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Effect of nitrogen supply on the C/N balance in the lichen *Evernia prunastri* (L.) Ach.

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Abstract: This study investigated the relationship between nitrogen and carbon in the thalli of the lichen *Evernia prunastri*, with an emphasis on the role of the C/N balance in the response of this species to N excess. For this purpose, thalli collected at a remote site were sprayed for 5 weeks with NH_4NO_3 (0.05 and 1 M) and $(\text{NH}_4)_2\text{SO}_4$ (0.025 and 0.5 M) solutions, and later analyzed for their C and N content. The results showed that the total N content of thalli increased and the total C content decreased along with increasing N concentration in the treatment solutions. Consequently, the C/N ratio also significantly decreased in treated samples. The Fv/Fm ratio, taken as a vitality index, turned out to be positively correlated with C content and negatively correlated with N content. It is suggested that N stress is caused by the lack of compensation for elevated ammonium levels with higher C assimilation. It is concluded that *E. prunastri* is a suitable bioindicator of N pollution and that the C/N ratio deserves further investigation as a promising tool for early warning of N excess.

Key words: Ammonium, biomonitoring, fertilization, physiological parameters, symbiosis

1. Introduction

The ecological success of symbiotic forms of life is based on a well-defined balance between the symbiotic partners. Lichens, with their estimated 17,300 species distributed all over the world (1), are a prime example of this. Lichenized fungi are considered to be nutritionally specialized organisms adsorbing mineral nutrients and nitrogen from the environment and deriving carbon from the photosynthetic partner, either an algal or a cyanobacterial photobiont (2).

The survival of both partners would be impossible without an equilibrated distribution of nutrients and assimilated C between them (3), such that the decline and the disappearance of some lichen species in particular conditions are regarded as the consequence of a rupture of this balance occurring when one of the partners is favored by environmental factors (4). For example, it has been observed that lichens tend to disintegrate in the case of nutrient-rich supplies (5).

Moreover, a regulated C/N economy seems to exist in lichens (6): in the case of increasing N content, an increased C assimilation is necessary to store the N in forms that are not toxic, such as amino acids and in particular arginine (7). The role of C is to provide both C skeletons for amino acid synthesis and energy for increased respiration (8).

On these bases, an uncontrolled assimilation of N has also been suggested as a possible factor capable of perturbing lichen symbiosis (8,9).

Less productive lichens are therefore assumed to be more susceptible to high N levels than many highly productive species (10). Nitrogen pollution is currently considered a major threat to human health and the stability of ecosystems (11,12). Reactive forms of N are derived mainly from agricultural practices and combustion of fossil fuels (13), and the strict connection of these activities with human daily life makes it difficult to decrease reactive N emissions.

Agricultural areas and urban environments are characterized by a shift in lichen flora from acidophilous to nitrophilous species with important loss of biodiversity due to the disappearance of N-sensitive species (14). The fruticose species *Evernia prunastri* (L.) Ach. responds significantly to several N compounds, providing a suitable tool for studying the effects of N on lichen physiology (15–17). Low N tolerance in this lichen has been partially explained by its high cation exchange capacity (18), which allows the entrance of a greater amount of ammonium into its cells than in tolerant species like *Xanthoria parietina* (L.) Beltr. (19). Comparing sensitive and tolerant species, some authors have suggested that *E. prunastri* may also be

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unable to develop and/or activate defensive mechanisms against N once it has penetrated the cells, such as polyamine production (17) or other metabolic pathways hypothesized for *X. parietina* (16,20).

In a previous paper, we showed that *E. prunastri* thalli treated separately with ammonium nitrate (0.05 and 1 M) and ammonium sulfate (0.025 and 0.5 M) for 5 weeks underwent partial or almost total inhibition of the photosynthetic apparatus, as indicated by the Fv/Fm values (16). In this work, we investigated the role of the C/N balance in the response of *E. prunastri* to N excess in order to 1) find out if there is a relation between C/N and lichen vitality (expressed as Fv/Fm), and 2) verify the suitability of the C/N ratio as an early physiological indicator of N stress.

2. Materials and methods

2.1. Lichen sampling

Thalli of *E. prunastri* (L.) Ach., an epiphytic green algal (*Trebouxia*) lichen species, were used in our experiments. *Evernia prunastri* is a fruticose acidophytic species readily disappearing under conditions of N pollution (14,19).

Lichen samples were collected in an unpolluted area of central Italy (Ancaiano, Siena, Tuscany), transferred to the laboratory in paper bags, and left overnight to acclimate in a climatic chamber at 15 ± 2 °C and $55 \pm 5\%$ relative humidity for a photoperiod of 12 h at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ photons of photosynthetically active radiation.

