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Characterization of Photobiont Associations in Lichens Using Carbon Isotope Discrimination Techniques

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I. Introduction

Each lichen comprises a symbiotic association between photobiont (chlorophyte algae and/or cyanobacteria) and mycobiont partners. Uptake of CO_2 by lichens may be relatively transient, a function of thallus water content, and regulated by variations in atmospheric humidity, dewfall, and precipitation. Without the possibility of regulating water content by stomata, poikilohydric lichens must steer between Scylla and Charybdis: sufficient water to reactivate photobiont photosynthesis and CO_2 uptake limited by diffusion in a water-saturated thallus.

While groups of lichens may be readily distinguished in terms of life-form, as well as the primary photobiont, some lichens containing a green algal phycobiont also have a subsidiary association with a cyanobacteria, which are limited to specialized structures (cephalodia). Major phycobiont genera include *Trebouxia* and *Myrmecia*, supplying the mycobiont with carbohydrates in the form of ribitol (Honegger, 1991). *Nostoc* is the most common cyanobiont genus, which furnishes the mycobiont with glucans and fixed nitrogen (Honegger, 1991). Work on the photosynthetic characteristics and water relations of lichens has shown that those primarily containing green algal phycobionts can be distinguished from those solely with cyanobacteria (cyanobiont) (Lange and Ziegler, 1986; Lange et al., 1988).

Associations with phycobiont alone (or containing cephalodia) can reactivate photosynthesis from water vapor in air, while the cyanobiont, surrounded by a gelatinous sheath, requires rewetting with liquid water (Lange et al., 1988; Bilger et al., 1989). These groups have previously also

been distinguished by means of carbon isotope ratio. Lichens classified to date as containing green algae have been shown to have carbon isotope composition ($\delta^{13}C$) values which range from -18 to $-34‰$ (Lange and Ziegler, 1986), and for the species listed in Lange (1988), the mean $\delta^{13}C$ was -29.6 ± 3.6 ($n = 16$). The $\delta^{13}C$ of cyanobiont lichens ranged from -14 to $-28‰$, with a mean value of $-23.5 \pm 1.7‰$ ($n = 20$; Lange, 1988). The lower carbon isotope discrimination (Δ) in the latter group has been related to a high diffusion resistance through the thallus depending upon water content (Lange and Ziegler, 1986; Lange, 1988). However, CO_2 compensation points suggest "C_4-like" characteristics, which have also been related to the action of a CO_2-concentrating mechanism (CCM) in the photobiont in lichens (Raven et al., 1990; Raven 1991; Griffiths et al., 1993). Such a CCM was originally reported for microalgae and cyanobacteria (Badger et al., 1978) and has been suggested to occur in endosymbiotic associations of fungus and cyanobacterium (Geosiphon pyriforme; Kluge et al., 1991).

In this chapter we reevaluate the variations in Δ for lichens and show that the following three groups may be distinguished on the basis of photobiont association: phycobiont, phycobiont plus cephalodia, and cyanobiont. In addition, on-line, instantaneous carbon isotope discrimination, thallus CO_2 compensation points, and maximum photosynthetic rates are used to characterize the various lichen groups. Finally we consider the interactions between diffusion resistance imposed by thallus water content, refixation of respiratory CO_2 derived from the mycobiont, and the possible activity of any CO_2-concentrating mechanism in the photobiont.

II. Carbon Isotope Discrimination and Gas Exchange Methodology

A. Lichen Material

The lichen species were collected in Portugal at Serra da Arrábida (mediterranean ecosystem), Serra de Aires e Candeeiros (oak forest), and in Jura, Scotland, for Ramalina fastigiata samples. At Serra da Arrábida the lichen material was collected on north and south slopes at different altitudes: north slope from 110 to 430 m and south slope from 150 to 420 m.

B. Carbon Isotope Discrimination in Organic Material

$\delta^{13}C$ and Δ were determined using standard mass spectrometric techniques at the University of Newcastle upon Tyne, United Kingdom, and the University of Utah. Calculations of Δ in organic material assumed a $\delta^{13}C$ of source air of $-8‰$ versus PDB standard, using the relationship of Farquhar et al. (1989).

