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A Role for Secondary Metabolites in Desiccation Tolerance in Lichens

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Abstract: In lichens, secondary metabolites have been shown to protect against biotic stresses such as pathogen attacks and grazing, and abiotic stresses such as ultraviolet (UV) and high photosynthetically active radiation (PAR). Lichen secondary metabolites are known to have strong antioxidant activity, and while theoretically they may have roles in tolerance to other abiotic stresses, these roles remain largely unclear. Here, we used the acetone rinsing method to harmlessly remove most of the secondary metabolites from the thalli of six lichen species. This enabled us to compare the effects of desiccation on thalli with and without the presence of secondary metabolites. Results showed that in general, the presence of lichen substances reduces the effects of desiccation stress. For all species, substances significantly improved the photosystem two (PSII) activity of the photobiont during either desiccation or rehydration. In the mycobiont, in four of the six species, the presence of substances reduced membrane damage, which was assessed by measuring ion leakage during rehydration following desiccation. However, in one species, secondary metabolites had no effect, while in another the presence of substances increased membrane damage. Nevertheless, it seems clear that in addition to their more established roles in protecting lichens against pathogen attacks and grazing, lichen substances can also play a role in aiding desiccation tolerance.

Keywords: lichen substances; desiccation; membrane damage; chlorophyll fluorescence

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1. Introduction

Lichens can successfully grow in a wide range of habitats, and in some extreme habitats can even dominate [1]. It has been suggested that the ability of lichens to colonize such environments is, at least in part, due to the presence of secondary metabolites that increase their ability to tolerate stress [2]. Good evidence has been presented suggesting that secondary substances can protect lichens against biotic stresses such as pathogen attacks (e.g., viruses, bacteria, and other fungi) and grazing, and abiotic stresses such as UV and high PAR (for review see [3]). The role of secondary metabolites in protecting lichens against other abiotic stresses such as temperature and desiccation remains much less clear. A common effect of abiotic stresses on all organisms is an increase in the formation of reactive oxygen species (ROS) [4]. When isolated from lichens, many secondary metabolites have been demonstrated to display very high antioxidant activity [5–7]. Some general correlations between the levels of lichen substances and abiotic stress tolerance have been reported. For example, in their natural habitat, Antarctic lichens are subjected to various stresses such as low temperatures, drought, high UV-B and solar irradiation, and long periods of darkness, especially during winter [8]. At the same time, Antarctic lichens have been shown to contain very high levels of lecanoric acid [8]. While there appear to be few direct demonstrations, in theory, it seems likely that lichen substances may act as a general protectant against ROS formed during abiotic stresses. The majority of lichen secondary

metabolites typically occur as hydrophobic crystals on the hyphal cell walls and are also extremely insoluble in water [9]. Furthermore, it has been observed that under certain conditions, rather than acting as antioxidants, they can behave as prooxidants (e.g., [10,11]). In particular, it has been shown that usnic acid treatment greatly increases oxidative stress in the free-living ascomycete *Candida albicans* [12]. Furthermore, even if beneficial to the mycobiont, it is uncertain how lichen substances on the surface of hyphae could protect the photobiont. Therefore, the role of secondary metabolites in the tolerance of lichens to abiotic stress remains unclear.

The main aim of the present study was to use the "acetone rinsing" method to test for the importance of lichen substances in the desiccation tolerance of a range of lichen species. It has been shown that acetone can be used to harmlessly remove most of these metabolites from the hyphal cell walls, allowing the effects of stress to be compared in thalli with and without substances [13]. To assess the tolerance of the photobiont to desiccation, photosynthesis was measured by testing the maximum potential quantum efficiency of PSII (F_V/F_M). Furthermore, the effect of substance removal on non-photochemical quenching (NPQ) was also tested. NPQ is an important mechanism used by photosynthetic organisms to harmlessly dissipate excess light energy [14]. The tolerance of the mycobiont to desiccation was assessed by measuring the membrane leakage of electrolytes. Results showed that while secondary metabolites generally appear to reduce the effects of desiccation stress, in some species the substances apparently act as prooxidants and exacerbate stress.

