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Comparative Stereological Study of the Photobiont of *Lasallia hispanica* (Frey) Sancho & Crespo and *Umbilicaria spodochroa* var. *Carpetana* Prov.

C. ASCASO and F. VALLADARES
Instituto de Edafologia y Biologia Vegetal, C.S.I.C., Serrano 115 bis
28006 Madrid, Spain
Tel. 34-1-2625020, Fax 34-1-4113077

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Abstract

A comparative study of the algal layer and the cells of the photobiont of two lichen species belonging to the *Umbilicariaceae* family, *Lasallia hispanica* and *Umbilicaria spodochroa* var. *carpetana*, has been carried out. We first tested whether any difference exists between the measurements made on fresh thalli and on samples processed for Transmission Electron Microscopy. When the samples were representative and large enough, no significant differences were found. In the present work, we have used, for the first time in lichens, stereological formulae to determine the value of some parameters in three dimensions through measurements made on sections: volume density (Vv), surface density (Sv) and numerical density (Nv) of the cells of the photobiont in relation to the algal layer. It was found that the average cell volumes of the *Trebouxia* cells were similar in both species. However, the percentage of the volume of the algal layer (Vv) occupied by the photobiont was higher in *U. s. carpetana* than in *L. hispanica*. Stereological methods have been also used to calculate the values of Vv and Sv of the cellular structures of the photobiont. The value of Vv for the chloroplast in relation to the whole cell was significantly higher in *U. s. carpetana* than in *L. hispanica* whereas the opposite was found for mitochondria. The Vv values for pyrenoid were similar in both lichens, whereas in *U. s. carpetana* this structure contained more pyrenoglobuli per μm^2 of section than in *L. hispanica*. Together, these results indicate that important differences exist between both species. A possible explanation for these differences might be the different ecological requirements of both lichens as well as the morphological

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differences of the thalli, although alternative explanations cannot be ruled out at present.

Keywords: *Lasallia hispanica*, *Umbilicaria spodochroa* var. *carpetana*, photobiont, *Trebouxia*, algal layer, algal cell, chloroplast, pyrenoid, stereology, volume and surface density

1. Introduction

The ecophysiology of various species of the lichen family *Umbilicariaceae* has been recently studied (Sancho and Kappen, 1989), allowing a better interpretation of anatomical and ultrastructural data.

Thallus morphology and anatomy has also been recently described in detail (Sancho and Crespo, 1989; Sancho and Balaguer, 1989). Several years before, Scott and Larson (1984) began with the comparative morphological studies in the family *Umbilicariaceae*, representing the first ultrastructural study of the group, although *Lasallia pustulata* had already been studied ultrastructurally at that time (Ascaso and Galvan, 1976; Peveling, 1977). The main ultrastructural characteristics of several *Trebouxia*-containing lichen species were already known by the end of the 1960's (Brown and Wilson, 1968; Jacobs and Ahmadjian, 1969; Peveling, 1969, 1973; Galun et al., 1970, 1971).

All these works represent descriptive and qualitative approaches. The first quantitative ultrastructural studies were carried out by Ascaso et al. (1985, 1986) and Scott and Larson (1986), both concerning species within the family *Umbilicariaceae*. Subsequently, the photobionts of other lichen species were studied from a quantitative ultrastructural perspective (Ascaso et al., 1988; Brown et al., 1988). These works involved quantification in two dimensions. The importance of taking into account the three-dimensional aspects of cellular structure has led to the creation of stereological techniques. Stereological mathematics is a branch of the geometric probability theory, described in detail for the first time by Weibel (1973). It is an extremely useful tool, provided that it is carefully adapted to the particular biological object of study (Weibel, 1981). An appropriate experimental design is essential (Gundersen and Osterby, 1981), especially with studies involving relatively small volumes and heterogeneous tissues (or pseudotissues), as is the case with plants and particularly with lichens.