In order to keep the lichens in the most natural conditions, 5 samples for each treatment were then fixed on a plastic net mounted on a wooden frame and exposed towards the northwest on the roof of the Department of Environmental Science of the University of Siena at ambient temperature and humidity, sheltered from direct rainfall. The experimental set up was intended to optimize environmental conditions for the thalli during the treatments, since photosynthetic pigments of *E. prunastri* may be altered when exposed to dry conditions and full sunlight (21,22) and photosynthetic processes may be more susceptible to treatments with salt solutions under full sunlight (23).

2.2. Nitrogen manipulation

Experiments were run for 5 weeks (5 March to 18 April 2007). Lichen thalli were hydrated by spraying until runoff with deionized water, alone or supplemented with treatment solutions. Spraying was done every Monday and Thursday in the morning to limit the rate of evaporation and prolong the effects of hydration.

During the first week, to ensure that the transplantation itself did not damage lichen thalli, samples were not treated with N, but with deionized water only. From the second week onward, spraying was done either with water (control samples) or with solutions (pH adjusted to 6.5) of NH_4NO_3 (0.05 and 1 M) and $(\text{NH}_4)_2\text{SO}_4$ (0.025 and 0.5 M).

These concentrations, based on the N uptake capacity of lichens (19), were chosen to ensure the same concentration of ammonium ions in the 2 treatments. A short piece of plastic tube was used during spraying to avoid intersample contamination.

Actual concentrations of NH_4^+ in the solutions were verified spectrophotometrically by the indophenol blue method (24) and were found to be within 3.8% of nominal concentrations. To avoid confusion, nominal concentrations are used throughout the text and figures.

2.3. C and N analysis

Five thalli were immediately analyzed for their C and N content to provide a reference level at the beginning of the experiment. After the treatments, lichen samples were air-dried to constant weight and carefully cleaned with nylon tweezers under a binocular microscope to remove as much extraneous material as possible. Samples were briefly washed in deionized water to remove excess N possibly deposited onto lichen surface.

Dried samples were analyzed in triplicate by direct total flash combustion using an elemental analyzer with a thermal conductivity detector (PerkinElmer, mod. CHN/O 200) in order to determine average total carbon (TC) and total nitrogen (TN). Recoveries and reproducibility were checked by analysis of procedural blanks and standard reference materials. Precision of analysis was estimated by the coefficient of variation of 10 replicates and was found to be within 5%.

2.4. Photosynthetic efficiency analysis

Every Tuesday and Friday, the days after the treatments, measurements of photosynthetic efficiency by the Fv/Fm chlorophyll *a* fluorescence method were taken for all the lichen thalli as a marker of lichen vitality (25). The Fv/Fm ratio was measured in dark-adapted samples at room temperature with the Plant Efficiency Analyzer Handy PEA (Hansatech Instruments Ltd.).

2.5. Statistics

Significance of differences ($P < 0.05$) between treatments and controls was checked by one-way analysis of variance (ANOVA), using the Dunnett test for post hoc comparisons. Prior to analysis, data not matching a normal distribution (Shapiro–Wilk W test at a 95% confidence interval) were transformed using the Box–Cox method. Homogeneity of variances was checked with the Levene test. Differences between samples sprayed with deionized water and treated with N were checked using the Student t-test.

Correlation coefficients between different variables were calculated by the Pearson *r* and considered significant when $P < 0.01$.

3. Results

The Table shows TC, TN, and their ratios in the lichen *E. prunastri* before and after the treatments. Prior to treatments, all samples had a similar C and N content.

Table. C and N content.

	H ₂ O (control)	NH ₄ NO ₃ , 0.05 M	NH ₄ NO ₃ , 1 M	(NH ₄) ₂ SO ₄ , 0.025 M	(NH ₄) ₂ SO ₄ , 0.5 M
C1	42.64 ± 0.84	42.20 ± 0.75	42.39 ± 0.42	42.04 ± 1.07	41.98 ± 0.50
C2	41.62 ± 0.83	39.88 ± 1.08	35.15 ± 3.17	40.19 ± 0.66	36.35 ± 2.97
N1	1.25 ± 0.18	1.12 ± 0.1	1.19 ± 0.08	1.50 ± 0.24	1.43 ± 0.10
N2	2.12 ± 0.09	2.54 ± 0.23	6.27 ± 1.68	2.60 ± 0.34	3.97 ± 2.21
C2/C1	0.98 ± 0.02	0.95 ± 0.04	0.83 ± 0.08	0.96 ± 0.04	0.76 ± 0.14
N2/N1	1.72 ± 0.25	2.33 ± 0.31	5.35 ± 1.73	1.75 ± 0.15	2.20 ± 0.93
C1/N1	34.81 ± 4.69	39.01 ± 3.62	35.86 ± 2.24	28.58 ± 3.84	29.64 ± 2.24
C2/N2	19.48 ± 0.62	15.76 ± 3.62	5.83 ± 1.96	15.66 ± 2.12	12.99 ± 5.66