2. Materials and Methods

2.1. Lichen Material

Thalli of *Crocodia aurata* (Ach.) Link, *Parmotrema perlata* (Huds.) M. Choisy, *Ramalina celastri* (Sprengel) Krog & Swinscow, and *Usnea undulata* Stirt. were collected from a small pocket of the Afromontane Forest in the Fort Nottingham Nature Reserve, KwaZulu Natal, South Africa. *Cladonia coniocraea* (Flörke) Sprengel was collected from a valley bushveld savanna at Cumberland Nature Reserve, Pietermaritzburg, KwaZulu Natal, South Africa. The lichens used in this study represent the most abundantly occurring Chlorophycean macrolichens in the region in which we work. In addition, *Cetraria islandica* (L.) Ach. was collected from the understory of a boreal forest on the outskirts of Syktyvkar, Komi Republic, Russia. From the literature, the main lichen substances in these species have been reported to be calycin and pulvinic acid for *Crocodia aurata* [15]; salizanic and stictic acid for *Parmotrema perlata* [16]; usnic acid for *Ramalina celastri* and *Usnea undulata* [17,18]; and fumarprotocetraric acid for *Cladonia coniocraea* and *Cetraria islandica* [19,20].

After collection, thalli were cleared of debris and allowed to air dry at room temperature overnight, and then stored at −24 °C. In general, storage was just for a few days, but in the case of *Cetraria islandica* it was c. 6 weeks. The day before experimentation, thalli were removed from the freezer, hydrated by being placed on wet filter paper in cool (15 °C), dim (30 µmol m^{-2} s^{-1}) conditions for 24 h, and then used immediately.

2.2. Acetone Rinsing

To assess the effect of lichen substances on desiccation tolerance, substances were removed using the "acetone rinsing" technique [13,21]. Briefly, before acetone rinsing, thalli were initially left overnight over silica gel to ensure they were completely dry. They were then gently shaken in 100% acetone for 10 min. The acetone was then discarded, and the process repeated twice. After acetone rinsing, the thalli were left at room temperature overnight to allow residual acetone to evaporate. When done in this way, acetone rinsing has only a small effect on the vitality of the thalli. For example, chlorophyll fluorescence measurements indicated that rinsing caused only slight reductions in F_V/F_M at the start of the experiments.

2.3. Chlorophyll Fluorescence Measurements

To assess the effects of desiccation on the photobiont, the maximum potential quantum efficiency of PSII (F_V/F_M) was measured through chlorophyll fluorescence using a Hansatech FMS2 (Hansatech Instruments, King's Lynn, England) fluorometer. After a dark adaptation period of 10 min, material was given a flash of light (8000 μmol m^{-2} s^{-1} for 0.8 s), and F_V/F_M was measured, where

F_M = maximum fluorescence, and

F_V = variable fluorescence, calculated as $F_M - F_o$, with F_o = minimal fluorescence yield of the dark-adapted state. Very occasionally, thalli with anomalous values of F_V/F_M at the start of the experiment were discarded. Immediately after measurement of F_V/F_M the actinic light (25 μmol m^{-2} s^{-1}) was then switched on, and when the fluorescence signal was stable (typically after 1 min) an additional flash of light was given, enabling estimation of $F_{M'}$, the maximal fluorescence yield of the light-adapted state. NPQ was then calculated using the formula of [22]:

$$NPQ = (F_M - F_{M'})/F_{M'}$$

2.4. Desiccation-Induced Ion Leakage

Ion leakage was used as an index of membrane damage using a modification of the method of [23]. Desiccated lichens were immersed in a beaker containing 100 mL distilled water and stirred with a magnetic stirrer. The electrical conductivity (Cv, μS cm^{-1}) of the water was measured at intervals using a water conductivity meter (Mettler-Toledo AG, Schwerzenbach, Switzerland). To assess the conductivity remaining in the thalli at the end of each experiment (Cf), the material was boiled in 5 mL of water for 30 min, 5 mL added, and conductivity was measured. The conductivity of the mean of several blanks (containing just distilled water) was subtracted from the measured solution conductivities. Ion leakage was calculated as the percentage of the total conductivity lost, calculated as: $Cv \times 100/(Cf + Cv)$.

