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Influence of Invertebrate Feeding on the Lichen *Cladonia pocillum*

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Abstract

Using scanning electron microscopy, it was demonstrated that moth larvae consumed upper cortex and algal layers of the lichen *Cladonia pocillum*, but not the medulla. Feeding usually occurred on primary squamules leaving podetia intact. Decrease in the algal complement of squamules was confirmed by significantly lower chlorophyll *a*, chlorophyll *b*, and total carotenoids. Decreased F_v/F_m in consumed squamules showed that the status of photosystem II was also altered. However chlorophyll *a/b* and the integrity of chlorophyll a were not changed significantly. Respiration, as indicated by dehydrogenase activity of the mycobiont hyphae in medulla, was not changed by feeding. The presence of fumarprotocetaric acid in the medulla was confirmed using TLC. Although fumarprotocetraric acid is considered an antiherbivore compound, it does not provide total protection for *C. pocillum*, as it is located only in medullar hyphae.

Keywords: Lichenivory, pigment content, fumarprotocetraric acid, dehydrogenase activity, chlorophyll *a* fluorescence, SEM

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1. Introduction

Lichens offer a variety of protective niches for invertebrates, and the ecology of lichen-invertebrate associations is relatively well documented (Gerson and Seaward, 1977). Lichen thalli serve as food for herbivorous species of invertebrates, lichenivory or lichenophagy having been documented previously by Gerson and Seaward (1977) and Rawlins (1984).

Lichenivorous invertebrates are found in the phylum Arthropoda, including both insects (e.g. orders Psocoptera and Lepidoptera) and non-insects (order Acari), as well as the phylum Mollusca (Broadhead, 1958; Gerson and Seaward, 1977; Rawlins, 1984; Seyd and Seaward, 1984).

Chemical defense in lichens includes production of secondary metabolites. The role of lichen secondary chemistry in deterring grazing activity is well documented and several studies have confirmed a negative correlation between presence of some secondary metabolites in lichens and degree of feeding (Lawrey, 1980; 1983; Giez et al., 1994; Hesbacher et al., 1995). However, this mechanism of protection is not totally effective. Secondary metabolites are produced by the fungal partner of the lichen symbiosis (the mycobiont), but lichenivores preferentially feed on the photobiont (algae, or cyanobacteria) and lichenophagy would thus affect the sensitive balance between symbionts. Other than the detailed work of Broadhead (1958) on the feeding preferences of Psocoptera, virtually nothing is known about the effects of invertebrate feeding on the separate components of a lichen. Fröberg et al. (2001) demonstrated that photobiont cells of *Xanthoria parietina* partly withstood enzyme activity in the digestive tract of a snails.

The main aim of this paper was to determine how the lichen *Cladonia pocillum* is affected by moth larvae feeding. We used scanning electron microscopy to address whether the algal layer was preferably consumed and examined selected ecophysiological parameters such as pigment content, and functioning of photosystem II. Possible effects of lichenivory on respiration were assessed by measuring dehydrogenase activity. We also determined whether the fumarprotocetraric acid was associated with the extent of feeding in *C. pocillum*.

2. Material and Methods

Lichen material

Samples of *Cladonia pocillum* (Ach.) Grognot, both control and populations grazed by moth larvae, were collected from coastal sand dunes of Lake Huron in Pinery Provincial Park, 8 km south of Grand Bend (43'15 81'50), Ontario, Canada in April and June 2002. This locality is rich in Lepidoptera, many of which are

potentially lichen feeders, especially in semiarid and xeric habitats (Rawlins, 1984). Twenty samples with podetia 1–1.5 cm high, each consisting approximately 80 cm^2 of the lichen mat, were randomly selected from areas exposed to direct sun. Macroscopic foreign material adhering to surfaces (mosses, soil particles, detritus) was removed with forceps. Experiments were conducted within 2–3 days of collection.

SEM studies

Samples of dry lichens were directly mounted onto aluminum stubs with adhesive carbon disk (Soquelec Ltd.). All mounted samples were dried at 35°C for 3 days prior to microscopy and sputter-coated with a thin film of gold for approximately 6 min on a Hummer VI Sputter Coater. Specimens were examined under the LEO 440, software-controlled scanning electron microscope at the University of Western Ontario Surface Science Center.