C. On-Line, Instantaneous Carbon Isotope Discrimination

Photosynthesis in stored lichen material was reactivated using standard techniques by spraying with deionized water, with thalli acclimated for 3 days and gently cleaned of soil or any other debris prior to experimenta-

tion, under a photosynthetic photon flux density (PPFD) of 30 μmol m^{-2} s^{-1} at a temperature of 15°C. For measurement of CO_2 uptake and respiration, pieces of lichen thalli, weighing approximately 0.5 g dry wt, were placed in a water-jacketed Plexiglas chamber at 15°C and PPFD of 120 μmol m^{-2} s^{-1}. For each experiment thallus water content was determined from the mean fresh weight before and after the CO_2 gas exchange measurements, and the oven-dried weight at the end of the experiment and was expressed as a percentage of thallus dry weight. CO_2 gas exchange was determined using an ADC LCA2 IRGA in differential mode, and the air was supplied using two mass flow controllers (Brooks Instruments, Cheshire, UK), from a cylinder of CO_2 (Distillers Ltd., UK) and from CO_2-free compressed air. Photosynthetic maximum rates (V_{max}, nmol CO_2 g^{-1} dry wt s^{-1}) in the light were determined 15–20 min after chamber closure, over a period of about 90 min, with measurements of dark respiration used to calculate gross thallus photosynthetic rate. The CO_2 was collected for isotopic analysis downstream of the cuvette in a liquid N_2 cold trap in a cryodistillation line, as described by Griffiths *et al.* (1990). Collections were made over 10–15 min, with CO_2 repurified to remove H_2O and N_2O by passage through two −90°C cold traps and over a reduced copper furnace at 600°C. Samples of reference CO_2 were collected at regular intervals and were purified and analyzed in the same way. The precision of these measurements as determined on 10 replicate CO_2 samples was 22.0 ± 0.2‰.

During on-line discrimination, the CO_2 leaving the cuvette is enriched in $^{13}CO_2$, and the discrimination expressed by the thallus is derived using the expression of Evans *et al.* (1986), whereby

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{\delta_o - \xi(\delta_o - \delta_e)}.$$

The concentration difference of CO_2 external (c_e) and leaving the chamber (c_o) was maintained at 2–3 Pa with the value of ξ calculated from

$$\xi = \frac{c_e}{c_e - c_o}.$$

Measurements of the isotopic composition of the CO_2 entering and leaving the cuvette (δ_o, δ_e, respectively) were made as described above. It is important to note that the source CO_2 used in the majority of this study had a $\delta^{13}C$ value of −22‰, as opposed to bulk-air CO_2 which is currently −8‰. It should be noted that the use of a commercial source of CO_2 ($\delta^{13}C \approx$ −22‰) for gas exchange measurements could in theory increase instantaneous Δ by up to 4‰ (I.R. Cowan, personal communication), although no differences were noted in on-line Δ when source CO_2 was varied from −22 to −42‰ in a later part of this study.

D. Thallus CO_2 Compensation Point

Sections of lichen thallus were placed in a glass water-jacketed chamber (volume, 100 cm^3; temperature, 15°C; and PPFD, 120 μmol m^{-2} s^{-1}) as part of a closed system gas exchange system. CO_2 uptake was determined

using an ADC 225 mk III IRGA, with an internal purge system used to reduce CO_2 partial pressure initially to speed the approach of the steady-state compensation point and prevent large changes in thallus water content.

III. Characterization of Three Lichen Groups as Determined by Photobiont Association

A. Carbon Isotope Discrimination in Organic Material

The three groups of lichens characterized in this study (Table I) form part of a broad survey of Δ, from which selected or related species are subsequently used for more detailed studies of gas exchange and on-line discrimination characteristics. The associations with green algae as phycobiont (G), taken from a total of 8 genera, all contained chlorophyte algae in the genus *Trebouxia*, one of the most commonly found phycobiont genera. For those lichens with green algal phycobiont which also associate with cyanobacteria (G + cephal), the cephalodia may form an integral structure within the medulla or be associated with the thallus surface (James and Henssen, 1975). The cyanobacteria contribute little fixed carbon to the association but are differentiated to fix atmospheric nitrogen. There are some 21 genera containing cephalodia, including *Peltigera* (James and Henssen, 1975), although *Lobaria* was the only genus represented at the main study site in Serra da Arrábida (Table I). Many of these genera also contain lichen associations where cyanobacteria are the only photobiont (cyanobiont, CB), with *Nostoc* found in the lichens sampled as part of this survey (Table I).

