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Development and Growth of the Lichen *Rhizocarpon geographicum*

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Abstract

The development of new areolae on the marginal hypothallus of the lichen *Rhizocarpon geographicum* (L.) DC was studied after complete or partial removal of the central areolae. New areolae developed slowly on the isolated hypothalli over two years. Development was similar when the areolae were completely removed and when the central areolae were separated from the marginal hypothallus by "moats" 2 to 5 mm in width. However, in intact thalli, the marginal areolae developed rapidly during Jan.–June 1986 but showed periods of retreat from the margin during Oct.–Dec. 1985 and July–Sept. 1986. These results suggested that primary areolae may develop from free-living algal cells trapped by the hypothallus while secondary areolae may develop from zoospores produced in the thallus. Complete removal of the areolae resulted in no measurable radial growth of the marginal hypothallus over 18 months. Removal of the central areolae to within 1 and 2 mm of the hypothallus significantly reduced growth. These results suggest that the areolae may supply the hypothallus with carbon for growth. When the marginal hypothallus was experimentally removed a new hypothallus developed within one year. Regeneration occurred initially by retreat of the marginal areolae and later by new hyphal growth. The concentration of ribitol, arabitol and mannitol was measured in the areolae and marginal hypothallus on four occasions in 1985/6 in a population growing on a steep south facing rock surface. The three carbohydrates were present in significantly higher concentration in the areolae than in the hypothallus. Hence, the slow growth rate of this species may result from inhibited transport of carbohydrate from areolae to hypothallus.

Keywords: *Rhizocarpon geographicum*, carbohydrates, gas chromatography, hypothallus, areolae

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1. Introduction

Rhizocarpon geographicum (L.) DC is a crustose lichen comprising discreet areolae, containing the alga *Trebouxia*, on a black hypothallus. The fungal hypothallus extends beyond the areolae to form a marginal ring up to 2 mm in width. This marginal hypothallus grows slowly with annual radial growth rates from 0.02 to 2 mm/yr (Hale, 1983; Armstrong, 1983; Innes, 1985). There are few published data on the development and growth of crustose lichens of this type. This paper describes first, the development of new areolae on the marginal hypothallus of intact thalli and after the areolae have been completely or partially removed. Second, the areolae were removed to determine if they supply the marginal hypothallus with carbon for growth. Third, the levels of the major carbohydrates were measured in hypothallus and areolae to determine if inhibited transport of carbohydrate to the marginal hypothallus could explain the low growth rates of this species.

2. Materials and Methods

All experiments were made in the field in an area of Ordovician slate rock in South Gwynedd, Wales (SN 6196), U.K. Unless otherwise stated, the experiments were made using thalli of *R. geographicum* 3 to 5 cm in diameter, each on a piece of slate, which were collected from several south facing rock surfaces (Armstrong, 1974). The thalli were placed on horizontal boards in the field. Thalli were collected one year before the start of the experiments.

Development of new areolae on isolated hypothalli

The aim of this experiment was (1) to study the development of areolae on isolated hypothalli and; (2) to test whether the presence of areolae close to, but separated from, the hypothallus influenced development. Twelve thalli 3 to 5 cm in diameter were used. The remainder of the slate surface was scraped with a scalpel to remove other lichen thalli and free-living algae. Three treatments with 4 replicate thalli in each were set up on 1 April 1985: (1) areolae were completely removed; (2) and (3) the marginal areolae were removed to leave "moats" 2 or 5 mm wide between the inner edge of the hypothallus and the outer edge of the areolae. Areolae were removed under a dissecting microscope with a scalpel and fine needle. All visible islands of areolae on the inner margin of the hypothallus were removed. Thalli were allocated to treatments at random and placed at least 30 cm apart on a large horizontal board in the field. The width of the developing areolae was measured at 6 monthly intervals at 10 points around each hypothallus and then averaged for each thallus. The data were analysed by a 1-way analysis of variance.

The development of areolae in intact thalli

The development of areolae in intact thalli was studied by measuring the radial growth of the edge of the areolae at 10 points around each of six thalli. The growth of the hypothallus was also measured. The pieces of slate were prepared as described above. Measurements were made at three monthly intervals from 1 Oct. 1985 to 1 Jan. 1987. Hypothallus growth was measured as the advance of the hypothallus tip over distances of 1 mm marked on the rock (Armstrong, 1975). Areolae growth was measured by comparing the hypothallus widths at the selected points on two successive occasions and taking into account the radial growth of the hypothallus. The growth increments of hypothallus and areolae were averaged for each thallus. The data were analysed by a two factor split plot analysis of variance with growth of areolae and hypothallus as the major factor and seasonal trend as the minor factor.