Pigment analysis and measurement of chlorophyll a integrity

To assess the effect of larval feeding on pigments and chlorophyll integrity, 20 mg of lichen sample was weighed in a conical centrifuge tube. Samples were then extracted in the dark for 1 h at 65°C in 3 ml of DMSO in the presence of polyvinylpolypyrrolidone (2.5 mg ml^{-1}) to minimize chlorophyll degradation (Ronen and Galun, 1984; Barnes et al., 1992). Extracts were then allowed to cool to ambient temperature, diluted 1:1 with fresh DMSO, and the absorbance, a reflection of turbidity, was checked at 750 nm with a Shimadzu spectrophotometer to be certain that it was always less than 0.01. To assess chlorophyll, the absorbance of the extracts was read at 665.1, 649.1, 435 and 415 nm and to determine the major primary carotenoids, absorbance was read at 480 nm (total carotenoids). All measurements were performed by Shimadzu UV 160 double-beam spectrophotometer. Chlorophyll *a*, chlorophyll *b*, chlorophyll *a+b* and total carotenoids were calculated using equations derived from specific absorption coefficients for pure chlorophyll *a* and chlorophyll *b* in DMSO (Wellburn, 1994).

The ratios of optical densities at 435 and 415 nm (OD 435/OD 415), termed the phaeophytinization quotient, were interpreted as reflecting the ratio of chlorophyll *a* to phaeophytin *a* and provided an indication of integrity of photobiont chlorophyll (Ronen and Galun, 1984). Five replicates were used.

Activity of photosystem II

Prior to measurement of chlorophyll *a* fluorescence, lichens were immersed

for 20 min in distilled water and stored for 24 h in a dark chamber with 100% air humidity. Before measurement of fluorescence, samples were dark-adapted for 30 min (Backor et al., 2003). The potential quantum yield of photosystem II (PSII) was measured using a Plant Stress Meter (PSM Mark II, Biomonitor, SCI AB) with sensor dia 5 mm, and results were expressed as F_v/F_m, calculated as the maximal fluorescence (F_m) less the minimal fluorescence (F_o), divided by F_m of dark adapted plants, i.e., $(F_m - F_o) / F_m = F_v/F_m$. Chlorophyll fluorescence parameters were determined in three separate positions on each measured sample, and the mean value was used as one observation. This was replicated ten times.

Assessment of dehydrogenase activity

To assess the effect of feeding on thallus dehydrogenase activity of lichen, 20 mg (± 1 mg) of air dw sample was weighed in a 15 ml plastic centrifuge tube (VWR brand). A modified method of Lin et al. (2001) was used in this study, where water for homogenization was substituted by DMSO. Into each tube 2 ml of 0.6% tetrazolium red (TTC) in 0.05 M phosphate buffer (pH 6.8) containing 0.005% Triton X 100 was pipetted. Tubes were kept in the dark for 20 h at 25°C to allow for red formazan (TPF, triphenyl formazan) production. Lichens were then rinsed 3 times in 10 ml distilled water. To extract water-insoluble formazan, 2 ml of dimethyl sulfoxide (DMSO) was used, which permeates mycobiont hyphae. After incubation at 65°C in the dark for 2 h, extraction of formazan was terminated by adding glass beads and vortexed for 30 s. Formazan was extracted by adding 4 ml of n-hexane, vortexing for 15 s and centrifuging at 400 g for 10 min. Absorbance of the supernatant was then measured at 485 nm by Shimadzu UV 160 double-beam spectrophotometer. Five replicates were used.

Determination of secondary products

All samples were dried at 35°C for 24 h prior to extraction of secondary compounds. Acetone (1.5 ml) was added to 20 mg pre-weighted lichen samples ina graduated microcentrifuge tube (VWR Scientific). At the end of incubation period (1.5 h at 25°C), the acetone extract was pipetted from lichen fragments and another volume of acetone was added. Extraction was repeated at least three times. Acetone extracts were collected and evaporated, and the residues were dissolved with fresh 1.5 ml acetone.