The present paper applies stereological techniques on two lichens of the *Umbilicariaceae* family to obtain a preliminary quantitative description of the algal layer as a whole and of the ultrastructure of its component algal cells. The correlation of this type of data with physiological studies may allow a better understanding of the lichen symbiosis.

2. Materials and Methods

Collection and preparation of material

Thalli of *Lasallia hispanica* (Frey) Sancho and Crespo and *Umbilicaria spodochroa* var. *carpetana* Prov. were collected in moist condition at El Escorial (Madrid, Spain) in January 1990. Half of the collected samples were sectioned fresh with a freezing microtome and stained with lactophenol cotton-blue. The remaining samples were fixed and embedded in Spurr's resin (Spurr, 1969; Ascaso et al., 1986). From these embedded samples, semithin and ultrathin sections were obtained. The semithin sections were stained with Methylene Blue and examined with light microscopy. The ultrathin sections were obtained with a diamond knife and stained with Reynolds (1963) lead citrate for transmission electron microscopy.

Sampling and photographic data

Three samples from each of three different thalli of both lichen species were taken from the zone intermediate between the umbilicus and the margin. In the study of the algal layer, 8979 algal cells in 240 sections were measured (120 for each lichen species studied; of these, 60 from fresh sectioned thalli and 60 from embedded thalli). The average surface area of these sections was 4500 μm^2. In the ultrastructural study of the photobiont cells, 60 photographs of algal cells of each species were taken at random.

Measurement and analysis of data

Quantitative measurements were made with a semiautomatic image analyzer MOP-Videoplan (Kontron). Stereological parameters were calculated according to the following formulae (Weibel, 1973, 1979).

- Volume density ($\mu m^3/\mu m^3$)
  VV = particle area/reference area
- Surface density ($\mu m^2/\mu m^3$)
  $Sv = (4/\pi) \times$ (part. perimeter/ref. area)
- Numeric density (μm^{-3})
  $Nv = (K/\beta) \times (Na^3/Vv)^{1/2}$

where "K" and "β" are constants (in the case of a sphere, their values are 1.5 and 1.382, respectively), and "Na" is the absolute number of particles per reference area.

For calculation of cellular volume, the Lindberg and Worwerk (1972) formula was used:

$$V_{est} = ((6\pi)^{1/2} + 3.4(s^2/a^2 - 0.2))a^{3/2}$$

The data are represented by the mean and their respective coefficients of variation (namely the standard deviation divided by the mean).

3. Results

Cellular volume of the photobiont

The cellular volume of the photobiont (estimated from the average cell surface area and its standard deviation) varied between 278 and 332 μm^3 in *Lasallia hispanica* and between 279 and 300 μm^3 in *Umbilicaria spodochroa* var. *carpetana* (Table 1). It is of interest to note that values obtained from fresh and embedded materials were similar.

Algal layer

Figure 1A shows the algal layer of *L. hispanica*. Table 2 contains the measurements and the values of the stereological parameters obtained from similar sections of both species.

Table 1. Diameter, area and volume of the cells of the photobiont of *Lasallia hispanica* and *Umbilicaria spodochroa* var. *carpetana*. n_1 = number of algal cells measured in *L. hispanica*; n_2 = number of algal cells measured in *U. s. carpetana*. The coefficient of variation is indicated in parentheses.

	Lasallia hispanica			*U. s. carpetana*		
	Area μm^2 ⊘ μm		Volume μm^3 ⊘ μm	Area μm^2 ⊘ μm		Volume μm^3 ⊘ μm
Light microscopy (×1000)						
• Fresh thalli	26.98	(.23)	331.84	27.38	(.22)	299.79
n = 1310	5.9		8.6	5.9		8.3
• Embedded thalli						
n_1 = 1972	27.41	(.60)	277.79	27.90	(.43)	278.97
n_2 = 2314	5.9		8.1	5.9		8.1
Transmission electron microscopy (×11715)						
n = 60	29.73	(.58)	301.97	30.81	(.20)	283.99
	6.1		8.3	6.3		8.1

