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RESEARCH ARTICLE

Intra- and inter-specific variations in chitin in lichens along a N-deposition gradient

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Abstract The mechanisms of nitrogen (N) tolerance in lichens are not yet fully understood. Here, we investigated how the increase of chitin content is related with N excess at inter- and intra-specific levels, by using species with differing ecological N tolerances (the tolerant *Xanthoria parietina* and *Parmotrema hypoleucinum* and the sensitive *Evernia prunastri* and *Usnea* sp.) and thalli of *X. parietina* and *P. hypoleucinum* from sites with different availabilities of N of agricultural origin (livestock), as confirmed by lichen N content and $\delta^{15}N$. Nitrogen, chitin (N-containing compound), and ergosterol contents were measured in lichen thalli. Nitrogen and chitin contents were higher in tolerant species than those in sensitive ones (inter-specific level) and in thalli collected from the N-polluted site than in thalli from the clean site (intra-specific level). We suggest that chitin contributes to N stress tolerance in lichens, and that excess N can be partially stored as chitin (non-toxic form) in the cell walls of tolerant species.

Keywords Ammonia · Ammonium · Cell membrane · Cell wall · Ergosterol · Isotopic signature · Stress response · *Xanthoria parietina*

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Introduction

It is well established that lichens reflect the atmospheric concentration of nitrogen (N) they experience, accumulating it over time. Spatial variations in lichen N content reflect the amount of deposited N, particularly ammonium/ammonia (NH_4^+/NH_3), although with differences according to species' functional groups (Gaio-Oliveira et al. 2001; Branquinho et al. 2010; Nielsen et al. 2014). The nitrogen isotopic signature ($\delta^{15}N$) of lichens has also been successfully used to monitor environmental N sources and pathways in several studies. Lichen $\delta^{15}N$ in agricultural regions of Europe varies between − 13.3 and − 1.5 (Boltersdorf and Werner 2013). This variability depends on the lichen species and the N origin and prevents the direct comparison between atmosphere and ecosystems. Nevertheless, the $\delta^{15}N$ values in lichens showed a significant correlation with N content and land use (Boltersdorf and Werner 2013; Boltersdorf et al. 2014).

Lichen communities are known as one of the ecosystem components most sensitive to N, even if lichen species vary widely in terms of N tolerance. Some are favored by high levels of N, whereas others disappear as soon as N availability increases, to concentrations which are still relatively low (Nimis and Martellos 2008; Pinho et al. 2011).

The response of lichens to excess N integrates physiological and ecological mechanisms, as shown for example by the tolerant species *Xanthoria parietina*. In fact, although more tolerant than other species as *Evernia prunastri*, *X. parietina* is also affected by high N concentrations ($[NH_3]$ up to 95.6 μg m^{-3}) (Munzi et al. 2014). Nevertheless, its higher threshold for N toxicity (Munzi et al. 2013) and the lack of competition from more sensitive species when N availability increases allow *X. parietina* to spread extensively in N-rich environments such as rooftops (Gaio-Oliveira et al. 2005) or trees at the borders of agricultural fields (Pinho et al. 2009).

This interaction between physiological (photosynthetic efficiency, membrane and chlorophyll integrity, etc.) and ecological aspects (i.e., the capacity to cope with high levels of N) highlights the need to understand better the N–lichens relationship and to clarify the mechanisms involved and their possible synergism. Mechanisms providing N tolerance in lichens have been investigated by many authors. Besides constitutive characteristics (Hauck 2010), other inducible metabolic mechanisms seem to be developed in tolerant species, as suggested by their greater tolerance when growing with high N availability (Silberstein et al. 1996; Munzi et al. 2011, 2013). However, a definitive explanation of N tolerance in lichens is still lacking (Hauck 2010; Munzi et al. 2013, 2014).

Since the fungal partner constitutes around 90% of lichen biomass, and represents the first site of contact for external elements, it must be assumed that the mycobiont acts as a barrier between the environment and the algal partner (located beneath the cortex), and conducts the majority of N absorption from external N resources (Dahlman 2003), as has been previously suggested for other pollutants (e.g., Cu) (Branquinho et al. 2011). Consequently, the mycobiont response to high N likely shapes the N tolerance of the lichen, and lichens with the same alga but different fungi can have different N tolerances. For example, *E. prunastri* and *X. parietina* are two green algal lichens with *Trebouxia* as a photobiont, but the former shows a greater sensitivity than the latter to N in both the laboratory (Pirintsos et al. 2009) and the field (Pinho et al. 2009, 2011).