2.5. Experimental Treatments

Apart from the 10 min dark adaption period needed for measuring fluorescence parameters, all experiments were carried out at low laboratory lighting (c. 5 μmol m^{-2} s^{-1}). For fluorescence measurements, lichen material was desiccated by placing 20 × 2 cm discs or 1 cm thallus segments (10 for the control and 10 with substances removed) in a desiccator over silica gel in the dark. Measurements of F_V/F_M and NPQ were taken at the start of the experiment and at intervals for 60 min. Material was then stored over silica gel in the dark at 20 °C for 2 weeks, and then hydrated by adding liquid water in the dark. Fluorescence measurements were taken at intervals during the following 2 h. In practice, the requirement for a 10 min dark adaptation period meant that much of the actual desiccation and rehydration was carried out in the dark. For ion leakage measurements, 10 replicates of c. 200 mg dry mass (5 for the control and 5 with substances removed) were placed in a desiccator over silica gel for 2 weeks, as for fluorescence measurements. Material was rapidly hydrated by adding liquid water; conductivity measurements were taken at 2 min intervals for 30 min. To test for any effects of lichen substance removal on drying rates, the water contents of replicate thalli were determined by weighing five replicates of c. 200 mg blotted material that had been hydrated (placed on wet filter paper) for 24 h. Material was then placed over silica gel, and fresh mass was determined while air drying at 10 min intervals for 60 min, and then material was dried overnight in an oven at 80 °C for 48 h to determine dry mass. Water content at each sampling interval was obtained by subtracting dry mass from the fresh mass and expressed as g H_2O g^{-1} dry mass. In all cases, storage for 2 weeks reduced the relative water contents of the material to c. 2%.

2.6. Statistical Analyses

All data were subjected to two-way analysis of variance (ANOVA), with time as one factor and the presence or absence of lichen substances as the second factor, using the

data analysis package supplied with Microsoft Excel and after normal tests for normality and homogeneity of variance. In all cases time as a factor was highly significant, and therefore only the significance of the presence or absence of lichen substances is presented in the Section 3.

3. Results

Figure 1 shows the changes in water content that occurred during desiccation in the lichens used in this study. Lichen substance removal had little effect on the maximum water holding capacity of most species, although it was slightly reduced in *Crocodia aurata*. Rates of drying were similar in thalli with and without substances. While substance removal had a significant effect on drying rates in *Cladonia coniocraea*, *Parmelia perlata*, and *Usnea undulata* (Table 1), the differences in the actual rates of drying were small. The response of photobionts to desiccation was assessed by measuring the maximum potential quantum efficiency of PSII (F_V/F_M) during a desiccation–rehydration cycle (Figure 2). During desiccation, the F_V/F_M of four species was significantly more sensitive to desiccation following the removal of lichen substances (Table 1). For *R. celastri* and *Cladonia coniocraea*, F_V/F_M declined at a similar rate in thalli with and without lichen substances. During rehydration following two weeks of desiccation, the initial values of F_V/F_M and general recovery from desiccation were significantly lower following lichen substance removal in four species, but similar in *U. undulata* and *Crocodia aurata* (Table 1). Slow drying increased NPQ in all species, in thalli with or without lichen substances—the only exception being *P. perlata* with substances removed, where no increase occurred (Figure 3, Table 1). Fully desiccated material of all species had low values of NPQ. During rehydration following desiccation, NPQ initially increased, and then declined. For *R. celastri* substance removal had no effect, while in *U. undulata* the removal of lichen substances increased NPQ during rehydration (Figure 3, Table 1). In the other four species, substance removal reduced NPQ during rehydration (Figure 3, Table 1).

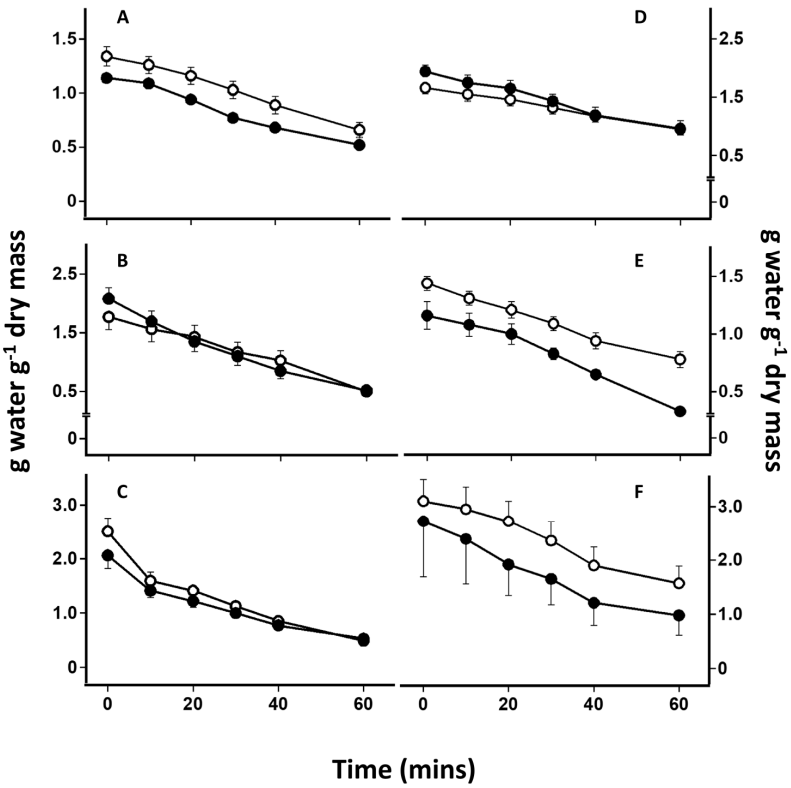

Figure 1. The effect of desiccation on the water content (g water g^{-1} dry mass) of control (open symbols) and acetone-rinsed (closed symbols) samples of six lichens. (**A**) *Cetraria islandica*; (**B**) *Parmotrema*