The influence of experimental removal of areolae on growth

On 1 April 1984 4 treatments with 6 replicate thalli in each were set up: (1) control with no areolae removed; (2) and (3) the central areolae were removed to within 1 or 2 mm of the marginal hypothallus; and (4) the areolae were completely removed as described above. Within 1 mm of the hypothallus, scattered islands of areolae were present while within 2 mm the areolae were more continuous. The growth of the hypothallus was measured using previous methods (Armstrong, 1975) at 10 points around each thallus at intervals of three months until Sept. 1985. The growth increments were averaged for each thallus. The total growth of the thalli over 18 months was analysed by a 1-way analysis of variance with comparisons among means made by "t" tests. In addition, the three monthly growth increments were analysed by a two factor split plot analysis of variance with degree of areolae removal as the major treatment and seasonal trend as the minor factor. Data from treatment 4 were excluded since no growth was recorded.

Regeneration of hypothallus after experimental removal

The aim of this experiment was to determine whether the marginal hypothallus regenerated after experimental removal. Regeneration may occur by new hyphal growth from the edge of the areolae or by retreat of the existing areolae. On 1 April 1986 the hypothallus was removed with a scalpel to the edge of the areolae from 8 thalli. Marks were scratched on the rock 1 mm from the edge of the areolae at 10 points around each thallus. At three monthly intervals the width of the developing hypothallus was measured at each of the ten points. The distance from the 1 mm marks to the tip of the areolae was also measured. The distances were compared with the hypothallus widths to determine whether the hypothallus developed from new hyphal growth or by retreat of the areolae. The growth increments were averaged for each thallus.

Levels of carbohydrates in hypothallus and areolae

A population of *R. geographicum* on a steep, south facing rock surface at the site was studied. Four replicate samples of areolae and hypothallus were collected on each of 4 occasions: 1 Nov. 1985; and 1 Feb.; 1 April and 1 July 1986. Between 10 to 20 thalli were required to yield 1 replicate sample. On each occasion, random samples of *R. geographicum* thalli were collected on slate fragments and taken to the laboratory near the site. Samples of areolae and marginal hypothallus (10–50 mg) were obtained by scraping the margin and centre of the thallus with a scalpel under a dissecting microscope. Care was taken to avoid contaminating the hypothallus samples with areolae. Samples were stored in 80% ethanol in a refrigerator. Analysis was carried out within 1 week of collection.

Carbohydrates were determined by gas chromatography. Samples of 3.5 to 40 mg dry weight were extracted with three changes of 80% ethanol. The extracts were reduced to near dryness under reduced pressure and made up to 2 ml with distilled water. Extracts were then shaken with Amberlite IR120 (H^+) and IR45 (OH^-) ion exchange resins (BDH U.K.) for 20 min to remove amino and organic acids (Whipps and Cooke, 1978). Extracts were then transferred to new flasks and reduced to dryness under reduced pressure. Extracts were silylated with bis(trimethysilyl) trifluoroacetamide (BSFTA) and trimethylchlorosilane (TMCS) in anhydrous pyridine. After 18 hr the resulting carbohydrate (TMS) derivatives were separated on a 25 m×0.32 mm "Flexsil" capillary column overlain with 0.4 μ OV1 liquid phase (Phase Separations, U.K.). One μl aliquots of each sample were initially injected onto a precolumn of silanised glass beads (100 mesh) and then split in the ratio 1:4 to avoid overloading the capillary column. Operating conditions of the Varian 3700 gas chromatograph were: injection temperature 150° C, oven temperature 150–270° C rise/min; FID detector temperature 320° C. Characterisation and quantification of unknown derivatives was performed

Table 1. Development of new areolae on the hypothallus of *Rhizocarpon geographicum* after removal or separation of the areolae

	Areolae removed	Size of moat between areolae and hypothallus 2 mm	5 mm
Mean width of areolae after 2 yrs	0.20	0.18	0.29

Analysis of variance: F (treatments) = 0.72, ($P > 0.05$).

by comparison and chromatography with known carbohydrate standards.

