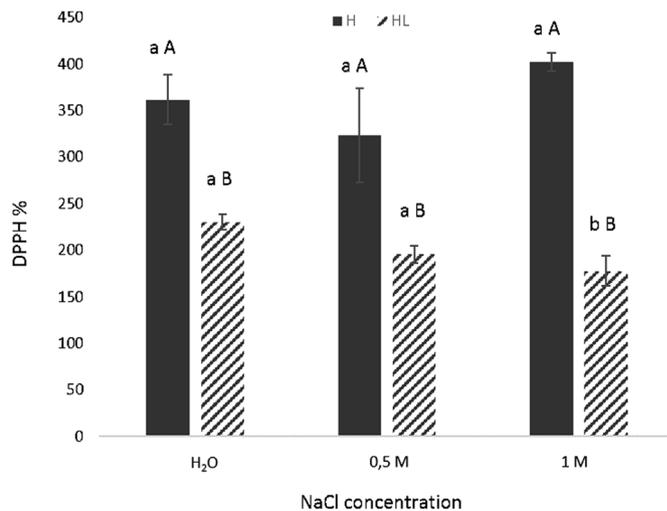


Fig. 5. Antioxidant activity of hairy thalli and hair-less ones after having been sprayed with different NaCl concentrations. The lower value indicates higher antioxidant activity. Values are means \pm standard error (n = 3) and are expressed in mg of the dry weight needed to reduce by at least 50% 1 mg of the stable free radical DPPH. Lower case letters denote significant differences between treatments means; upper case denotes sample sets means ($p < 0.05$). The solid black bar = hairy thalli (C); the striped bar = hair-less thalli (HL).

less thalli at the end of dehydration, along with increases in MDA and H₂O₂ concentrations. These results suggest that the presence of hair protects the thalli during dehydration from oxidative (and photo-oxidative) stress and the lipid peroxidation generated by free radicals. ROS may accumulate in lichen tissues and represent a major cause of damage during desiccation, especially in photosynthetic organisms (Le Devehat et al., 2014). When desiccated in the light, chlorophyll molecules continue to be excited, but the energy not used in carbon fixation can cause singlet oxygen to form (Kranmer et al., 2008). Moreover, in hairless thalli the amount of oxidative stress markers increased, and triggered an antioxidative response in our samples. Actually, an increase of carotenoid concentrations observed, as a sudden consequence of hair removal and could be linked to the photoprotective role of hair in *S. villosa*. Furthermore, an alteration of chlorophylls content in hairless thalli (likely determined by chlorophyll degradation as a consequence of photo-oxidation) occurred also to control samples. In addition, an increase in the antioxidant response was suggested also by

the low DPPH levels measured in hairy thalli. However, this response was not sufficient to counteract oxidative stress, as shown by the increase of MDA concentrations found in hairless thalli with respect to hairy ones, suggesting that the presence of hair protected the thallus. In effect, the levels of H₂O₂ in hairless thalli were higher and contributed to the oxidative stress involved in these processes. Therefore, the presence of hair is seen to regulate water absorption and dehydration in order to keep the thallus photosynthetically active, even when water content is low, and at the same time to slow the onset of oxidative stress.

Spraying *S. villosa* thalli with two different NaCl concentrations increased Na accumulation in the thallus, in a different manner depending on the presence of hair. The amount of Na in hairy thalli and in hair-less thalli after salt treatment was significantly lower than hair-less ones, confirming that the presence of hair served to repulse excess water droplets, as well as the salt particles therein. These results were also confirmed by lower intracellular Na concentrations in hair-less thalli after salt treatment, suggesting that the cell wall also contributed to salt exclusion from the cytosol. Indeed, the cell wall was seen to act as an immobilizing filter in hairy thalli and in hair-less thalli after salt treatment, prevent a high amount of Na from entering the cell. In samples exposed to the highest NaCl concentration, observed changes in vitality depended mainly on the intracellular Na uptake, as proved in Matos et al. (2011). At the highest level of salt treatment, the performance of hairy thalli was also affected (reduction of F_v/F_m values), suggesting that even if hair could act as a barrier to salt entering, some Na still penetrates, compromising the activity of the samples. By contrast, in hair-less thalli all physiological parameters investigated were affected at both salt concentration levels. As demonstrated by Delmail et al. (2013), the first consequence of salt exposure is faster dehydration in hair-less samples. Furthermore, our study found that a reduction of F_v/F_m values at 0.5 M and 1 M NaCl impacts on photosynthetic performances.