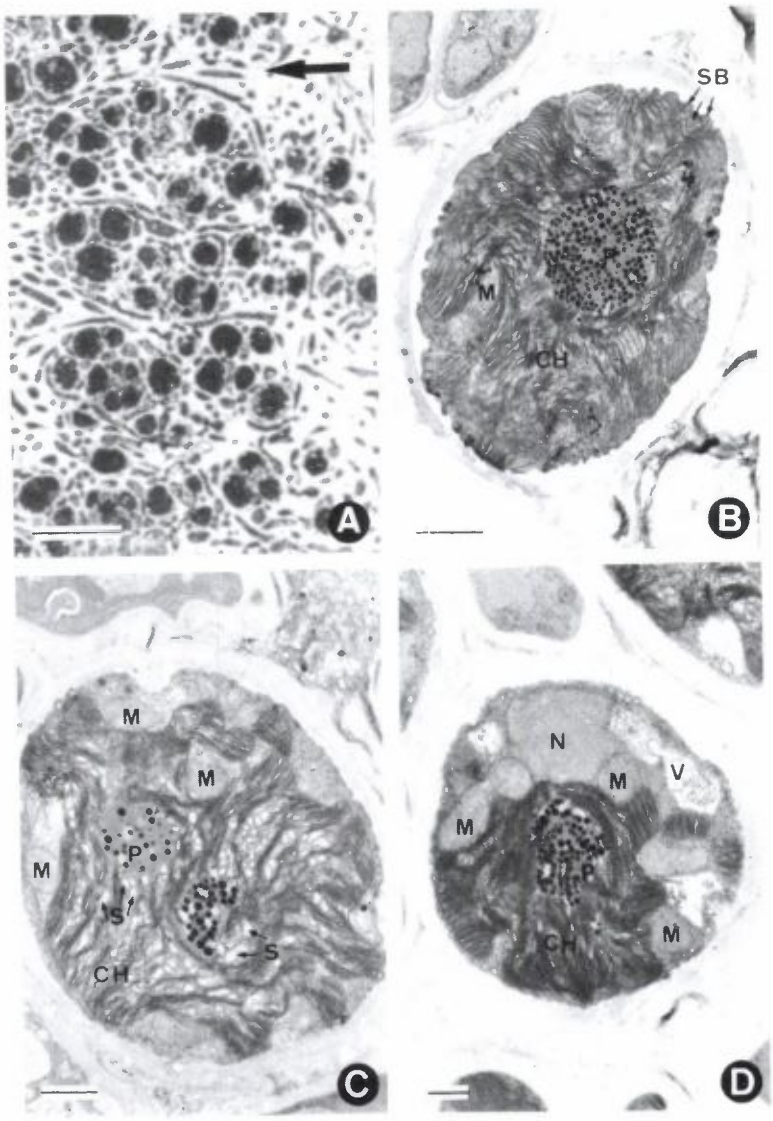

Figure 1. Algal layer of *Lasallia hispanica* (A). Semithin section from embedded thallus sample. Bar = 20 μm. For orientation the arrow points towards the upper surface. Transmission electron micrographs of the photobiont of *Umbilicaria spodochroa* var. *carpetana* (B) and *Lasallia hispanica* (C, D). Bar = 1 μm. P = pyrenoid; CH = chloroplast; N = nucleus; M = mitochondria; S = starch; SB = storage bodies; V = multivesicular complexes

Table 2. Thickness of the thallus and algal layer, and stereological parameters of the algal layer of *Lasallia hispanica* and *Umbilicaria spodochroa* var. *carpetana*. The upper values were obtained from fresh thalli and the lower values were obtained from embedded thalli. The thickness values were obtained from fresh thalli (155 measurements). The coefficient of variation is indicated in parentheses.