perlata; (**C**) *Ramalina celastri*; (**D**) *Usnea undulata*; (**E**) *Cladonia coniocraea*; (**F**) *Crocodia aurata*. Thalli were desiccated over silica gel for 60 min. The error bars indicate the standard error of the mean, $n = 5$.

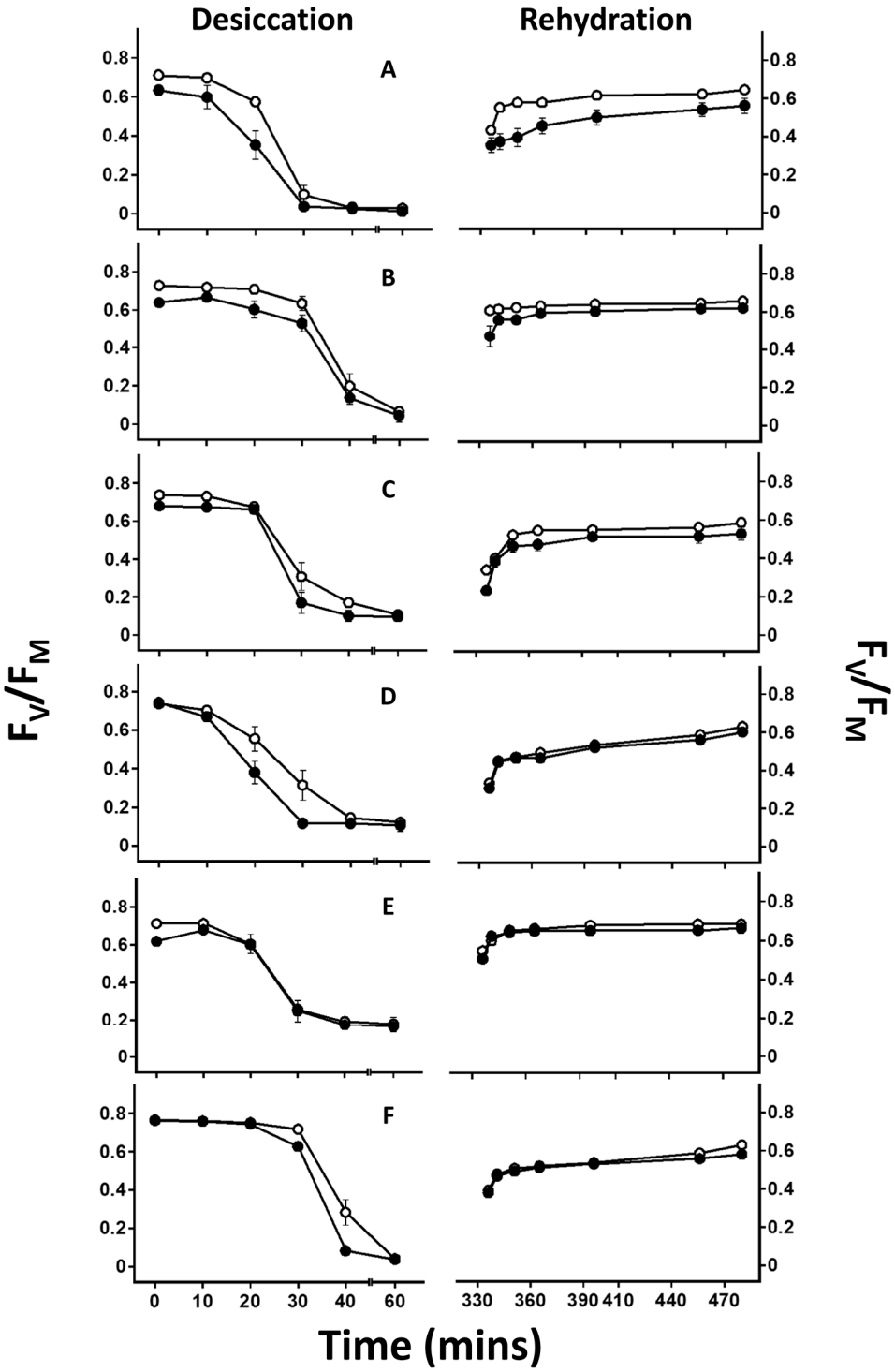

Figure 2. The effect of desiccation over silica gel for 2 weeks and rehydration in liquid water on the maximum potential quantum efficiency of photosystem two (PSII) (F_V/F_M) in control (open symbols) and acetone-rinsed (closed symbols) samples of six lichens. (**A**) *Cetraria islandica*; (**B**) *Parmotrema perlata*; (**C**) *Ramalina celastri*; (**D**) *Usnea