The salt treatment also caused a significant decrease in photosynthetic pigments in hair-less thalli. Confirming that the impairment of photosynthetic pigments (degradation of Chl a and Chl b, increase of carotenoids in control samples) could be linked to the photoprotective role of hair in *S. villosa*. Nevertheless, hair-less thalli showed a high antioxidant activity after salt treatments, presumably scavenging the ROS accumulated in lichen tissues. Indeed, salt treatments when combined with dehydration activated physiological processes involved in desiccation tolerance that facilitate survival in a desiccated state, as well as the rapid re-establishment of normal physiological activity upon rehydration (Kranner et al., 2008; Nardini et al., 2013).

5. Conclusions

The study highlighted the first time the relevance of hair as a strategic morphological trait in lichens facing extreme environments. The presence of hair in *S. villosa* was found to improve the performance of this species. Our results suggest that hair could offer a passive, but selective, water control. Hair was found to not only increase the thallus surface and promote water absorption when water availability was low, and could also repel excessive water to avoid oversaturation. Furthermore, hair was seen to favor a slower dehydration of the algal layer; keeping the photosynthetic process active, even at low water content. We can also conclude that in the presence of salt, hair activates a significant stress avoidance mechanism, which, by preventing salt from entering, allows the thallus to tolerate that presence.

Author statement

EB, RB, CG and IC designed the work. EB and RB collected the samples. EB, IC, LL, SP, CT and PV, acquired and analyzed the data. EB and CG drafted the manuscript. RB, IC, AC, LL, LP and AP critically revised the article. All the authors approved the version of the

manuscript to be published.

Acknowledgments

SEM imaging took place in M.E.M.A., Department of Earth Science, University of Florence and authors thank the technician L. Chiarantini for processing the scanning electron micrographs. We acknowledge Jana Scmazzon for English revision.