Fungal cell walls and plasma membranes represent the boundary between the external environment and the cellular compartment and can have a major role in regulating N uptake and/or exclusion, acting as a physical and/or chemical barrier. Chitin, a polymer of b-1,4-linked *N*-acetyl-glucosamine residues ($C_8O_6H_{15}N$), is a major constituent of fungal cell walls (Griffin 1994), while ergosterol ($C_{28}H_{44}O$) is a main constituent of fungal plasma membranes (Griffin 1994; Smith and Read 1997; Ekblad et al. 1998).

The fungal cell wall is a complex organelle that is a composite of glucan and chitin fibers held together by proteins and mannan. The content and localization of chitin vary among the fungi. Although the primary role of chitin appears to be related to its role in structural integrity (including responses to environmental changes and replication), other roles have been hypothesized (Gottlieb et al. 1991). One of the cell wall's compensatory mechanisms activated in yeasts when exposed to cell wall-perturbing agents or cell wall mutations is the remodeling of the cell wall to combat cell lysis. This includes a strong increase in chitin content, reaching up to 20% of the cell wall dry mass (Aguilar-Uscanga and François 2003; Klis et al. 2002), increasing cell wall strength. Similarly, ergosterol plays an essential role in bulk membrane function, affecting membrane rigidity, fluidity, and permeability (Parks and Casey 1995), and is probably involved in other crucial specific cellular processes (Fumiyoshi and Toshiki 2009).

Crittenden et al. (1994) found that chitin concentrations in *X. parietina* and other lichen fungi when grown in pure culture were directly proportional to N supply. Previous experiments have shown a positive linear increase of chitin and ergosterol with N thallus concentration in 75 lichen species (Palmqvist et al. 2002), and suggested that species exposed to high amounts of N invest more into metabolically inactive biomass in relation to plasma membrane surface area (Sundberg et al. 1999). However, in these studies, chitin and ergosterol were compared only at the inter-specific level and only used as indirect markers of mycobiont activity, fungal biomass (chitin), and fungal respiration (ergosterol), without considering any possible role of these molecules in N tolerance.

In the present work, we hypothesize that the higher chitin content in lichen exposed to high N availability is related to the capacity of tolerant species to store toxic N in non-toxic secondary metabolites (Silberstein et al. 1996; Palmqvist et al. 1998).

If the hypothesis is correct, then fungal cells of tolerant lichen species should have higher contents of chitin (due to more microfibrils per unit volume of wall or thicker walls) and ergosterol than sensitive species, and moreover, tolerant species should increase their N, ergosterol, and chitin contents under high N availability.

Material and methods

To test our hypothesis, we measured and compared the contents of N, chitin, and ergosterol in the thalli of species with various N ecological tolerances (Nimis and Martellos 2008) and in the thalli of the same species exposed to different N concentrations. Different N availabilities at the two sites were estimated by measuring the N content (Branquinho et al. 2010) and N isotopic signature of the samples (Boltersdorf et al. 2014 and references therein).

Lichen sampling

In April 2014, dry thalli of lichen species were collected from *Quercus suber* stems from two sites with differing N availabilities at Companhia das Lezírias, Portugal (Table 1). The two sites, both Mediterranean cork oak woodland areas, differ because one hosts a cattle barn, while the other is devoid of agricultural activities. Nitrogen availability was indirectly evaluated by measuring the N contents of lichen thalli as surrogates of atmospheric N availabilities (Olsen et al. 2010), based on the ability of lichens to reflect local N depositions originating from agriculture (Branquinho et al. 2010; Boltersdorf et al. 2014). The natural N stable isotopes' signature of the lichens was used to confirm the different N sources at the two sites and to identify the preeminent N form available to lichens from the atmosphere, based on the observation that

Table 1 Description of sampling site in terms of N availability and number of observations for each species (NA = not available)

Site	*Xanthoria parietina*	*Parmotrema hypoleucinum*	*Evernia prunastri*	*Usnea* sp.
1. Grazing excluded since 1998 (low N availability)	9	9	9	9
2. Cattle barn, 300 cows (high N availability)	3	3	NA	NA

atmospheric N originating from agriculture is mainly in the inorganic reduced form (NH_3/NH_4^+) and has a distinctive range of isotopic signatures that is depleted in ^{15}N (Boltersdorf et al. 2014).