To determine the constituents present in acetone extracts, c. 50 μl of acetone extract prepared as above was applied by 5 μl glass pipettes (Drummond) at 1.2 cm intervals across the short side of 10 × 20 cm pre-coated plates Merck silica gel

60 F-254 with a fluorescent indicator. The standardized method for identification of lichen products (Culberson and Kristinsson, 1970; Culberson, 1972) was used.

Three solvents systems: A, B and C were used. In solvent B methyl tert-butyl ether was used as a substitute for diethyl ether (Culberson and Johnson, 1982). Plates were viewed under short- and longwave UV light using IS-1000 gel documentation system (Canberra-Packard Canada Ltd.) consisting of an Electronic Multiwave Transilluminator, digital camera and computer with imaging software (Alpha-Imager 2000I Version 3.3b). Plates were visualized with a spray of 10% sulfuric acid and heated for 30 min at 110°C. Each sample was analyzed in triplicate on each plate, in each solvents system. Pure substances (atranorin, usnic acid) and microextracts of herbarium specimens with TLC determined chemistry (e.g. didymic acid, fumarprotocetraric acid, lobaric acid, norstictic acid) were run as standards.

Statistical analysis

One-way analysis of variance and Tukey's pairwise comparisons (MINITAB Release 11, 1996) were applied to determine the significance ($P<0.05$) of differences in all measured parameters.

3. Results

Fig. 1 shows scanning electron micrographs of the surface of primary squamules of *Cladonia pocillum*, and Fig. 2 demonstrates the surface after feeding by invertebrates. Feeding damage was largely restricted to the upper cortex and algal layer, with the medulla left intact. Podetia lacked visible evidence of damage.

Table 1 shows chlorophyll content and total carotenoid concentration in primary squamules and podetia of *C. pocillum* before and after feeding. Chlorophyll *a*, chlorophyll *b*, total chlorophyll content, and total carotenoids were not significantly altered in podetia after feeding, however, all of these parameters were significantly decreased in grazed squamules. In unaffected thalli, podetia contained significantly more chlorophyll *a* than primary squamules. Podetial carotenoids and chlorophylls were less affected by grazing than squamular segments (Tables 1 and 2).

Chlorophyll *a*/*b* ratio and integrity of chlorophyll *a* were unchanged by lichenivory (Table 2). All measured fluorescence parameters were significantly lowered in squamules affected by feeding, with the highest F_v/F_m ratios in unaffected lichen samples, both primary squamules and podetia (Table 3).

Values of dehydrogenase activity were higher in podetia than in squamules in both control and grazed lichens (Table 4).