References

- Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E., 2001. The effect of drought and ultra-violet radiation on growth and stress markers in pea and wheat. *Plant Cell Environ.* 24, 1337–1344.
- Banchi, E., Carniel, F.C., Montagner, A., Petruzzellis, F., Pichler, G., Giarola, V., Bartels, D., Pallavicini, A., Tretiach, M., 2018. Relation between water status and desiccation-affected genes in the lichen photobiont *Trebouxia gelatinosa*. *Plant Physiol. Biochem.* 129, 189–197.
- Benesperi, R., Lastrucci, L., Nascimbene, J., 2013. Human disturbance threatens the red-listed macrolichen *Seiophora villosa* (Ach.) Fröden in coastal *Juniperus* habitats: evidence from western peninsular Italy. *Environ. Manag.* 52, 939–945.
- Bianchi, E., Paoli, L., Colzi, I., Coppi, A., Gonnelli, C., Lazzaro, L., Loppi, S., Papini, A., Vannini, A., Benesperi, R., 2019. High-light stress in wet and dry thalli of the endangered Mediterranean lichen *Seiophora villosa* (Ach.) Fröden: does size matter? *Mycol. Prog.* 18, 463–470.
- Castro, J., Zamora, R., Hódar, J.A., Gómez, J.M., Gómez-Aparicio, L., 2004. Benefits of using shrubs as nurse plants for reforestation in Mediterranean mountains: a 4-year study. *Restor. Ecol.* 12, 352–358.
- Cowan, I.R., Lange, O.L., Green, T.G.A., 1992. Carbon-dioxide exchange in lichens: determination of transport and carboxylation characteristics. *Planta* 187, 282–294.
- del Prado, R., Sancho, L.G., 2007. Dew as a key factor for the distribution pattern of the lichen species *Teloschistes lacunosus* in the Tabernas Desert (Spain). *Flora* 202, 417–428.
- Delmail, D., Grube, M., Parrot, D., Cook-Moreau, J., Boustie, J., Labrousse, P., Tomasi, S., 2013. Halotolerance in lichens: symbiotic coalition against salt stress. In: *Ecophysiology and Responses of Plants under Salt Stress*. Springer, New York, pp. 115–148.
- Esseen, P.A., Olsson, T., Coxson, D., Gauslaa, Y., 2015. Morphology influences water storage in hair lichens from boreal forest canopies. *Fungal Ecol* 18, 26–35.
- Fernández-Marín, B., Becerril, J.M., García-Plazaola, J.I., 2010. Unravelling the roles of desiccation-induced xanthophyll cycle activity in darkness: a case study in *Lobaria pulmonaria*. *Planta* 231, 1335–1342.
- Fletcher, A., 1976. Nutritional aspects of marine and maritime lichen ecology. In: Brown, D.H., Hawksworth, D.L., Bailey, R.H. (Eds.), *Lichenology: Progress and Problems*. Academic, New York, pp. 359–384.
- Green, T.G.A., Sancho, L.G., Pintado, A., 2011. Ecophysiology of desiccation/rehydration cycles in mosses and lichens. In: Lüttge, U., Beck, E., Bartels, D. (Eds.), *Desiccation Tolerance*. Springer, Berlin Heidelberg, pp. 89–120.
- Green, T.G.A., Brabyn, L., Beard, C., Sancho, L.G., 2012. Extremely low lichen growth rates in Taylor Valley, Dry Valleys, continental Antarctica. *Polar Biol.* 35, 535–541.
- Hamlett, C.A., Shirtcliffe, N.J., Pyatt, F.B., Newton, M.I., McHale, G., Koch, K., 2011. Passive water control at the surface of a superhydrophobic lichen. *Planta* 234, 1267–1274.
- Hatano, T., Kagawa, H., Yasuhara, T., Okuda, T., 1988. Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chem. Pharm. Bull.* 36, 2090–2097.
- Honegger, R., 2003. The impact of different long-term storage conditions on the viability of lichen-forming ascomycetes and their green algal photobiont. *Trebouxia* spp. *Plant Biol.* 5, 324–330.
- Kärnefelt, I., 1989. Morphology and phylogeny in the *Teloschistales*. *Cryptogam. Bot.* 1, 147–203.
- Kranner, I., Beckett, R., Hochman, A., Nash III, T.H., 2008. Desiccation tolerance in lichens: a review. *Bryologist* 111, 576–593.
- Lange, O.L., Meyer, A., Zellner, H., Ullmann, I., Wessels, D.C.J., 1990. Eight days in the life of a desert lichen: water relations and photosynthesis of *Teloschistes capensis* in the coastal fog zone of the Namib Desert. *Madoqua* 17, 17–30.
- Lange, O.L., Green, T.A., Melzer, B., Meyer, A., Zellner, H., 2006. Water relations and CO₂ exchange of the terrestrial lichen *Teloschistes capensis* in the Namib fog desert: measurements during two seasons in the field and under controlled conditions. *Flora* 201, 268–280.
- Larson, D.W., 1987. The absorption and release of water by lichens. In: In: Peveling, E. (Ed.), *Progress and Problems in Lichenologies in the Eighties*, Biblioteca Lichenologica, vol. 25. J. Cramer, Berlin, pp. 351–360.
- Le Devehat, F., Thüs, H., Abasq, M.L., Delmail, D., Boustie, J., 2014. Oxidative stress regulation in lichens and its relevance for survival in coastal habitats. *Adv. Bot. Res.* 71, 467–503.
- Malaspina, P., Giordani, P., Pastorino, G., Modenesi, P., Mariotti, M.G., 2015. Interaction of sea salt and atmospheric pollution alters the OJIP fluorescence transient in the lichen *Pseudevernia furfuracea* (L.) Zopf. *Ecol. Indic.* 50, 251–257.
- Matos, P., Cardoso-Vilhena, J., Figueira, R., Sousa, A.J., 2011. Effects of salinity stress on cellular location of elements and photosynthesis in *Ramalina canariensis* Steiner. *Lichenologist* 43, 155–164.