Variations in the levels of carbohydrates in areolae and hypothallus at each sampling period were statistically tested by a two factor split plot analysis of variance with thallus location as the major factor and type of carbohydrate as the minor factor. Comparisons between means were made using the standard errors appropriate to the split plot design (Ridgman, 1975).

3. Results

The areolae developed slowly on isolated hypothalli over 2 years. Development was similar with and without the presence of mature areolae in the centre (Table 1). Development occurred as follows: (1) small islands of areolae appeared on the inner margin of the hypothallus within 6 months; (2) the islands slowly enlarged while the hypothallus extended around them; and (3) the islands coalesced within 2 years.

The three monthly radial growth increments of areolae and hypothallus in intact thalli during 1985/86 (Fig. 1) showed that the growth of the hypothallus was more uniform than that of the areolae. The areolae advanced rapidly in Jan.–June 1986 but retreated during Oct.–Dec. 1985 and July–Sept. 1986. Retreat of the areolae resulted in a black zone resembling the marginal hypothallus. The total growth of the areolae over 15 months was less than that of the hypothallus although the difference was not statistically significant. In addition, a plot of the three monthly growth increments (Fig. 2) indicated that growth of the hypothallus was negatively correlated with that of the areolae ($r = 0.4$, $P < 0.05$) although the relationship was weak.

Complete removal of areolae resulted in no measurable growth of the hypothallus over 18 months (Table 2, Fig. 3). In addition, analysis of variance of the data showed (1) a reduction in growth compared with the control when the areolae were removed to within 1 mm (P (< 0.001) and 2 mm ($P < 0.05$)

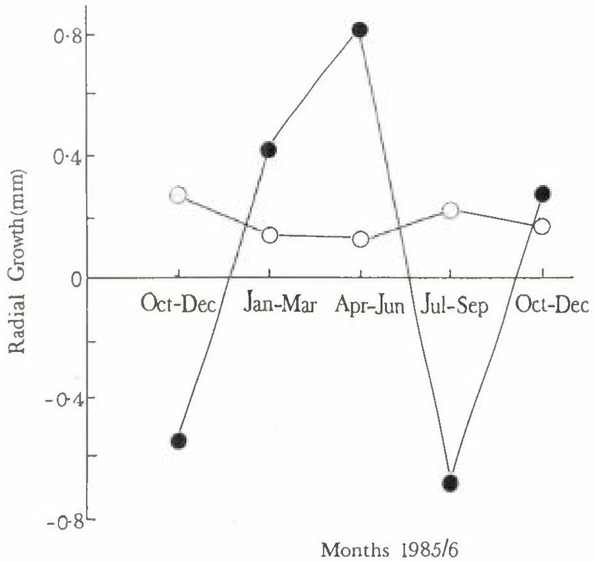

Figure 1. Radial growth of (o) hypothallus and (•) areolae in intact thalli of *Rhizocarpon geographicum*. Analysis of variances: Main effects Hypothallus/areolae F = 4.29 (P > 0.05), Seasonal Trend F = 3.17 (P < 0.05), Interaction F = 4.45 (P < 0.01).

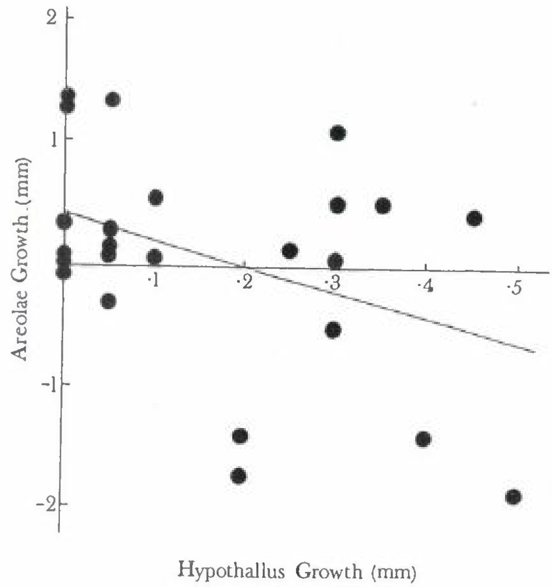

Figure 2. Correlation between the radial growth of the areolae and hypothallus in intact thalli of *Rhizocarpon geographicum*. Regression line Y = 0.41 – 2.1X, t = 2.08 (P < 0.05), r = –0.398 (P < 0.05).