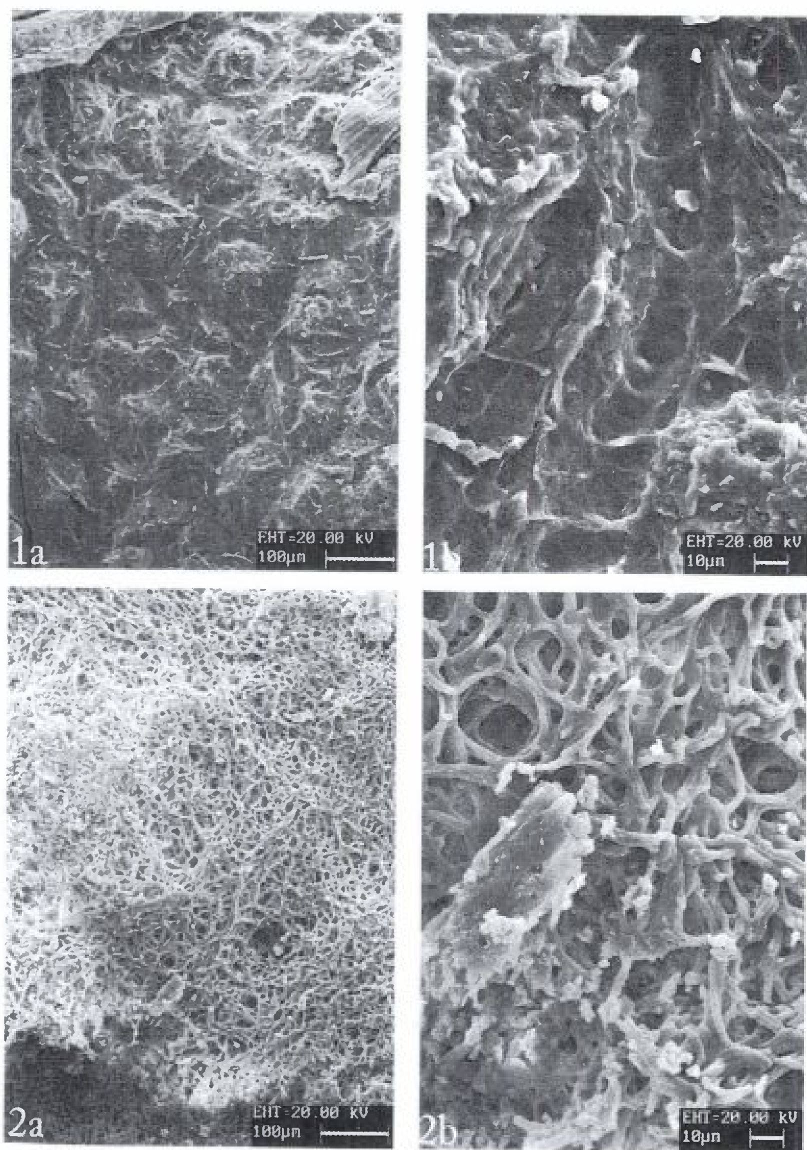

Figure 1. a) Scanning electron micrographs of the intact cortex of a primary squamule of *Cladonia pocillum*, b) detail view.

Figure 2. a) Scanning electron micrographs of primary squamules of *Cladonia pocillum* after feeding by invertebrates. Damage is largely restricted to the upper cortex and algal layer, hyphae of medulla are almost intact, b) detailed view.

Table 1. Clorophyll *a* (Chl a), chlorophyll *b* (Chl b), chlorophyll *a+b* (Chl a+b) and total carotenoids ($\mu g.g^{-1}$ dw) contents in *C. pocillum*, C-control, E-eaten, B-basal squamules, P-podetia, n = 5, X = mean values, SD = standard deviations. Values in each vertical column followed by the same letter do not differ significantly at P<0.05 by Tukey's pairwise comparisons.

	Chl a	Chl b	Chl a+b	Total carotenoids
		($\mu g.g^{-1}$)		
	X ± SD	X ± SD	X ± SD	X ± SD
C-B	537.0 ± 84.8 b	211.9 ± 35.3 a	748.9 ± 117.0 b	204.2 ± 36.9 a
C-P	813.0 ± 160.1 a	286.0 ± 66.9 a	1099.0 ± 222.9 a	270.5 ± 64.6 a
E-B	250.9 ± 60.6 c	95.7 ± 11.5 b	346.5 ± 70.0 c	91.1 ± 13.5 b
E-P	620.3 ± 200.5 ab	200.3 ± 54.3 a	820.6 ± 253.8 ab	210.1 ± 33.1 a
ANOVA	F = 14.22 P<0.000	F = 13.95 P<0.000	F = 14.55 P<0.000	F = 16.46 P<0.000

Table 2. Clorophyll *a/b* ratio (Chl a/b) and chlorophyll *a* degradation (OD 435/OD 415) in *C. pocillum*, C-control, E-eaten, B-basal squamules, P-podetia, n = 5, X = mean values, SD = standard deviations. Values in each vertical column followed by the same letter do not differ significantly at P<0.05 by Tukey's pairwise comparisons.

	Chl a/b X ± SD	OD435/415 X ± SD
C-B	2.55 ± 0.20 a	1.06 ± 0.05 a
C-P	2.87 ± 0.23 a	1.05 ± 0.09 a
E-B	2.61 ± 0.44 a	1.16 ± 0.21 a
E-P	3.07 ± 0.24 a	1.00 ± 0.13 a
ANOVA	F = 3.45 P = 0.042	F = 1.39 P = 0.282

However, we did not observe significant differences in dehydrogenase activity between intact and grazed basal squamules.