- Nardini, A., Marchetto, A., Tretiach, M., 2013. Water relation parameters of six *Peltigera* species correlate with their habitat preferences. *Fungal Ecol* 6, 397–407.
- Nascimbene, J., Nimis, P.L., Ravera, S., 2013. Evaluating the conservation status of epiphytic lichens of Italy: a red list. *Plant Biosyst.* 147, 898–904.
- Nash III, T.H., 2008. Lichen sensitivity to air pollution. In: Nash III T.H. (Ed.), *Lichen Biology*, second ed. Cambridge University Press, Cambridge, pp. 299–314.
- Nash III, T.H., Lange, O.L., 1988. Responses of lichens to salinity: concentration and time-course relationships and variability among Californian species. *New Phytol.* 109, 361–367.
- Paoli, L., Munzi, S., Pisani, T., Guttová, A., Loppi, S., 2013. Freezing of air-dried samples of the lichen *Evernia prunastri* (L.) Ach. ensures that thalli remain healthy for later physiological measurements. *Plant Biosyst.* 147, 141–144.
- Petruzzellis, F., Savi, T., Bertuzzi, S., Montagner, A., Tretiach, M., Nardini, A., 2018. Relationships between water status and photosystem functionality in a chlorolichen and its isolated photobiont. *Planta* 247, 705–714.
- Ros, R.M., Werner, O., 1997. Musgos y hepáticas del sureste arido español. *Quercus* 135, 31–35.
- Rundel, P.W., 1974. Water relations and morphological variation in *Ramalina menziesii* Tayl. *Bryologist* 77, 23–32.
- Smith, V.R., Gremmen, N.J.M., 2001. Photosynthesis in a sub-Antarctic shore-zone lichen. *New Phytol.* 149, 291–299.
- Smith, C.W., Aptroot, A., Coppins, B.J., Fletcher, A., Gilbert, O.L., James, P.W., Wolseley, P.A., 2009. *The Lichens of Great Britain and Ireland*. The British Lichen Society, London.
- Søchting, U., Frödén, P., 2002. Chemosyndromes in the lichen genus *Teloschistes* (*Teloschistaceae*, *Lecanorales*). *Mycol. Prog.* 1, 257–266.
- Taiti, C., Giorni, E., Colzi, I., Pignattelli, S., Bazihizina, N., Buccianti, A., Luti, S., Pazzagli, L., Mancuso, S., Gonnelli, C., 2016. Under fungal attack on a metalliferous soil: ROS or not ROS? Insights from *Silene paradoxa* L. growing under copper stress. *Environ. Pol.* 210, 282–292.
- Upreti, D.K., Divakar, P.K., Shukla, V., Bajpai, R., 2015. *Recent Advances in Lichenology: Modern Methods and Approaches in Biomonitoring and Bioprospection*, vol. 1 Springer.
- Wang, W., Vinocur, B., Altman, A., 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218, 1–14.
- Wellburn, A.R., 1994. The spectral determination of chlorophyll *a* and chlorophyll *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolutions. *J. Plant Physiol.* 144, 307–313.
- Zedda, L., Rambold, G., 2015. The diversity of lichenised fungi: ecosystem functions and ecosystem services. In: Upreti, D., Divakar, P., Shukla, V., Bajpai, R. (Eds.), *Recent Advances in Lichenology*. Springer, New Delhi, pp. 121–145.