Table I   List of Lichen Species, Photobiont Type, and Their Algal Genus, upon Which the Carbon Isotope Discrimination Analyses Were Performed[a]

Lichen genera	Photobiont	Algae genera
Anaptichia	Phycobiont	*Trebouxia*
Evernia	Phycobiont	*Trebouxia*
Hypogymnia	Phycobiont	*Trebouxia*
Parmelia	Phycobiont	*Trebouxia*
Physcia	Phycobiont	*Trebouxia*
Ramalina	Phycobiont	*Trebouxia*
Usnea	Phycobiont	*Trebouxia*
Xanthoria	Phycobiont	*Trebouxia*
Lobaria	Phycobiont + cephal	*Myrmecia* + *Nostoc*
Leptogium	Cyanobiont	*Nostoc*
Lobaria	Cyanobiont	*Nostoc*
Nephroma	Cyanobiont	*Nostoc*
Peltigera	Cyanobiont	*Nostoc*

[a] Phycobiont + cephal = phycobiont + cephalodia

A number of species from each of these groups were collected from the Macchia vegetation and oak trees at the Serra de Arrábida, Portugal. Carbon isotope discrimination was lowest in the phycobiont associations, with a mean value of 13.4 ± 1.5‰ for 18 species (Fig. 1), at the bottom of the range normally associated with C_3 higher plants. There were also statistically significant differences within this group for two well-represented genera, *Parmelia* and *Ramalina* (Fig. 1, species number 4–7 and 8–14, respectively). The three species of *Lobaria* (G + cephal) showed the highest discrimination, with a mean value of 23.2‰, while lichens with cyanobacteria as cyanobiont formed an intermediate group in terms of Δ, with a mean value of 15.2‰ for the 3 species sampled (Fig. 1). The distinction between Δ in these two groups (G + cephal and CB) corresponds with the $\delta^{13}C$ data in Lange and Ziegler (1986) and Lange (1988).

B. Gas Exchange and Instantaneous Discrimination in Lichens

Carbon isotope discrimination in organic material was reanalyzed for the specific samples used to determine gas exchange and instantaneous dis-
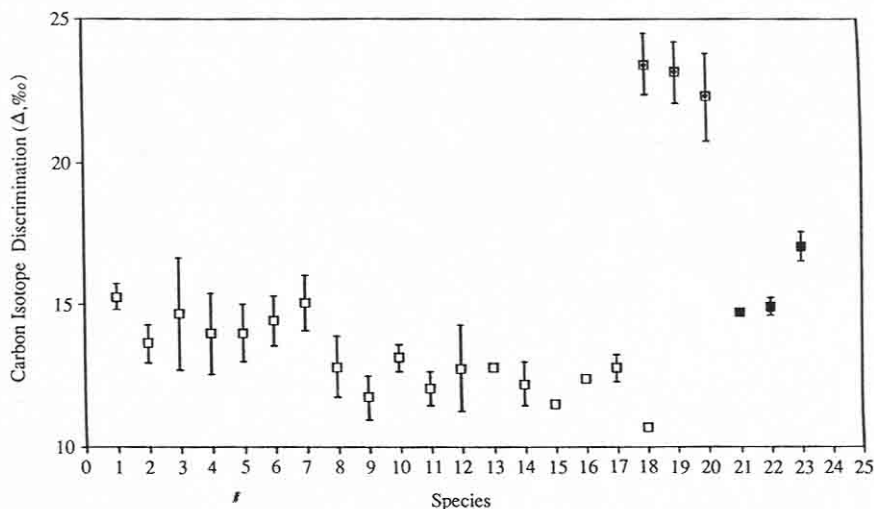