Mean % weight values (\pm standard deviation, N = 5) of total C before (C1) and after (C2) the treatments, total N before (N1) and after (N2) the treatments, and their ratios in thalli of *Evernia prunastri* sprayed twice a week for 5 weeks with deionized water (control), ammonium nitrate at 0.05 and 1 M, and ammonium sulfate at 0.025 M and 0.5 M. Values in bold differ from the control (one-way ANOVA, Dunnett test, $P < 0.05$); values in italics differ from those measured before the treatments (Student t-test).

Total N content increased in all treated samples, while TC decreased in the case of NH₄NO₃ at 1 M and (NH₄)₂SO₄ at 0.5 M. Prior to treatments, the C/N ratio ranged between 28.58 \pm 3.84 and 39.01 \pm 3.62 and significantly decreased in all treated samples.

Figure 1 shows the correlation curves between the potential quantum yield of PSII (Fv/Fm) in *E. prunastri* and the total content of C (Figure 1a) and N (Figure 1b) after the same treatments. Fluorescence values increased with increasing C content ($R = 0.97$, $P < 0.01$) and decreased with decreasing N content ($R = -0.85$, $P < 0.01$). The C/N ratio was positively correlated with Fv/Fm ($R = 0.91$, $P < 0.01$).

4. Discussion

High levels of N are toxic for many organisms. One defensive mechanism against N excess known for higher plants and lichens is to assimilate N on C skeletons in order to avoid the accumulation of toxic concentrations of free intracellular ammonium and nitrate (7,26). Our findings suggest that this protection mechanism does not occur in the lichen *E. prunastri*, thus helping to explain its well-known sensitivity to N excess.

Energetic costs of nitrate reduction, ammonium assimilation processes, and transportation are supported by the photosynthetic activity of the photobiont and the cellular sugar stocks, such as mannitol reserves, made available by the mycobiont (19). A rising chlorophyll *a* concentration has been found in fertilized samples of *Hypogymnia physodes* (L.) Nyl. and *Platismatia glauca* (L.) W.L.Culb. & C.F.Culb. supplied with different

concentrations of NH₄NO₃, suggesting that these species respond to an increased demand of C with increased photosynthetic activity (8). In contrast, Gaioliveira et al. (19) reported unaltered concentrations of chlorophyll *a* in *E. prunastri* irrespective of N dosage of the treatment, while Frati et al. (27) found a significant decrease in the concentration of chlorophyll *a*, chlorophyll *b*, and carotenoids, as well as chlorophyll degradation to pheophytin, concomitant with N accumulation in transplants of *E. prunastri* close to a pig stock farm. This is consistent with our results, since the decreased C content observed in treated thalli of *E. prunastri* suggests a lower accumulation of C through absorption processes along with the occurrence of oxidative phenomena in the tissues concomitant with CO₂ release. Moreover, the fluorescence values used as indicators of the overall vitality of the lichens (16) increased with increasing C content and decreased with increasing N content.

In the balance between the 2 symbionts, highly to moderately N-tolerant species react to the N supply by investing energy and resources in the growth of the photobiont, to support a higher efficiency of light energy conversion and N assimilation (6). Dahlman et al. (8) found that thalli of fertilized *H. physodes* and *P. glauca* had N concentrations 2- to 3-fold higher than control thalli and a 2-fold higher soluble carbohydrates concentration. In contrast, sensitive species exhibit a partial or total inhibition of PSII caused by N fertilization (Figure 1b), lacking the C assimilation necessary for N conversion in nontoxic forms.