Fumarprotocetraric acid (FMPC) was the only detectable secondary metabolite by TLC. We confirmed its presence in both primary squamules and podetia of control and grazed *C. pocillum*.

Table 3. Chlorophyll *a* fluorescence (minimal (F_0), maximal (F_m), variable (F_v) and potential quantum yield of photosystem II (F_v/F_m) in *C. pocillum*, C-control, E-eaten, B-basal squamules, P-podetia, n = 10, X = mean values, SD = standard deviations. Values in each vertical column followed by the same letter do not differ significantly at P<0.05 by Tukey's pairwise comparisons.

	Chl a fluorescence			
	F_0	F_v	F_m	F_v/F_m
	X ± SD	X ± SD	X ± SD	X ± SD
C-B	0.510 ± 0.091 a	0.770 ± 0.183 a	1.271 ± 0.272 a	0.601 ± 0.025 a
C-P	0.509 ± 0.052 a	0.750 ± 0.059 ab	1.257 ± 0.111 a	0.597 ± 0.014 a
E-B	0.338 ± 0.128 b	0.243 ± 0.116 c	0.581 ± 0.231 b	0.409 ± 0.068 c
E-P	0.515 ± 0.113 a	0.596 ± 0.167 b	1.104 ± 0.276 a	0.532 ± 0.024 b
ANOVA	F = 7.47	F = 30.29	F = 19.41	F = 53.47
	P = 0.001	P<0.000	P<0.000	P<0.000

Table 4. Absorbance of formazan indicating dehydrogenase activity in 20 mg *C. pocillum*, C-control, E-eaten, B-basal squamules, P-podetia, n = 5, X = mean values, SD = standard deviations. Values in each vertical column followed by the same letter do not differ significantly at P<0.05 by Tukey's pairwise comparisons.

	Dehydrogenase activity A / 20 mg X ± SD
C-B	0.123 ± 0.037 b
C-P	0.314 ± 0.097 a
E-B	0.132 ± 0.019 b
E-P	0.261 ± 0.039 a
ANOVA	F = 14.11 P<0.000

4. Discussion

Our results confirmed previous reports that invertebrates preferentially feed on upper cortex and algal layer of lichens (Broadhead, 1958; Rawlins, 1984; Hesbacher et al., 1995). We showed this by SEM as well as by documenting a

decrease of chlorophyll and total carotenoids in primary squamules. This would suggest removal of some photobiont cells from the thallus. A decrease in F_v/F_m ratios in primary squamules demonstrated feeding also reduced significantly the operation of photosystem II. Podetia of *C. pocillum* were in most respects not affected by grazing, perhaps because they are higher than the base and therefore less accessible to predators.

The relative parameters such as chlorophyll *a/b* ratio and ratio of chlorophyll *a* to phaeophytin were unchanged. These parameters may reflect the physiological status of lichen photobionts under stress (Garty et al., 2000; Backor and Váczi, 2002; Backor et al., 2003), but a decrease in the total number of cells by feeding can also be symptomatic of lichen damage.

Mycobiont hyphae in medulla remained after feeding, as was evident from SEM, and the unchanged dehydrogenase activity of the thallus following grazing suggested maintenance of respiratory activity in damaged thalli and probably most of the mycobiont mass (Towill and Mazur, 1975).

Fumarprotocetraric acid, confirmed in our material by TLC, is a typical secondary metabolite of *C. pocillum* (Brodo et al., 2001). Fumarprotocetraric acid has been previously linked in the literature to lichenivory; for example, it may cause an increase in the larval period of the moth *Spodoptera litoralis* (Giez et al., 1994). In addition, some secondary compounds play important roles in food selection by lichenivores (Lawrey, 1980; 1983; Hesbacher et al., 1995). Fumarprotocetraric acid may protect against grazing from at least some moths, however, only in the part of lichen where it is present (e.g. medulla). Hesbacher et al. (1995) found that larvae of the moth *Eilema complanata* fed on cortical and algal layers of *Cladonia pyxidata* only, as medulla contained fumarprotocetraric acid. Our results for *C. pocillum* are in accordance with these findings.