Figure 1. The effect of altitude and north/south exposure in carbon isotope discrimination in a range of lichen species was studied in Serra da Arrábida. Using a two-way layout ANOVA model (analysis of variance), it was found that 90% of the total variability present on the data could be explained by species alone. The most important difference was found between *Lobaria* spp. (23.2 ± 2.2‰) and all the other genera. Significant differences were also found between *Ramalina* spp. (12.5 ± 1.0‰) and *Parmelia* spp. (14.2 ± 1.1‰) but never within each genus. Species list: 1, *Anaptychia ciliata*; 2, *Evernia prunastri*; 3, *Hypogymnia physodes*; 4, *Parmelia caperata*; 5, *Parmelia hypotropa*; 6, *Parmelia reticulata*; 7, *Parmelia sulcata*; 8, *Ramalina calicaris*; 9, *Ramalina canariensis*; 10, *Ramalina everneoides*; 11, *Ramalina farinacea*; 12, *Ramalina fastigiata*; 13, *Ramalina lusitanica*; 14, *Ramalina pusilla*; 15, *Physcia adscendens*; 16, *Physcia leptalaea*; 17, *Usnea* sp.; 18, *Xanthoria parietina*; 19, *Lobaria amplissima*; 20, *Lobaria laetivirens*; 21, *Lobaria pulmonaria*; 22, *Leptogium furfuraceum*; 23, *Nephroma laevigatum*; 24, *Peltigera canina*.

crimination characteristics. This data, presented as part of Table II, independently confirmed the three groups identified in Fig. 1. However, there were additional differences between the groups in terms of on-line Δ values, maximum photosynthetic rates (V_{max}), and thallus CO_2 compensation point when measured at optimal thallus water content (Table II).

The low values of Δ in organic material of phycobiont lichens (15.0‰) contrasted with the higher values of on-line, instantaneous Δ found for the same group (22.2‰; Table II). In terms of higher plant photosynthesis, while instantaneous Δ is suggestive of the C_3 pathway, the thallus compensation point (1.98 Pa) is more likely to be associated with a CCM. Lichens containing green algae plus cephalodia were consistently "C_3-like" with high values of instantaneous Δ and organic material Δ (26.7 and 24.7‰, respectively) as well as CO_2 compensation point (Table II). Maximum photosynthetic rates were on average slightly higher for the phycobiont plus cephalodia group (4.73 as opposed to 3.09 nmol CO_2 g^{-1} dry wt s^{-1}) (Table II). In constrast, organic material Δ was low in cyanobiont associations, with instantaneous values lower than those in organic material Δ (12.0 and 16.3‰, respectively), and the thallus compensation point was the lowest of the three groups (Table II). The responses of lichens containing cyanobacteria consistently suggested the activity of a CCM, while the average maximum rate of net CO_2 uptake (13.89 nmol CO_2 g^{-1} dry wt s^{-1}) was higher than that in the phycobiont groupings.

C. Contribution of Thallus Respiration to Instantaneous Discrimination

It is important to note that measurements of photosynthetic characteristics of lichens reflect a combination of photobiont and mycobiont processes. Rates of dark respiration may be equivalent to 50‰ of net CO_2 uptake, and if the thallus desiccates, a high proportion of the recently fixed carbon may be respired quite rapidly (Farrar, 1975; Lange and Ziegler, 1986; Lange, 1988). Since most of the respiration is derived from the mycobiont, it is likely that similar rates occur in the light. Accordingly, estimates of thallus CO_2 compensation point (see Table II) are likely to overestimate the value expressed by the photobiont, because of respiratory CO_2 release by the fungal partner. In the same way, the on-line instantaneous isotope signature of CO_2 leaving the gas exchange cuvette will be altered and reflects the proportion of gross photosynthetic processes. In effect the phycobiont is supplied with CO_2 from two different sources: one representing ambient CO_2 ($\delta^{13}C$ of -8‰), the other from respiratory CO_2 (similar to whole thallus $\delta^{13}C$).

Respiration was shown to comprise 50 and 31‰ of net CO_2 uptake for *Lobaria pulmonaria* and *Lobaria scrobiculata*, respectively (Lange and Ziegler, 1986), although this proportion was reduced when measured under the experimental conditions used in this study to 23 and 15‰ of gross photosynthetic rate (data not shown). The proportion of respiratory CO_2 leaking out of the thallus, and the carboxylation strength of the photobiont, will be

Page 206, second paragraph, third line: "50‰" should read "50%"; last paragraph, first line: "50 and 31%" should read "50 and 31‰"; fourth line: "23 and 15%" should read "23 and 15‰."