Xanthoria parietina (L.) Th.Fr. is a ubiquitous species, widely distributed and well known for being very tolerant to eutrophication (Nimis and Martellos 2008; Pinho et al. 2011). *Parmotrema hypoleucinum* (J.Steiner) Hale, although considered oligotrophic, is a common species in Portugal whose presence is not limited by high N availability even in urban, agricultural, and industrial areas (e.g., Pinho et al. 2008; Sérgio et al. 2016); *Evernia prunastri* (L.) Ach. is a moderately sensitive species found at sites without or with weak eutrophication (Nimis and Martellos 2008); *Usnea* is a genus which diminishes with increasing levels of N (Nimis and Martellos 2008; Pinho et al. 2011). The metabolic plasticity of *X. parietina* and *P. hypoleucinum* allows them to grow in both N-poor and N-rich conditions (sites 1 and 2; Table 1), while *E. prunastri* and *Usnea* were only present at the site with the lowest N availability (site 2; Table 1).

All lichen samples were collected from the trunk at between 100 and 180 cm height (three thalli from site 1 and nine thalli from site 2 for each species due to lichen frequency), on the same day, and transported in paper bags to the laboratory, where they were cleaned of impurities, freeze dried, and stored at − 20 °C until analyses were performed. The youngest parts of the collected thalli (up to 1 cm from the margin for foliose lichens and up to 2 cm from the lacinia's tip in fruticose species) were used for analyses.

Ergosterol quantification

The ergosterol quantification was performed according to Dahlman et al. (2002) with small changes: ergosterol was extracted from 10 to 25 mg pulverized, freeze-dried samples with 1 m ethanol (SIGMA), in the dark, for 30 min with orbital agitation; then, the extract was centrifuged (5000 rpm, 15 min) and the supernatant collected, in amber vials, for immediate HPLC-UV analysis, performed with a Shimadzu (Japan) LC-6A pump and a Shimadzu (Japan) SPD-6AV UV-Vis detector. Data were recorded and analyzed using a generic signal recorder software (Azur, Datalys, France); separation was achieved with a Merck LiChroCART 250–4.6 mm Purospher STAR reversed-phase 18e (5 μm) column kept at 25 °C. The mobile phase was 100% methanol (SIGMA) at a flow rate of 1 mL min^{-1}, and detection was performed at the wavelength of maximum absorbance (280 nm), determined by spectrophotometric assays on standard solutions of ergosterol (SIGMA). The injection volume was 20 μL.

Quantification was achieved through external calibration, using standard solutions of ergosterol (SIGMA) in ethanol (six levels, encompassing samples' chromatographic response range, $R^2 = 0.9968$). The mean relative standard deviation of the quantification was 11%.

Chitin quantification

Chitin was quantified using a method adapted from Dahlman et al. (2002). Prior to extraction, samples of 10–25 mg pulverized, freeze-dried lichens were suspended in 1 mL 0.2 M NaOH then incubated on a rotary agitator at room temperature, for 6 h; after centrifugation at maximum speed for 15 min, another 1 mL 0.2 M NaOH was added to the pellet before incubation overnight at 100 °C to remove amino acids and proteins. After centrifugation (max. speed, 15 min), the pellet was suspended in 1 mL 6 M HCl and incubated at 100 °C for 5 h to hydrolyze chitin; after cooling to room temperature and centrifuging (max. speed, 15 min), the supernatant, containing any glucosamine, was collected in a new Eppendorf vial, then evaporated under a vacuum. Two hundred microliters of water (Milli-Q), 250 μL FMOC-Cl (15 mM in acetone) derivatization reagent, and 50 μL borate buffer (1 M, pH 6.3) were added to the extract. After mild agitation and incubation for 10 min, excess FMOC-Cl was removed by two-phase partitioning with 1 mL heptane, repeated twice, and the samples were injected into the HPLC within 10 min.