Table 2. Mean radial growth (mm/18 months of *Rhizocarpon geographicum* with areolae with areolae experimentally removed

(1)	(2)	(3)	(4)
Control	2 mm of areolae	1 mm of areolae	Areolae completely removed
1.35	0.78	0.19	0

Analysis of variance (excluding treatment 4): F (treatments) = 4.65 ($P < 0.05$). Standard error of the difference between two means = 0.24. Comparisons among means (2) v (1) t = 2.37 ($P < 0.05$); (3) v (1) t = 4.83 ($P < 0.001$).

of the hypothallus (Table 2); and (2) that growth varied significantly with season ($P < 0.001$) (Fig. 3) with peaks of growth in the July–Sept. periods. These results suggest that more than a 2 mm wide marginal ring of areolae may be necessary for the hypothallus to grow at rates similar to those of intact thalli.

After removal of the marginal hypothallus (Fig. 4) regeneration occurred uniformly over 1 year (Fig. 4A). The new hypothallus developed as follows (Fig. 4B): (1) during April–Sept. the areolae retreated with little new hyphal growth; and (2) during Oct.–March hyphal growth was more rapid and the areolae advanced. Hence, regeneration occurred initially by retreat of the areolae and later by new hyphal growth.

Ribitol, arabitol and mannitol were present in significantly higher concentrations in the areolae than in the hypothallus (Fig. 5) particularly on 1 Nov. ($P < 0.05$), 1 Feb. ($P < 0.05$) and 1 July ($P < 0.001$). Ribitol showed little variation between sampling times in hypothallus or areolae. Arabitol was present in the areolae at higher concentration than mannitol from 1 Nov. to 1 April, but on 1 July the concentration of mannitol slightly exceeded that of arabitol.

4. Discussion

Little is known about the reproduction of *R. geographicum* in the field. This species lacks an obvious method of vegetative reproduction and so may develop from spores. In the experiment on cleaned pieces of slate development of new areolae on isolated marginal hypothalli may have occurred from "pioneer" algal cells present in the hypothallus or from zoospores produced by the central areolae. However, development was similar whether or not mature areolae were present in the centre. Hence, if zoospores were formed in the areolae then they were unable to cross the "moats" to colonize the hy-

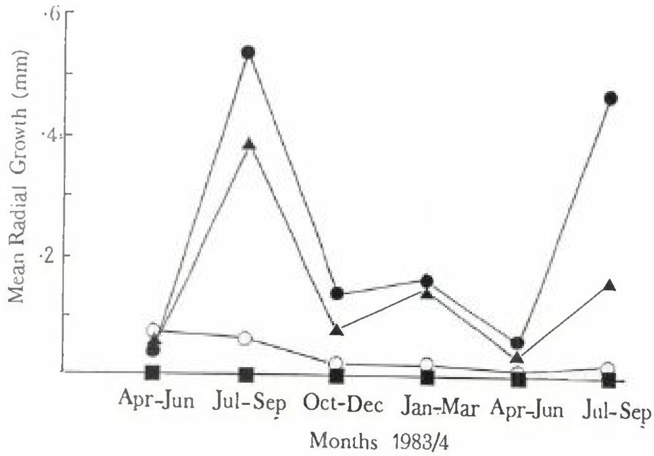

Figure 3. Seasonal variation in the radial growth of *Rhizocarpon geographicum* with the areolae experimentally removed. (•) Control, Areolae removed to within 2 mm (▲) and (○) 1 mm of the hypothallus, (■) areolae completely removed. Analysis of variance: Treatments F = 17.31 ($P < 0.001$), Seasonal trend F = 7.77 ($P < 0.001$), Interaction F = 2.18 ($P > 0.05$).