Table II Carbon Isotope Discrimination in Organic Material (Δ), Instantaneous Carbon Isotope Discrimination (Δ_i), Maximum Photosynthetic Rate (V_{max}), and Compensation Point (Comp. pt.) for Different Lichen Associations with Phycobiont (G), Phycobiont + Cephalodia (G + cephal), and Cyanobiont (CB)

Group	Species	Δ (‰)	Δ_i (‰)	V_{max} (nmol CO_2 g^{-1} dry wt s^{-1})	Comp. pt. (Pa)
G	*Evernia prunastri*	16.7	22.4	3.72	2.50
	Parmelia reticulata	16.3	20.0	3.60	1.93
	Ramalina fastigiata	12.1	22.9	1.96	1.52
	Mean	15.0 ± 2.5	22.2 ± 0.5	3.09 ± 1.14	1.98 ± 0.49
G + Cephal	*Lobaria amplissima*	23.5	28.2	3.03	7.53
	Lobaria pulmonaria	24.0	27.1	6.00	5.35
	Sticta aurata	26.5	33.8	5.18	5.67
	Mean	24.7 ± 1.6	26.7 ± 3.6	4.73 ± 1.52	6.18 ± 1.12
CB	*Lobaria scrobiculata*	16.7	11.3	8.84	1.51
	Peltigera canina	15.9	12.6	18.88	1.35
	Mean	16.3	12.0	13.89	1.43

related to the magnitude of the diffusion resistance operating within the thallus. Interpretation of on-line discrimination in lichens should be viewed with caution until the contribution from respiratory processes can be quantified.

IV. Evaluation of Lichen Physiology Using Carbon Isotope Discrimination and Gas Exchange Techniques: Discussion

It is apparent that the two groups of lichens previously identified in terms of carbon isotope composition should now be revised. Highest carbon isotope discrimination is shown by those associations consisting of phycobiont + cephalodia, while those comprising phycobiont or cyanobiont alone show lower discrimination. The morphological and physiological correlates of these separate categories remain to be determined with particular reference to the different rehydration mechanisms found in phycobiont and cyanobiont lichens (Lange and Ziegler, 1986; Lange, 1988; Lange et al., 1986, 1989). More detailed analyses of ultrastructural modifications to the mycobiont during cycles of (de)hydration, and also in response to aging across a thallus, are required. Phycobiont cells tend to be smaller with higher photosynthetic activity in actively growing regions, and the mycobiont may be denser in old regions of the thallus (Hill, 1985). This may be related to a 3–4‰ gradient in $\delta^{13}C$ with age across large thalli of *L. pulmonaria* (C. Máguas and E. Brugnoli, unpublished data).

There are difficulties in characterizing the *in vivo* photosynthetic competence of the photobiont when lichenized. It has been assumed that thallus respiration rates were constant under light and dark conditions and that photosynthetic responses and CO_2 compensation points were true indicators of photobiont respiratory activity. Another possible approach would be to evaluate the photosynthetic characteristics of photobiont cells when cultured *in vitro*, although carboxylation conductance and source–sink relationships may change photobiont responses in the absence of the mycobiont partner (Richardson, 1973). Finally, the large proportion of respiratory CO_2, which may be fixed in the light, could alter the on-line discrimination signal and the relationship between carboxylation strength and diffusion resistance within the thallus now needs to be investigated.

Although the traditional view is that diffusion resistance predominantly regulates Δ in lichens (Lange and Ziegler, 1986; Lange, 1988; Lange et al., 1988) we may also interpret the data presented above in terms of the activity of a CO_2-concentrating mechanism in the photobiont (Badger et al., 1978; Raven et al., 1990; Raven, 1991; Griffiths et al., 1993; Palmqvist et al., 1993). In theoretical terms, it is not possible to distinguish between a low Δ associated with either diffusion resistance or any CO_2-concentrating mechanism activity (Sharkey and Berry, 1985; Raven, 1991). Despite this difficulty, changes in instantaneous Δ were consistent with the induction of such a mechanism in the chlorophyte *Chlamydomonas reinhardtii* (Sharkey

and Berry, 1985). In addition, low organic material Δ was associated with the induction/repression of a CO_2-concentrating mechanism in *Chlorella emersonii* (Beardall *et al.*, 1982).

Measurements of on-line discrimination and CO_2 compensation points are consistent with the operation of a CO_2-concentrating mechanism in cyanobiont lichens. Free-living cyanobacteria have an obligate requirement for a CO_2-concentrating mechanism in view of the kinetic properties of cyanobacterial RuBisCO (Espie *et al.*, 1991; Raven, 1991). Such a mechanism could account for the C_4-like Δ characteristics and thallus compensation points in the lichenized cyanobacteria (Table II, Fig. 1).