	L. hispanica	*U. s. carpetana*
Thallus thickness (μm)	386.4 (.10)	302.7 (.13)
Algal layer thickness (μm)	64.3 (.12)	76.6 (.16)
% of thallus thickness occupied by algal layer	16.6	25.3
$Vv_{algal\ cell,algal\ layer}$ ($\mu m^3/\mu m^3$)	.169(.18) .188(.20)	.216(.18) .231(.17)
$Sv_{algal\ cell,algal\ layer}$ ($\mu m^2/\mu m^3$)	.166(.25) .170(.24)	.204(.21) .200(.15)
$Na_{algal\ cell,algal\ layer}$ ($\mu m^{-2} \times 10^{-4}$)	81 (0.30) 78 (.32)	93 (.22) 86 (.23)
$Nv_{algal\ cell,algal\ layer}$ ($\mu m^{-3} \times 10^{-4}$)	20 (.50) 18 (.51)	21 (.37) 18 (.26)
Number of cell sections measured in each case	2314 1972	2426 2264

The thickness of the algal layer varied significantly between the two species examined ($p < 0.01$), with values of 76.6 μm in *U. s. carpetana* and 64.3 μm in *L. hispanica*. The total thallus thickness was less in *U. s. carpetana* (302.7 μm) than in *L. hispanica* (386.4 μm). Thus, the algal layer represents 25.3% of the total thallus thickness in *U. s. carpetana*, whereas that of *L. hispanica* represents only 16.6% of total thallus thickness. Furthermore, the Vv of the algal cell related to the algal layer in *U. s. carpetana* ($Vv_{Algalcell,algallayer}$ 0.216–0.231 $\mu m^3/\mu m^3$) was significantly higher than that in *L. hispanica* ($Vv_{algalcell,algallayer}$ 0.169–0.188 $\mu m^3/\mu m^3$). Once more the values obtained from fresh and embedded materials are similar.

Ultrastructure of the photobiont

The measurements and values of the stereological parameters that follow have been obtained from cellular sections of the photobiont of both lichen species (Figs. 1B and 1C). Absolute values of the measurements of the cellular structures are listed in Table 3.

In terms of the morphometric parameter surface area (Table 3) both mitochondria (average surface area per section 0.24 μm^2) and cytoplasmic storage bodies (average surface area per section 0.01 μm^2) have identical values in the two species. However, the number of mitochondria and storage bodies observed per μm^2 of cell section were very different in both species. These morphometric data, as well as those related with other cellular structures like chloroplast or nucleus (Fig. 1D), can be more effectively interpreted if stereological parameters are also considered (Table 4).

The percentage of the total cell volume occupied by the chloroplast was greater in the photobiont of *U. s. carpetana* (59%) than in *L. hispanica* (52%). Nevertheless, the surface density of the chloroplast ($SV_{chloroplast,cellwall}$), that is, the number of μm^2 of surface represented by the organelle in relation to one μm^3 of the total cell, was larger in *L. hispanica* (Table 4). The fact that the chloroplast of the photobiont of *L. hispanica* occupied a lesser percentage

Table 3. Absolute number, average number per cell section, average surface area and number pre μm^2 of the organelles of the photobiont of *Lasallia hispanica* and *Umbilicaria spodochroa* var. *carpetana*

	Lasallia hispanica				*U. s. carpetana*			
	Abs. Num.	Av. Num.	Av. Surf. (μm^2)	Num. per μm^2	Abs. Num.	Av. Num.	Av. Surf. (μm^2)	Num. per μm^2
Protoplast	60	1	23.35	–	60	1	23.41	–
Chloroplast	60	1	15.84	–	60	1	18.33	—
Pyrenoid	47	.78	2.08	0.26	54	.90	1.97	0.029
Nucleus	20	.33	1.70	.011	31	.52	1.22	.017
Mitochondria	257	4.28	.24	.144	145	2.42	.24	.078
Storage bod.	289	4.82	.01	.162	618	10.30	.01	.334
Starch	283	4.72	.02	.159	–	–	–	–
Prot. bodies	4	.07	.51	.002	–	–	–	–
Vesic. compl.	108	1.80	.30	.061	115	1.92	.17	.062
Mielin bodies	120	2.00	.07	.067	113	1.88	.08	.061