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REFERENCES

Backor, M., Fahselt, D., Davidson, R., and Wu, C.T. 2003. Effects of copper on wild and tolerant strains of the lichen photobiont *Trebouxia erici* (Chlorophyta) and possible

tolerance mechanisms. *Archives of Environmental Contamination and Toxicology* **45**: 159–167.

Backor, M. and Váczi, P. 2002. Copper tolerance in the lichen photobiont *Trebouxia erici* (Chlorophyta). *Environmental and Experimental Botany* **48**: 11–20.

Barnes, J.D., Balaguer, L., Manrique, E., Elvira, S., and Davison, A.W. 1992. A reappraisal of the use of DMSO for the extraction and determination of chlorophylls a and b in lichens and higher plants. *Environmental and Experimental Botany* **32**: 85–100.

Broadhead, E. 1958. The psocid fauna of larch trees in northern England. An ecological study of mixed species populations exploiting a common resource. *Journal of Animal Ecology* **27**: 217–263.

Brodo, I.M., Sharnoff, D., and Sharnoff, S. 2001. *Lichens of North America*.Yale University Press, New Haven & London. 795 pp.

Culberson, C.F.1972. Improved conditions and new data for the identification of lichen products by a standardized thin-layer chromatographic method. *Journal of Chromatography* **72**: 113–125.

Culberson, C.F. and Johnson, A. 1982. Substitution of methyl tert-butyl ether for diethyl ether in the standardized thin-layer chromatographic method for lichen products. *Journal of Chromatography* **238**: 483–487.

Culberson, C.F. and Kristinsson, H. 1970. A standardized method for the identification of lichen products. *Journal of Chromatography* **46**: 85–93.

Fröberg, L., Björn, L.O., Baur, A., and Baur, B. 2001. Viability of lichen photobionts after passing through the digestive tract of a land snail. *Lichenologist* **33**: 543–545.

Garty, J., Weissman, L., Tamir, O., Beer, S., Cohen, Y., Karnieli, A., and Orlovsky, L. 2000. Comparison of five physiological parameters to assess the vitality of the lichen *Ramalina lacera* exposed to air pollution. *Physiologia Plantarum* **109**: 410–418.

Gerson, U. and Seaward, M.R.D. 1977. Lichen-invertebrate associations. In: *Lichen Ecology*. M.R.D. Seaward, ed. Academic Press, London, pp. 69–119.

Giez, I., Lange, O.L., and Proksch, P.1994. Growth retarding activity of lichen substances against the polyphagous herbivourous insect *Spodoptera littoralis. Biochemical Systematics and Ecology* **22**: 113–120.

Hesbacher, S., Giez, I., Embacher, G., Fiedler, K., Max, W., Trawöger, A., Türk, R., Lange, O.L., and Proksch, P. 1995. Sequestration of lichen compounds by lichen-feeding members of the Arctiidae (Lepidoptera). *Journal of Chemical Ecology* **21**: 2079–2089.

Lawrey, J.D. 1980. Correlations between lichen secondary chemistry and grazing activity by *Pallifera varia. The Bryologist* **83**: 328–334.

Lawrey, J.D. 1983. Lichen herbivore preference: a test of two hypotheses. *American Journal of Botany* **70**: 1188–1194.

Lin, C.H., Chen, B.S., Yu, C.W., and Chiang, S.W. 2001. A water-based triphenyltetrazolium chloride method for the evaluation of green plant tissue viability. *Phytochemical Analysis* **12**: 211–213.

Rawlins, J.E. 1984. Mycophagy in Lepidoptera. In: *Fungus-Insect Relationships*. Q. Wheeler, and M. Blackwell, eds. Columbia University Press. New York. pp. 382–423.

Ronen, R. and Galun, M. 1984. Pigment extraction from lichens with dimethyl sulfoxide (DMSO) and estimation of chlorophyll degradation. *Environmental and Experimental Botany* **24**: 239–245.

Seyd, E.L. and Seaward, M.R.D. 1984. The association of oribatid mites with lichens. *Zoological Journal of the Linnean Society* **80**: 369–420.

Towill, L.E. and Mazur, P. 1975. Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. *Canadian Journal of Botany* **53**: 1097–1102.

Wellburn, A.R. 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolutions. *Journal of Plant Physiology* **144**: 307–313.