undulata*; (**E**) *Cladonia coniocraea*; (**F**) *Crocodia aurata*. The error bars indicate the standard error of the mean, $n = 10$.

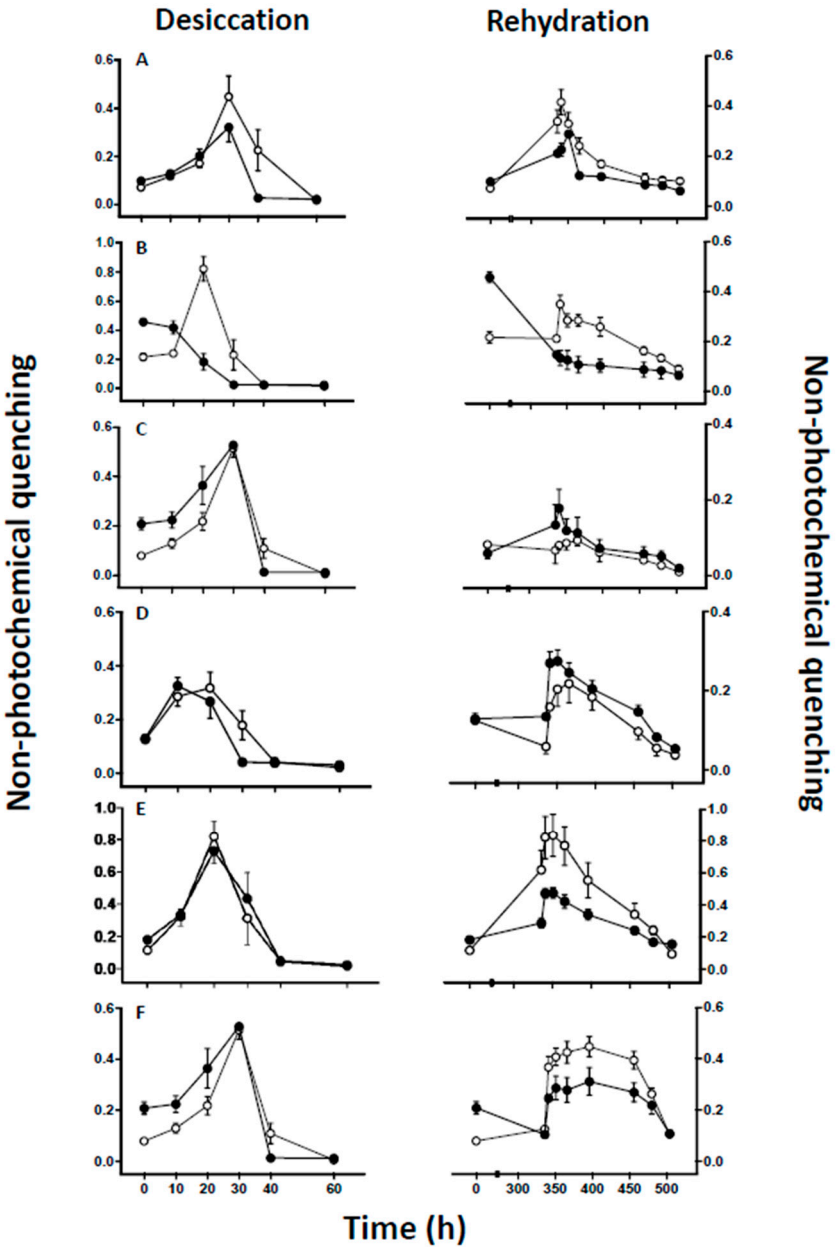

Figure 3. The effect of desiccation on nonphotochemical quenching (NPQ) at 25 μmol m^{-2} s^{-1} in control (open symbols) and acetone-rinsed (solid symbols) samples of (**A**) *Cetraria islandica*; (**B**) *Parmotrema perlata*; (**C**) *Ramalina celastri*; (**D**) *Usnea undulata*; (**E**) *Cladonia coniocraea;* and (**F**) *Crocodia aurata* following 2 weeks of desiccation followed by rapid rehydration. The error bars indicate the standard error of the mean, $n = 10$.