Cyanobacteria isolated from lichens did have the capacity to induce a CO_2-concentrating mechanism when cultured *in vitro* (Griffiths *et al.*, 1993). The Δ of organic material from cultures of *Nostoc* was lower than that of *Coccomyxa sp.* (the dominant phycobiont in *Peltigera aphthosa*), suggesting that the Chlorophyte mechanism was less efficient or more easily repressed (Griffiths *et al.*, 1993). However, the only measurement of the *in vitro* fractionation factor for cyanobacterial RuBisCO is lower than that for Chlorophytes (Guy *et al.*, 1987), which would also result in lower Δ in cyanobiont associations.

Having observed the higher values of V_{max} for cyanobiont associations (when expressed per unit thallus dry weight), this evidence intuitively would not seem to be consistent with higher diffusion resistances. A more detailed analysis of field and laboratory studies showed that cyanobiont lichens have generally higher maximum rates of CO_2 uptake at higher thallus water contents that found for phycobiont lichens (Griffiths *et al.*, 1993).

While variations in the expression of a CO_2-concentrating mechanism could account for the different categories of CO_2 compensation points found for the three lichen groups (Table II), the extent of photorespiration *in vivo* remains to be determined. Further work is also required to investigate the basis for the consistent C_3-like characteristics of phycobiont plus cephalodia lichens, as determined from organic material Δ, instantaneous Δ, and CO_2 compensation point (Fig. 1, Table II) as compared to the lower organic Δ of those solely containing a phycobiont. It is tempting to speculate that the improved nitrogen supply from cephalodia could alter photosynthetic characteristics in lichenized phycobionts, as found for *C. emersonii* (Beardall *et al.*, 1982).

In order to place these observations in the context of the ecological niches occupied by each group, we contend that cyanobiont associations are *more* often restricted to moist, shaded habitats (James and Henssen, 1975; Millbank, 1985; Honnegar, 1991). While this observation may be criticized in that cyanobiont lichens are found in exposed, desert areas (but usually where runoff of water occurs following rainfall) we suggest that these are the exceptions. Thus the potential for a higher thallus water content and need for liquid water to reactivate photosynthesis provides a more stable microclimate for the operation of the cyanobacterial CO_2-concentrating

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mechanism. While the strategy for CO_2 uptake following rehydration from atmospheric water vapor is excluded from cyanobiont lichens, the higher carboxylation conductances, low photorespiration, and proportionally lower dark respiration rates result in higher rates of CO_2 assimilation over longer periods, once rewetted. The CO_2 uptake characteristics of phycobiont lichens, which are found in more exposed habitats where photosynthesis is more transiently reactivated, are more C_3-like with low carboxylation conductances. While the variation in gas exchange characteristics between phycobiont ± cephalodia and cyanobiont lichens will provide intriguing lines of research in the future, carbon isotope discrimination techniques have proved to be a powerful means of distinguishing the three groups of lichens.

V. Summary

Analysis of the $\delta^{13}C$ values of organic material in lichens shows that there are three categories, with carbon isotope discrimination related to photobiont association. Assuming a source air of $\delta^{13}C$ of $-8‰$, discrimination (Δ) was lowest in lichens containing only a single photobiont, such as the groups with green algal phycobiont ($13.4‰$) or cyanobiont ($15.2‰$), and highest in associations containing phycobiont together with cyanobacteria in cephalodia ($23.2‰$). Additional measurements of on-line discrimination, photosynthetic rates, and CO_2 compensation points are presented for each group, and consideration is given to the problems associated with the likely occurrence of respiration rates derived from the mycobiont in the light.

Lichens containing phycobiont plus cephalodia were consistently C_3-like, with high on-line discrimination and CO_2 compensation points. These lichens had maximum photosynthetic rates slightly higher than those of lichens with a single phycobiont (4.73 and 3.09 nmol CO_2 g^{-1} dry wt s^{-1}), although in the latter group on-line discrimination and CO_2 compensation points were lower. Maximum photosynthetic rates were higher in cyanobiont lichens (13.89 nmol CO_2 g^{-1} dry wt s^{-1}), which suggests that diffusion resistances are not higher in cyanobiont lichens. When compared with low CO_2 compensation points and low on-line discrimination, these high carboxylation conductances are more likely to be associated with the activity of a CO_2-concentrating mechanism in lichens solely containing cyanobacteria.

Acknowledgments

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