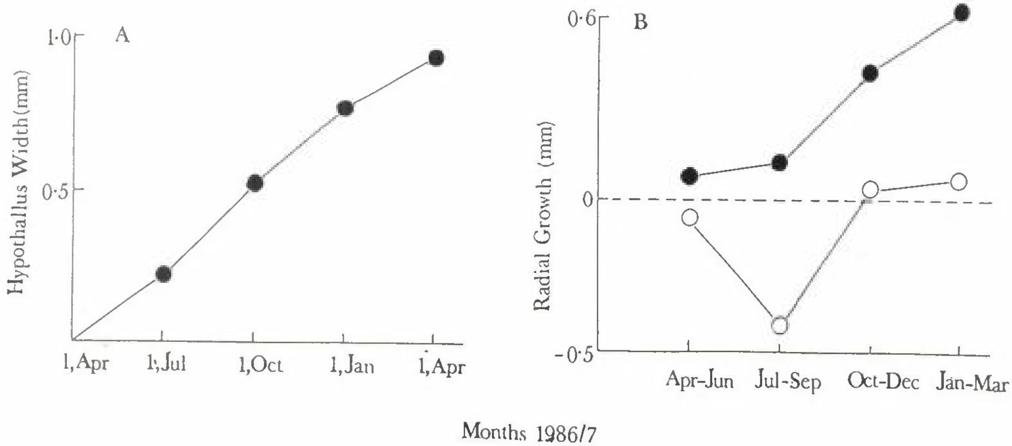

Figure 4. Regeneration of the hypothallus of *Rhizocarpon geographicum* after experimental removal. A. hypothallus width B. (•) hyphal growth, (○) areolae growth or retreat.

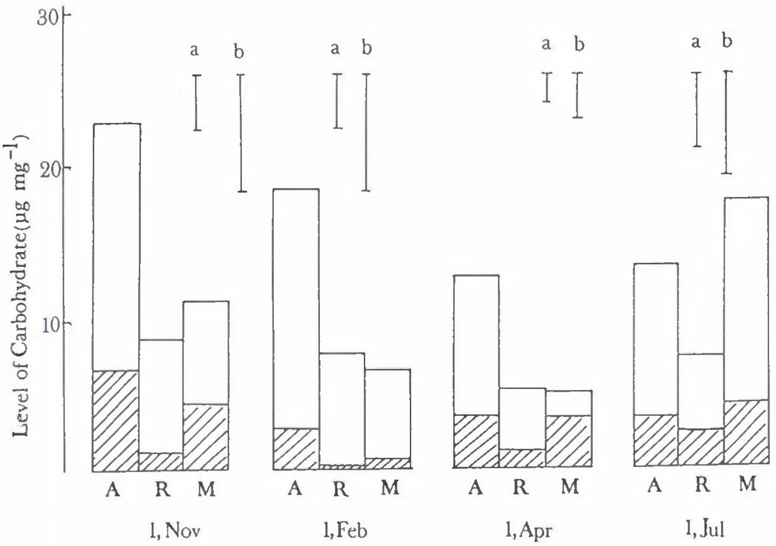

Figure 5. The concentration (µg/mg of extracted tissue) of (A) arbabitol, (R) ribitol and (M) mannitol in the areolae (total height of histograms) and hypothallus (hatched histograms) of *Rhizocarpon geographicum* at 4 sampling times during 1985/86. Analysis of variance: 1 Nov. Thallus location $F = 11.25$ ($P < 0.05$), Carbohydrate $F = 28.03$ ($P < 0.001$), Interaction $F = 7.41$ ($P < 0.01$); 1 Feb. Thallus location $F = 9.16$ ($P < 0.05$), Carbohydrate $F = 21.96$ ($P < 0.001$), Interaction $F = 10.18$ ($P < 0.01$); 1 April Thallus location $F = 125.92$ ($P < 0.001$), Carbohydrate $F = 38.72$ ($P < 0.001$), Interaction $F = 20.05$ ($P < 0.001$); 1 July Thallus location $F = 24.65$ ($P < 0.001$), Carbohydrate $F = 3.97$ ($P < 0.05$), Interaction $F = 2.19$ ($P < 0.05$). a = L.S.D. for comparisons between carbohydrates, b = L.S.D. for comparisons between hypothallus and areolae.

pothallus. In the likely absence of free living algal cells on the slate fragments and active zoospores it is probable that the areolae developed from "pioneer" algal cells already present in the hypothallus. In the field, it is more likely that primary areolae result from the trapping of free-living algal cells rather than from zoospores produced by neighbouring thalli. In addition, the data suggest that secondary areolae may develop from "pioneer" algal cells in the marginal hypothallus. These cells may be pushed forwards by special hyphae as suggested by Nienburg (1926) or they may originate from zoospores (Slocum, Admadjian and Hildreth, 1980). Since the thalli were growing on cleaned pieces of slate it is less likely that free living algal cells were trapped by the advancing hypothallus. The rapid colonization of the hypothallus observed in intact thalli suggests that the areolae may develop from zoospores which swim along the hyphae into the marginal hypothallus.