HPLC-UV analysis was performed with a Shimadzu (Japan) LC-6A pump and a Shimadzu (Japan) SPD-6AV UV-Vis detector; data were recorded and analyzed using generic signal recorder software (Azur, Datalys, France); separation was achieved with a Merck LiChroCART 250–4.6 mm Purospher STAR reversed-phase 18e (5 μm) column kept at 25 °C. The mobile phase was 100% methanol (SIGMA) at a flow rate of 1.4 mL min^{-1}, and detection was performed at the wavelength of maximum absorbance (242 nm), determined by spectrophotometric essays on standard solutions or glucosamine (SIGMA). The injection volume was 20 μL. Samples were analyzed in triplicate.

Chitin was quantified through external calibration, using standard solutions of glucosamine (SIGMA) in water, which was derivatized as the sample (six levels, encompassing sample chromatographic response range, $R^2 = 0.9935$). The mean relative standard deviation of the quantification was 7.68%.

The nitrogen content of chitin was calculated as 6.3% by weight, based on the chemical formula (Dahlman et al. 2003).

Stable isotopes and total N

The ratios of N stable isotopes of the samples were determined at the Stable Isotopes and Instrumental Analysis Facility (SIIAF) of the Centre for Ecology, Evolution and Environmental Changes (cE3c), University of Lisbon, Portugal. The $^{15}N/^{14}N$ ratio was determined by continuous flow isotope mass spectrometry (CF-IRMS) (Preston and Owens 1983), on a Sercon Hydra 20–22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyzer for online sample preparation by Dumas combustion. The major mass signals of N were used to calculate total N abundance, using Sorghum Flour Standard OAS and Wheat Flour Standard OAS (Elemental Microanalysis, UK) as elemental composition reference materials. One composed sample per species from each site was analyzed.

Data analyses

Data qualifications of normality and homogeneity of variance were tested using the Kolmogorov–Smirnov test and the Levene test, respectively. Significant differences between mean values were analyzed using a one-way variance analysis (ANOVA) followed by Tukey's post hoc test (significance level at $p < 0.05$). Pearson correlation coefficients were calculated to study the relationships between chitin or ergosterol and total N in lichen tissue.

Results

Table 2 shows ergosterol, chitin, and total N contents, the N content of chitin, and N isotopic signatures measured in lichen samples from site 1. Chitin content varied markedly and significantly between species: the lowest content was observed in the most sensitive species, *E. prunastri* and *Usnea* sp., and the highest content in the most tolerant one, *X. parietina*. Ergosterol content was also higher in tolerant species, but varied much less than chitin, and a significant difference was only found between the most tolerant and the most sensitive species.

Within species (Table 2), ergosterol did not change between the two sites while chitin content varied significantly both in *X. parietina* and *P. hypoleucinum*, increasing near the cattle barn.

Total N and chitin concentrations (Fig. 1a) were positively correlated in all the samples, while ergosterol concentration varied inter-specifically, but not intra-specifically (Fig. 1b). Chitin increased linearly with thallus N content, by around 6-fold. Lichen samples from the site with the highest N availability had higher concentrations of N and chitin than samples of the same species from site with low levels of N (Table 2, Fig. 1a).

Generally, ergosterol and chitin contents increased with increasing lichen tolerance to N (Fig. 1), reflecting species distribution in functional groups related to N tolerance.

Discussion

Tolerant lichen species showed constitutively higher contents of chitin and ergosterol than sensitive species, while growing in N-rich environments increased their content of chitin but not of ergosterol. We suggest that this pattern is associated with the species' ability to live in N-rich environments.

The values of ergosterol and chitin concentrations we observed are in line with data for green algal lichens: ergosterol in temperate/subtropical green algal lichens ranged between 0 and 1.5 mg g^{-1}, while values of chitin were mostly between 0 and 20 mg g^{-1} DW (Palmqvist et al. 2002).

The nitrogen content of thalli of *X. parietina* from site 1 was similar to background values previously observed for that species (Frati et al. 2007; Olsen et al. 2010). Therefore, the higher N contents found at site 2 (Table 2), increases of 24 and 45% in *P. hypoleucinum* and *X. parietina*, respectively, confirmed a greater availability of N at site 2 than at site 1. Moreover, N contents found in lichen tissues from site 2 (Table 2) are in agreement with those of previous studies conducted in predominantly agricultural and highly urbanized areas (Boltersdorf et al. 2014 and references therein). Finally, the nitrogen isotopic signature confirmed the existence of different N sources at the two sites and is compatible with agricultural activities as the main N source affecting the sampled lichens at site 2 (Boltersdorf et al. 2014).