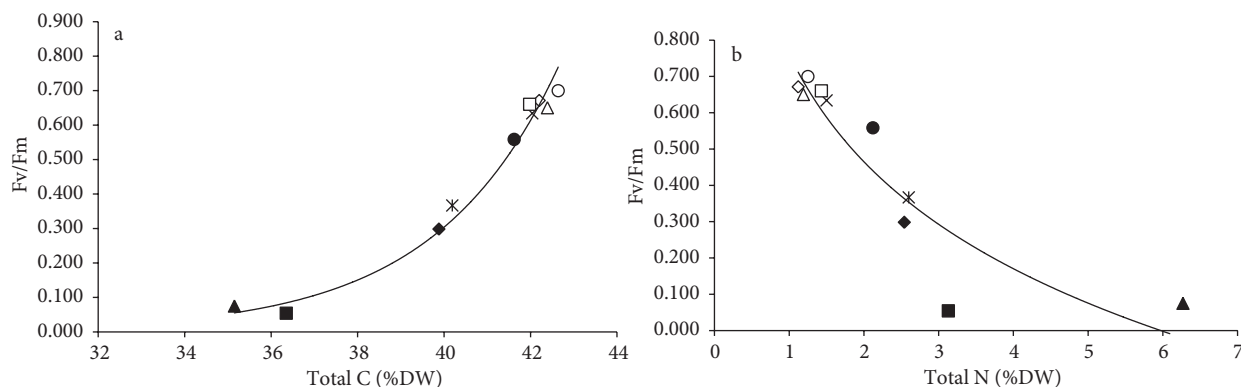


Figure 1. Correlation a) between Fv/Fm and total C (% dry weight [DW]) and b) between Fv/Fm and total N (%DW) in samples of *E. prunastri* treated with N solutions. Circle = control; diamond = 0.05 M; x = sulfate, 0.025 M; square = sulfate, 0.5 M; triangle = nitrate, 1 M; empty symbols = before the treatments; filled symbols = after the treatments.

Total N concentrations in untreated thalli were consistent with several studies that indicated that the background N content of *E. prunastri* is approximately 10 mg/g dry weight (6,18,28). Increasing N content in control samples can be explained by exposure for 5 weeks to an urban environment that, even if only moderately polluted (29), presents a concentration of N compounds higher than the rural background (30,31).

Samples treated with NH_4NO_3 at 0.05 M and $(\text{NH}_4)_2\text{SO}_4$ at 0.025 M did not show any significant difference from the control, probably because the N concentration of the solutions was low enough to be hidden by the atmospheric deposition. The lack of significance of the N increment in thalli supplied with $(\text{NH}_4)_2\text{SO}_4$ at 0.5 M can be attributed to intraspecific variability, as witnessed by the high value of the standard deviation. In contrast, the N load supplied as NH_4NO_3 at 1 M clearly caused a significant N accumulation in the lichens.

The ratio of ammonium ions between the solutions NH_4NO_3 at 1 M and $(\text{NH}_4)_2\text{SO}_4$ at 0.5 M was 1:1, but the ratio of TN was 2:1. Thus, it is not surprising that the amount of N in thalli treated with NH_4NO_3 at 1 M was higher than in thalli treated with $(\text{NH}_4)_2\text{SO}_4$ at 0.5 M, even if among the inorganic compounds it is known that the affinity for ammonium and its uptake rate are much higher than for nitrates (32,33).

The observed changes in C and N contents after the treatments made the C/N values significantly decrease, both in samples supplied with N and in those supplied with deionized water (Table). Photosynthetic processes in sensitive lichens may be susceptible to osmotically induced dehydration (34). In addition to nitrogen stress, at the highest concentrations osmotic stress could also partially contribute to inhibiting photosynthetic processes. *Evernia prunastri* appeared susceptible to salt stress in the presence of high irradiance with approximately full sunlight (23);

therefore, to minimize this factor, our experiment was carried out under shaded conditions.

Vingiani et al. (35) reported a value of 51.96 for the C/N ratio ($C = 44.17$, $N = 0.85$) in nonexposed thalli of *Pseudevernia furfuracea* (L.) Zopf, which decreased to 42.34 ($C = 43.78$, $N = 1.034$) in thalli exposed in bags in the Naples urban area for 10 weeks and to 35.75 ($C = 42.87$, $N = 11.99$) in thalli exposed for 17 weeks. Satya and Upreti (36) showed that in *Rinodina sophodes* (Ach.) A.Massal. taken from a moderately polluted village in India, the C/N ratio was 15.29 ($C = 35.62$, $N = 2.33$).

The strong correlation of C and N content with Fv/Fm values (Figures 1a and 1b) means that N exposure did not merely change the C and N levels in the lichen, but also affected the health status of the lichen.

Several physiological parameters have been suggested as suitable early indicators of N stress (15,37), and in this light the C/N balance merits further investigation. The values measured in untreated lichens indicated a low intraspecific variability of this parameter in our samples, but a more comprehensive study on the influence of the species and the collection site is still lacking.

Assuming that different lichen species avoid N toxicity in different ways (38), it seems that *E. prunastri* is not provided with any of them. Recent studies have shown that this species cannot avoid N entrance into the cell owing to its high cation exchange capacity (18). It is unable to use/produce polyamines to neutralize N excess (17) and no repairing systems seem to be involved in its metabolism, unlike the N-tolerant *X. parietina* (20), and, as our results showed, it cannot compensate for elevated ammonium levels with higher C assimilation. All of these features indicate that this species can be used as a suitable bioindicator of N pollution.

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