Table 4. Volume density (Vv, $\mu m^3/\mu m^3$) and surface density (Sv, $\mu m^2/\mu m^3$) of the organelles in relation to the cell wall in the photobiont of *Lasallia hispanica* and *Umbilicaria spodochroa* var. *carpetana*. The coefficient of variation is indicated in parentheses.

	Lasallia hispanica		*U. s. carpetana*	
Protoplast/wall				
Vv	.778	(.09)	.763	(.08)
Sv	.871	(.25)	.829	(.21)
Chloroplast/wall				
Vv	.528	(.17)	.591	(.14)
Sv	1.081	(.26)	.974	(.21)
Pyrenoid/wall				
Vv	.067	(.60)	.059	(.62)
Sv	.253	(.61)	.228	(.63)
Nucleus/wall				
Vv	.016	(.65)	.022	(.69)
Sv	.069	(.67)	.110	(.71)
Mytochondria/wall				
Vv	.034	(.70)	.020	(.70)
Sv	.351	(.72)	.199	(.75)
Storage bod./wall				
Vv	.002	(.90)	.004	(.60)
Sv	.088	(.91)	.183	(.70)
Starch/wall				
Vv	.002	(.92)	0	
Sv	.078	(.93)	0	
Prot. bod./wall				
Vv	9×10^{-4}	(1.13)	0	
Sv	.009	(1.32)	0	
Vesic. comp./wall				
Vv	.012	(.90)	.011	(.88)
Sv	.112	(.93)	.144	(.89)
Mielin bod./wall				
Vv	.005	(1.02)	.006	(1.09)
Sv	.125	(.99)	.092	(1.03)
Number of cell sections measured	60		60	

of the cellular volume than that of *U. s. carpetana* while possessing a greater surface density, leads us to believe that the chloroplast is more lobed in the photobiont of *L. hispanica.*

The *Trebouxia* cells of both lichens have an "impressa" type of pyrenoid (sensu Friedl, 1989a, 1989b). In this organelle there are no differences between the two species neither in the volume nor in the surface density. However, the number of pyrenoglobuli within the pyrenoid is significantly greater in *U. s. carpetana* (Table 5). The ratio of the perimeter of the pyrenoid to its area in both lichens is greater than that predicted by calculations which assume a spherical shape (Table 5). This implies that the shape of the pyrenoid is irregular and lobed.

Although, as we have stated above, there were no differences in the average surface area of the mitochondria and of the storage bodies of both species, there were important differences in the values of the stereological parameters for these structures. The mitochondria occupied 3.4% of the cellular volume (with a surface density of 0.351 $\mu m^2/\mu m^3$) in *L. hispanica*, opposed to 2.2% of the cellular volume ($Sv_{mitochondria,cell\ wall}$ 0.199 $\mu m^2/\mu m^3$) in *U. s. carpetana.*

Table 5. Comparison of several parameters of the pyrenoid of the photobiont of *Lasallia hispanica* and *U. s. carpetana.* The coefficient of variation is indicated in parentheses.

	Lasallia hispanica	*U. s. carpetana*
Area (μm^2)	2.08 (.60)	1.97 (.59)
Diameter (μm)	1.63	1.58
Perim./area (μm^{-1})	3.43 (.54)	3.52 (.47)
Theoretical values (in a sphere case)		
a) Estimated $\varnothing$ ($4\varnothing/\pi$ (μm))	2.07	2.01
b) Perim/area (μm^{-1})	1.93	1.98
Average number of pyrenoglobuli per pyrenoid section	84.03 (.61)	127.30 (.57)
Number of pyrenogl. per μm^2 of pyrenoid	41.98 (.29)	69.45 (.30)
Number of pyrenoid measured	60	60

This fact, together with a similar value of the average section in both species, suggests that *L. hispanica* has a greater average number of mitochondria per photobiont cell.