Chitin can incorporate N in excess, since N represents around 6.3% of chitin's mass (Dahlman et al. 2003), and can make up 10–30% of the cell walls of filamentous fungi by dry weight (de Nobel et al. 2000).

Cell wall composition, in particular that of chitin, has an important role in maintaining the efficiency of fungal cells under stress conditions such as UV light, other radiation, and heavy metals (Feofilova 2010). Moreover, increased chitin synthesis and deposition in cell walls have been shown to be essential to the compensatory response to cell wall stress in *Saccharomyces cerevisiae* and other fungi, through the enhanced expression of the glutamine: fructose-6-phosphate

Table 2 Average values (±SD; $n = 9$ from site 1 and $n = 3$ from site 2) of ergosterol, chitin, and N content in chitin (mg g^{-1}) and values (composed samples) of total N content (mg g^{-1}) and $\delta^{15}N$ measured in samples from sites 1 and 2. Values followed by a different letter (comparing species from site 1) or symbol (comparing samples from sites 1 and 2) are statistically different (Tukey test, $p < 0.05$)

Sample	Ergosterol	Chitin	N	$\delta^{15}N$	N-chitin
Xp (site 1)	$0.99 \pm 0.12^{a,\#}$	$14.25 \pm 1.37^{a,\#}$	16.14	−7.78	$0.89 \pm 0.09^{a,\#}$
Ph (site 1)	$0.82 \pm 0.14^{ab,\#+}$	$10.23 \pm 0.7^{b,\$}$	12.07	−10.21	$0.66 \pm 0.04^{b,\$}$
Ep (site 1)	0.66 ± 0.05^{bc}	3.40 ± 1.42^{c}	12.34	−10.20	0.21 ± 0.09^{c}
Us (site 1)	0.54 ± 0.16^{c}	3.62 ± 2.92^{c}	10.53	−9.33	0.23 ± 0.18^{c}
Xp (site 2)	$0.95 \pm 0.06^{\#}$	$20.74 \pm 3^{+}$	23.37	−5.05	$1.31 \pm 0.19^{+}$
Ph (site 2)	$0.72 \pm 0.02^{+}$	$13.09 \pm 0.39^{\#}$	15.23	−7.68	$0.82 \pm 0.02^{\#}$

amidotransferase-encoding gene (Ram et al. 2004). In both yeasts and filamentous fungi, chitin contributes significantly to the mechanical strength of the cell wall, and when its synthesis is affected, growing hyphae tend to undergo lysis (Ram et al. 2004 and references therein). The activation of the chitin biosynthetic pathway in the fungal partner, in response to cell wall stress, could be one of the mechanisms developed by tolerant lichen species exposed to N excess. In fact, one of the consequences for sensitive lichens of exposure to N excess is damage to cell wall/membranes and a resulting electrolyte leakage (Munzi et al. 2009). Coherently with this hypothesis, in previous experiments, *X. parietina* from a site with a high N availability (ca. 52 kg ha^{-1} y^{-1}) showed a loss of intracellular potassium and magnesium much lower than *X. parietina* from a site with a low N availability (ca. 2 kg ha^{-1} y^{-1}) (Munzi et al. 2013).