Mycobiont responses to desiccation were tested by measuring ion leakage as a proxy for membrane damage from thalli desiccated for two weeks over silica gel (Figure 4). In all species, during rehydration following desiccation, ion leakage was initially rapid, and then gradually slowed. Removal of lichen substances had a variable effect on membrane damage. In *Cetraria islandica* substance removal had no significant effect on ion leakage (Table 1). In *P. perlata* ion leakage was significantly greater in thalli with lichen substances present, while in the other four species, ion leakage was significantly greater in thalli with lichen substances removed.

Table 1. Statistical analysis (ANOVA) of the effects of desiccation on photobiont responses (F_V/F_M), non-photochemical quenching (NPQ), and mycobiont response (ion leakage) in thalli of *Cetraria islandica*, *Parmotrema perlata*, *Ramalina celastri*, *Usnea undulata*, *Cladonia coniocreae*, and *Crocodia aurata*. For all comparisons, the error had 179 degrees of freedom. Significance: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Species	Water	F_V/F_M		NPQ		Ion
	Content	Desiccation	Rehydration	Desiccation	Rehydration	Leakage
Cetraria islandica	***	**	***	0.083	***	0.237
Parmotrema perlata	***	***	***	***	***	***
Ramalina celastri	0.190	0.552	***	0.686	0.132	***
Usnea undulata	*	***	0.852	0.388	***	***
Cladonia coniocraea	0.112	0.533	*	0.981	***	***
Crocodia aurata	0.204	***	0.136	*	***	***