Storage bodies were scarce in the photobionts of all lichen thalli studied. Only lipidic storage bodies were observed in the photobiont of *U. s. carpetana*, while both lipidic and starch storage bodies were seen in *L. hispanica*. The $Vv_{lip.\ bod.,wall}$ and $Sv_{lip.\ bod.,wall}$ is greater in *U. s. carpetana* than in *L. hispanica* (0.004 $\mu m^3/\mu m^3$ y 0.183 $\mu m^2/\mu m^3$ as compared to 0.002 $\mu m^3/\mu m^3$ y 0.088 $\mu m^2/\mu m^3$).

Structures similar in appearance and electron density to the pyrenoid matrix (which we are calling proteinaceous bodies) were observed only in the photobiont chloroplasts of *L. hispanica*. They were present in few of the chloroplasts examined, and occupied only 0.2% of the chloroplast volume. Vesicular complexes and myelin-like bodies were present in the algal cells of both lichens studied, also with low values of volume and surface density.

All these stereological parameters can similarly be calculated for another reference surface area other than the cellular wall, increasing in absolute value as the reference surface area diminishes, but conserving the proportions for each organelle. Figure 2 represents the volume density values of the organelles in relation to the protoplast (2A) and to the chloroplast (2B) for those structures comprised within this organelle.

4. Discussion

In the present work, the quantitative data obtained from thallus and algal layer thickness and from the cellular area of the photobiont have been compared between fresh and embedded samples. We have attempted to take into account alterations in the algal layer caused by embedding treatment, an issue which has long concerned morphologists. Our results indicate that when the sample is representative and large enough, no important quantitative differences are obtained between fresh and embedded lichen thalli. The difference in the diameters of the photobiont cells between fresh and embedded thalli found by Greenhalgh and Anglesea (1979) might have been due to an inappropriate sample size and by the different measurement method used with each material.

The photobiont cell diameters obtained in this work (8.1–8.6 μm) coincide with those obtained from embedded material by Greenhalgh and Anglesea (1979) and fall within the range of variation obtained by Anglesea et al. (1983) and Hill (1985). Taking into account our results along with those of the other authors cited, we may tentatively estimate the average volume of *Trebouxia* cells as 300 μm^3.