The areolae margin showed periods of advance and retreat which may be attributable to seasonal variation in zoospore production and algal mortality. Slocum et al. (1980) found that zoosporogenesis in *Parmelia caperata* was more frequent in Oct.–Dec. and April–June; a result consistent with this suggestion. The areolae can advance rapidly in favourable periods and could grow over the marginal hypothallus. Hence, *R. geographicum* may posess mechanisms to maintain a marginal hypothallus. The margin of the areolae retreated after removal of the marginal hypothallus. One interpretation of this result is that the areolae break down if they advance too close to the hypothallus tip; thus reestablishing the hypothallus. Alternatively, the marginal hypothallus may be maintained by its greater net growth over a period compared with that of the areolae.

In lichens with *Trebouxia* as the phycobiont, carbohydrate is released from the alga as ribitol and is then converted into arabitol and mannitol (Farrar, 1976a; 1973). The hypothallus may obtain carbon for growth (1) from the central areolae by transport through the hyphae; (2) from "pioneer" algal cells embedded in the hypothallus tip; (3) by leakage from the areolae followed by reabsorption (Farrar and Smith, 1976); or (4) from exogenous sources (Smith, 1978). Complete removal of areolae resulted in no measurable growth of the hypothallus over 18 months. This result suggests that growth of the hypothallus is dependent on carbohydrate supplied by the central areolae. The carbohydrate may be transported internally through the hyphae or externally by leakage and reabsorption. Ribitol was detected in low concentration in the marginal hypothallus of intact thalli which may reflect the presence of pioneer algal cells. However, the results suggest that little significant growth could occur from this source. In addition, ribitol has been recorded in leachates from lichens (Cooper and Carroll, 1978). Application of carbohydrates to isolated hypothalli could be made to test the ability of the hypothallus to use exogenous sources of nutrients.

Partial removal of the areolae also inhibited radial growth suggesting that more than a 2 mm outer ring of areolae may contribute carbon for radial growth. Hence, horizontal transport may occur over greater distances in *R. geographicum* than in some foliose lichens (Armstrong, 1979). Studies have suggested that *R. geographicum* grows differently than foliose lichens (Beschel, 1973; Mottershead and White, 1972; Miller, 1973, Armstrong, 1983). Benedict (1985) suggested that variations in the shapes of lichen growth curves are the result of differences in the width of the peripheral growth zone or annulus (Proctor, 1977). These differences determine the stage at which the sigmoidal course of lichen growth is interrupted by a linear growth phase. When the annulus is narrow, the linear phase begins early and truncates the sigmoidal growth pattern. A curve such as those shown by species of *Parmelia* (Armstrong, 1976b) and *Buellia canescens* (Proctor, 1977) would result. However, when the growth zone is wide the linear phase begins later after the sigmoidal pattern becomes established. The data now reported suggest that the peripheral growth zone in *R. geographicum* may be relatively wide; a result consistent with Benedict's hypothesis.

The slow growth of the marginal hypothallus may be attributable to (1) low rates of production of ribitol by the alga; (2) low rates of transfer to the fungus, first in the areolae and then in the hypothallus; or (3) the use of carbon for stress resistance (Farrar, 1976b) with little being allocated for growth processes. The major carbohydrates were present in higher concentration in the areolae than in the hypothallus. Although a concentration gradient between areolae and hypothallus may be necessary to drive transport, the data suggest that movement from one region to another may be inhibited. There may be no direct hyphal connections between the areolae and marginal hypothallus. In addition, inhibited transport may result from poor translocation in the hyphae or to the limited ability of the hypothallus to absorb leached nutrients. In addition, slow radial growth may result from the allocation of carbon compounds for stress resistance (Farrar, 1973; 1976b). Lichens with *Trebouxia* use arabitol and mannitol as carbohydrate reserves. Stressing such lichens causes the level of arabitol to fall faster than that of mannitol (Farrar, 1973). Hence, arabitol may function as a short term reserve while mannitol may have a more protective function. In *R. geographicum* the concentration of arabitol in the areolae was greater than that of mannitol from 1 Nov. to 1 April while on 1 July the level of mannitol was slightly higher than that of arabitol. The increase in mannitol, possibly synthesized from arabitol (Farrar, 1973; 1976b), may be a response to stress since south facing rocks in summer are subjected to increasing periods of drought and alternating wet and fast drying periods (Armstrong, 1975; 1976a). Hence, the low levels of carbohydrates in the hypothallus compared with the fungal component of the areolae, and the use of carbohydrate for stress resistance may explain, in part, the low growth rates of this species.

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