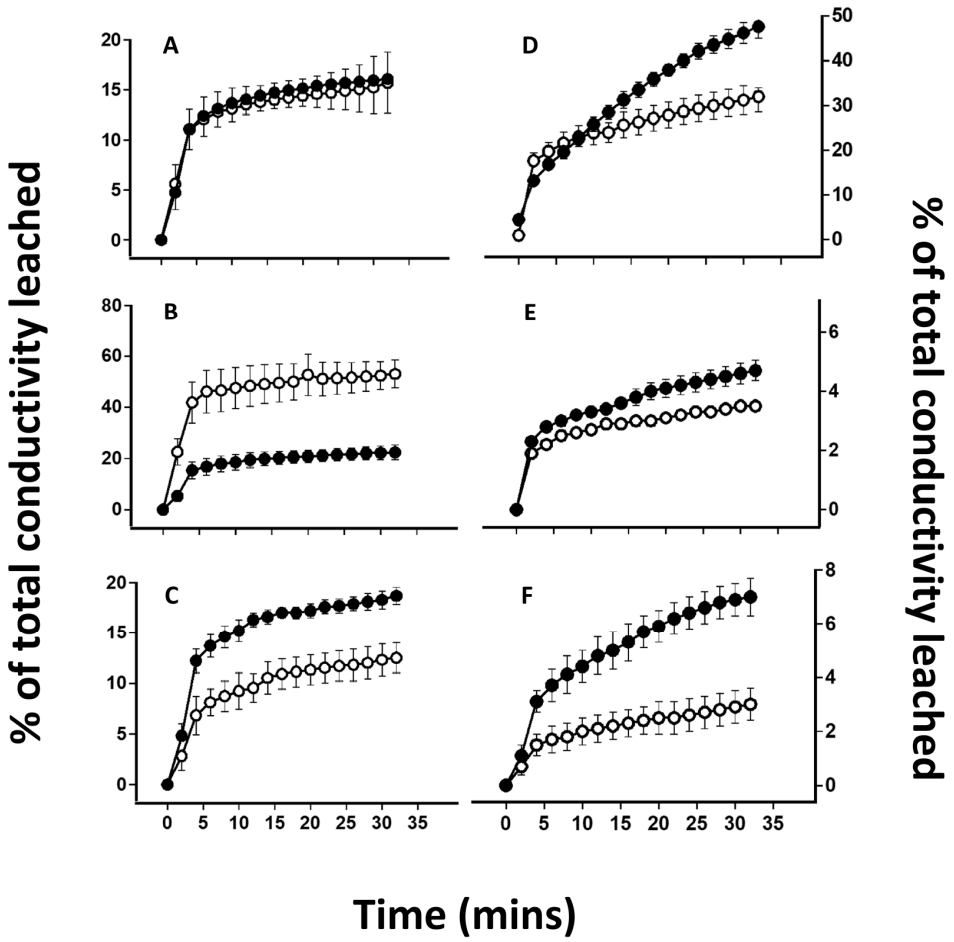

Figure 4. The effect of rehydration following desiccation over silica gel for 2 weeks on the percentage of total conductivity lost in control (open symbols) and acetone-rinsed (closed symbols) samples of six lichens. (**A**) *Cetraria islandica*; (**B**) *Parmotrema perlata*; (**C**) *Ramalina celastri*; (**D**) *Usnea undulata*; (**E**) *Cladonia coniocraea*; (**F**) *Crocodia aurata*. The error bars indicate the standard error of the mean, $n = 10$.

4. Discussion

The results presented here suggest that, in general, lichen substances reduce the effects of desiccation stress in both the photobiont and the mycobiont. For the photobiont, the presence of lichen substances improves PSII activity during either desiccation or rehydration, or both, for all species (Figure 2, Table 1). Interestingly, for the mycobiont, the presence of

lichen substances reduces desiccation-induced membrane damage in four of the six species studied, but shows no effect in *Cetraria islandica*, and actually increases membrane damage in *Ramalina celastri* (Figure 4, Table 1).

4.1. Effect of Lichen Substances on Rates of Desiccation

It was originally thought that lichen substance removal may increase rates of desiccation, possibly because of the substances in the upper cortex forming a structure analogous to the cuticle in higher plants. It has been proposed that when a thallus is wetted, lichen substances may restrict water entry into thalli, preventing excessive water uptake or "oversaturation" [14]. Oversaturation will have the effect of restricting CO_2 diffusion to the photobionts for photosynthesis. Theoretically, therefore, the removal of substances could cause increased total water uptake (on a g H_2O g^{-1} dry mass basis), reducing rates of drying. However, in practice, lichens with and without lichen substances had similar water contents at full turgor, and dried at similar rates (Figure 1). This suggests that the effect of lichen substances on the tolerance of thalli to desiccation is not simply a result of an effect on drying rate.

4.2. Effect of Lichen Substance Removal on Photobiont Sensitivity to Desiccation

For all species, the presence of lichen substances improves the PSII activity of the photobiont during either desiccation or rehydration, or in some cases both (Figure 2, Table 1). The most likely explanation for the improvement in PSII activity during desiccation or rehydration is that lichen substances have very high antioxidant activity [5–7]. Lichen substances are produced in high concentrations at considerable metabolic expense, suggesting that they play key roles in lichen biology [6]. In addition to their better documented roles in defense against pathogens and herbivores, they may increase desiccation tolerance by scavenging desiccation-induced ROS. While they are rather insoluble and occur as crystals on the surface of the mycobiont hyphae (see Section 1), lichen substances are nevertheless spatially well-positioned to intercept ROS, such as H_2O_2, on the surface of the surrounding fungal tissues that otherwise may diffuse from the mycobiont to the photobiont during stress. An alternative explanation could be that lichen substances increase thallus reflectance [21,24], potentially reducing any harmful effects of light during desiccation. While light can certainly increase the harmful effects of desiccation on poikilohydric organisms [14,25], here, desiccation and rehydration were deliberately carried out in the dark or under conditions of low laboratory light. Therefore, increased reflection is unlikely to be the explanation for the greater tolerance of thalli with substances present. The efficiency of lichen substances in reducing the effects of desiccation stress appears to differ between species. Lichen substance removal showed the least effect in *Cladonia coniocraea* (Table 1). This was surprising, because the major lichen substance present in this species has been reported to be fumarprotocetraric acid, which is also present in *Cetraria islandica*. However, in the present study no attempt was made to quantify the amount of lichen substances in the thalli used, and possibly the *C. coniocraea* used contained unusually low concentrations of fumarprotocetraric acid. Furthermore, most lichens contain a mixture of secondary substances [2], and it seems likely that the activity of one lichen substance may be modulated by the presence of other substances. While further work is needed to test these possibilities in more detail, the results presented here suggest that lichen substances exert a generally protective effect against desiccation stress in the photobiont.