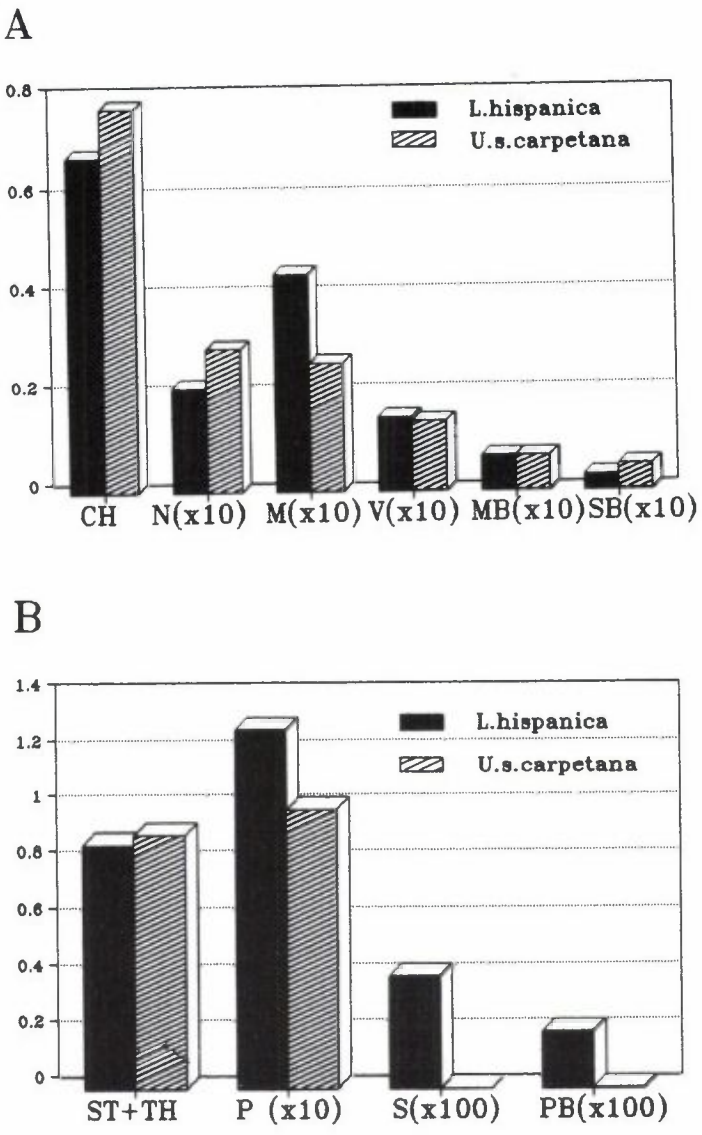

Figure 2. A. Volume density of the organelles in relation to the protoplast ($Vv_{organ.,protoplast}$) in both lichen species studied. B. Volume density of the organelles in relation to the chloroplast ($Vv_{organ.,chloroplast}$) in both lichen species studied. P = pyrenoid; CH = chloroplast; N = nucleus; M = mitochondria; S = starch; SB = storage bodies; V = multivesicular complexes; ST+TH = stroma and thylakoid membranes; PB = proteinaceous bodies; MB = myelin-like bodies.

The algal layer thickness of both lichen species studied was larger than that observed in *Parmelia saxatilis* (Greenhalgh and Alglesea, 1979) and in *Lobaria pulmonaria* (Hill, 1985). These data are not fully comparable due to preparative differences and to the fact that the vertical extension of the photobiont layer may vary considerably in different taxa.

The greater thickness of the algal layer in *Umbilicaria spodochroa* var. *carpetana* becomes more significant when one takes into account the lesser total thickness of the thallus in this species in comparison to that of *Lasallia hispanica*. The results obtained with the stereological methods, applied for the first time to lichens in the present work, shows that the photobiont occupies a greater percent volume of the algal layer in *U. s. carpetana* than in *L. hispanica*. Furthermore, *U. s. carpetana* has a photobiont with a larger chloroplast (Vv% chloroplast related to cell wall 59%, in contrast to 53% in *L. hispanica*). All these data would point to a larger photosynthetic efficiency in *U. s. carpetana*, which might be an important adaptation to the moderately illuminated habitats where this lichen is found. This preliminary hypothesis will have to be confirmed or refuted in further studies that take into account different environmental conditions and other physiological states. It must also be compared with studies on other lichens within the *Umbilicariaceae* family.

The volume and surface density of mitochondria, however, is greater in *L. hispanica* than in *U. s. carpetana*. Given that the study of lichen respiration is complicated by the presence and the interaction of two respiring symbionts, the implications of this difference in mitochondrial volume density will remain unclear until stereological studies of the mycobiont are developed. The thorough study of the chloroplast and the mitochondria, in direct relationship with photosynthesis and respiration, is an interesting field for future investigations combining ultrastructure and ecophysiology.