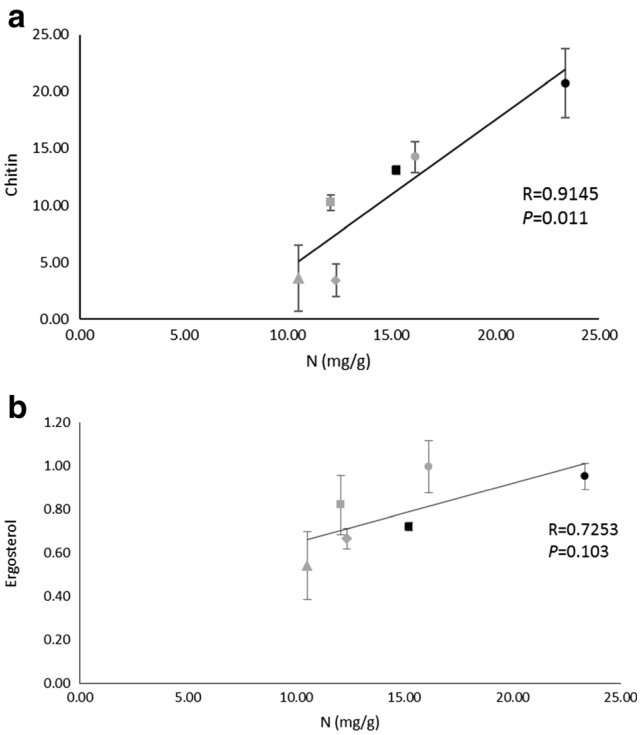

Fig. 1 Correlation (Pearson *r*) between N (mg g^{-1} of dry weight) and chitin (**a**) and ergosterol (**b**) concentrations (mg g^{-1} of dry weight) in thalli of *Xanthoria parietina* (circle), *Parmotrema hypoleucinum* (square), *Evernia prunastri* (diamond), and *Usnea* sp. (triangle) from site 1 (gray, $n = 9$), and site 2 (black, $n = 3$). Vertical bars represent standard deviation, n = number of thalli

A study by Feofilova (2010) showed that growth of *Aspergillus niger* on culture media with high N content led to high chitin–glucan ratios in its cell walls. Makkonen et al. (2007), studying the effect of nutrient enrichment on the anatomy of the lichen *Cladonia portentosa*, found that N content (% of dry weight) was positively correlated with the volume occupied by the inner fungal cortex in the lichen thallus, while the size and volume of algal cells were not affected by N supply. This increased expansion of the lichen area could be related to cell wall thickening and enhanced production of chitin, but further investigation is needed to confirm this.

The variation of total N content observed in our samples, however, cannot be fully explained by the increase in chitin content and the consequent storage of N in the cell walls. The difference between the N contents of *X. parietina* and *P. hypoleucinum* from sites 2 and 1 was one order of magnitude larger than the difference between the N contained in the chitin of the same samples (see Table 2). This difference could be partially explained by an increased production of proteins in lichens exposed to higher N concentrations, in agreement with observations in higher plants exposed to ammonium (Marino et al. 2016). This concurs with data from another (unpublished) experiment in which we found that the content of proteins in *X. parietina* at site 2 was 1.5-fold than that in *X. parietina* from site 1.

Due to the symbiotic nature of lichens, a response of the algal partner to increased N is to be expected. In a study conducted using 92 specimens of *X. parietina*, Beck and Mayr (2012) showed that the $\delta^{15}N$ values of the photobiont *Trebouxia decolorans* were more negative than those of the associated mycobiont. They interpreted this result as evidence that the *Trebouxia* photobiont can compete successfully with the mycobiont for available N. Therefore, the independent uptake of ammonia by the photobiont can contribute to explain the different N content in the samples.

Finally, Freitag et al. (2012) reported a change in lipid composition in the lichen *C. portentosa* in response to N deposition, showing how the metabolism can be affected, and that the amount of other N-containing metabolites can also change, contributing to explaining this difference in N content.

Further work is necessary to clarify in particular the interspecific response and the reported results provide an interesting insight that can help design future experiments.

Conclusions

A higher chitin content was associated with increasing environmental N availability and N tolerance in lichens, while ergosterol was not affected by atmospheric N concentration. This was evident at both inter- and intra-specific levels, since N-tolerant species showed a higher concentration of chitin than sensitive species, and thalli of highly and moderately tolerant species (*X. parietina* and *P. hypoleucinum*, respectively) collected from sites with high N availability displayed higher chitin contents than those from a site with low N availability. As in other fungi, this suggests that chitin is involved in maintaining the efficiency of fungal cells under N stress conditions.

Although the results are not conclusive regarding interspecific differences, since the ergosterol, chitin, and N contents of lichens could be related to a range of other factors such as morphology, N:P stoichiometry, and growth form, this study gives reliable indications on the intra-specific mechanisms of lichen response to increased N availability, contributing to integrate previous ecological, physiological, and metabolic observations.

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