As indicated above, all experiments were carried out under low laboratory lighting. However, in the field, lichens will often experience wetting and drying cycles under full or partial sunlight. As discussed by, e.g., [14], while desiccation will reduce carbon fixation by photobionts, lichen thalli will continue to absorb similar amounts of PAR. These conditions are conducive to the formation of ROS, which may cause photoinhibition. The most important mechanism used to reduce ROS formation during photosynthesis is probably the ability to "radiationlessly" dissipate excess light energy as heat through a process known as NPQ [14]. This cycle responds dynamically to changing light intensities; the carotenoid

violaxanthin is enzymatically converted to zeaxanthin in a pH-regulated process that occurs during increases in light intensity. In the results reported here, consistent with reports in the literature for other lichens [26], NPQ tended to increase during drying (Figure 3). Generally, lichen substance removal had little effect on the increase of NPQ during drying. However, interestingly, in four of the six species substance removal significantly reduced NPQ during rehydration (Figure 3, Table 1). The reasons for the effects of lichen substance removal on NPQ are unclear. It would seem unlikely that the substances, which occur as crystals on the mycobiont, have direct effects on photobiont NPQ. Rather, substance removal probably increases general oxidative stress during recovery from desiccation, which may result in the loss of components of the xanthophyll cycle. In the experiments reported here, any reduction in NPQ during rehydration would have no effect on photobiont health, as they were carried out under low light conditions. However, in the field, if rehydration occurred under sunny conditions, low NPQ would further promote oxidative stress and thereby exacerbate the harmful effects of desiccation on photobiont health. It would be interesting to repeat the present experiments in the presence of light. It seems likely that the negative effect of lichen substance removal on photobionts would be even greater in the presence of light.

4.3. Effects of Lichen Substance Removal on the Tolerance of the Mycobiont to Desiccation

In four out of the six species tested here, substance removal increased the susceptibility of the lichens to desiccation-induced membrane leakage (Figure 4, Table 1). Membranes are known to be a major site for oxidative damage during abiotic stress [18], and this is particularly true during desiccation stress in lichens [27–29]. Although lichen algae can be richer in ions than fungal cells [30], they represent no more than 10% of the total volume of a lichen [31]. Therefore, most ions lost will have originated from the mycobiont. It seems most likely that, as for the photobiont, in the mycobiont the generally protective effects of lichen substances on desiccation-induced membrane damage are likely to be due to their ability to act as antioxidants. However, in *Cetraria islandica* substance removal had little effect on membrane leakage, and in *Parmotrema perlata* substance removal actually improved tolerance. As discussed in the Introduction, lichen substances can play many roles in lichen biology, and in some circumstances can act as prooxidants. Thus, for example, while protecting against herbivory or pathogen attacks, the presence of a particular secondary metabolite may increase the susceptibility of a lichen to desiccation stress. Interestingly, the ability of substances to protect the mycobiont is not always correlated with their ability to protect the photobiont. For example, while increasing the sensitivity of the mycobiont of *P. perlata* (Figure 4), secondary metabolites reduced the sensitivity of the photobiont (Figure 2). Therefore, depending on the species, lichen substance removal may affect the photobiont and the mycobiont in different ways. Species varied considerably in their desiccation tolerance, which was assessed as the proportion of ions that leaked during rehydration following desiccation (Figure 4). Similar variations have been reported in a survey of the amount of K^+ lost into the intracellular space following desiccation stress in a range of lichen species [32]. In the present study, sensitivity to desiccation-induced ion leakage was not always consistent with the observed microhabitat of the collection site. For example, the classic shade species *Crocodia aurata*, which grows in a relatively moist microhabitat, was one of the most resistant. Other studies have shown that in lichens it is difficult to predict the amount of desiccation-induced ion leakage by simple visual inspection of its microhabitat [32]. For example, even the semi-aquatic species *Dermatocarpon fluviatile* and *Lichina pygmaea* display only intermediate values of ion leakage [32]. However, within a collection of individual species, ion leakage can provide a sensitive index for the effects of desiccation on membrane integrity, and results presented here suggest that lichen substances generally have a protective effect.

5. Conclusions

In this study we show that although some variation exists between species, removal of lichen substances generally increases the susceptibility of both the photobiont and the mycobiont to desiccation-induced damage. The most likely explanation for these results is that the substances can scavenge potentially harmful ROS formed during desiccation. Future work is needed to study the concentration dependence of any protective effects of lichen substances, which specific substance or combination of substances is most effective, and why, in some cases, lichen substances promote rather than reduce damage. Furthermore, lichens differing in thallus structure will differ in drying rates, and the efficacy of lichen substances at protecting against abiotic stress may depend on drying rates. It would be instructive to take a single species that responds well to substance removal, and then to dry this species at different rates with and without lichen substances. This would provide useful information as to whether the effects of substance removal may differ according to thallus structure. However, it seems clear that in addition to their more established roles in protecting lichens against pathogen attacks and grazing, lichen substances can also play a role in abiotic stress resistance.

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