The pyrenoid, an organelle present in the chloroplast of some lichenized algae such as *Trebouxia*, was linked to starch metabolism since the earliest investigations (Arnott et al., 1967; Fisher and Lang, 1971). This organelle, recently described in detail by Friedl (1989a, 1989b), has been studied in several lichen species with special attention to the pyrenoglobuli present in the matrix and in the starch surrounding it. In 1987, Brown et al. made a different kind of investigation of the pyrenoid, where the effect of dessication of the thallus on the structure of this organelle as well as its recovering capacity was revealed. Nonetheless, under conditions of only moderate dryness the structure of this organelle does not suffer changes; the study of the pyrenoglobuli may reveal differences. In the photobiont of *Lasallia pustulata* values of 121 pyrenoglobuli per μm^2 of pyrenoid have been obtained from samples processed after collection and values of 50 pyrenoglobuli/μm^2 have been obtained from

samples dehydrated for 2 days (Ascaso et al., 1986). In the present work the number of pyrenoglubli per μm^2 in both lichen species studied was calculated. In *U. s. carpetana*, significantly greater values of this parameter were obtained (69.44, in comparison to 41.98 μm^{-2} in *L. hispanica*). The values of *U. s. carpetana* were similar to those obtained in *Parmelia laevigata* 68.91 μm^{-2}) after a short dark treatment (Brown et al., 1988) but were different from those obtained in *P. laevigata* just after collection in the field (79.3 μm^{-2}; Ascaso et al., 1988). These results indicate that the study of the pyrenoglobuli presents complex problems. Their varied localization within the pyrenoid and the differences in their density and size need the development of quantification methodology complementary to that used hitherto by other authors.

According to previous accounts, both pyrenoglobuli and starch are associated with the pyrenoid. However, starch is present in the chloroplasts of vascular plants, which lack pyrenoids. Furthermore, in both lichen species studied here the pyrenoglobuli were always present, while starch was present in only one species. Therefore, we may conclude that the relation between the pyrenoid and starch is not as close as has been previously supposed.

The proteinaceous bodies look similar to the pyrenoid matrix but they do not have the spongy look of the matrix, which is perhaps caused by tubule intrusions. In a previous, unpublished study we found similar structures that apart from being completely separated from the pyrenoid, seem to be in a certain way related to it. This relation between the proteinaceous bodies and the pyrenoid can be defined not only by the similar aspect, but also because in some proteinaceous bodies we found pyrenoglobuli in their interior. This is the reason why we have tentatively named them proteinaceous bodies in spite that their biochemical nature that has not actually been studied. These structures are scarce in our material, being present only in the photobiont of *L. hispanica.*

The average number of cytoplasmic storage bodies calculated for the photobiont of *U. s. carpetana* is similar to the value obtained by Ascaso et al. (1986) in *Lasallia pustulata* stored for 2 days under dark and dry conditions. These values are greater than in *L. hispanica.* Nonetheless, the average surface area of these storage bodies, which like pyrenoglobuli are lipidic in nature, is less than corresponding values obtained in *Myrmecia* from *Lobaria amplissima* (0.3–1.1μm^2) (Ascaso et al., 1986) and in *Trebouxia* from *Parmelia laevigata* and *P. sulcata* (0.03–1.31μm^2) (Brown et al., 1988). Holopainen (1982) reported that the cytoplasmic storage bodies of *Trebouxia* (from *Bryoria capillaris* and *Hypogymnia physodes*) were larger in samples collected during the dry summer months; similarly, Peveling and Galun (1976) found an increase in the diameter of these storage bodies in *Coccomyxa* stored in dry conditions.

These data coincide with our preliminary observations of thalli of *L. hispanica* and *U. s. carpetana* collected during the dry summer months (unpublished results), and should help to explain the low values obtained with the same species collected in winter and in moist condition for the present study. In this way, the seasonal variation in the quantities of starch and other storage bodies seems to be an important factor which must be taken into account, as has been reported by Scott and Larson (1986) and by Fiechther and Honegger (1988).

Comparisons from one lichen species to another, even with the same genus of algal symbiont, needs to be made with care. It is possible that the differences observed in this comparative study may be due in part to the fact that the photobionts of the two lichens studied belong to distinct species of the genus *Trebouxia*.

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