

THREE-DIMENSIONAL QUANTITATIVE DESCRIPTION OF SYMBIONT ULTRASTRUCTURE WITHIN THE ALGAL LAYER OF TWO MEMBERS OF THE LICHEN FAMILY UMBILICARIACEAE

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Abstract: The ultrastructure of the symbionts within the algal layer of *Lasallia hispanica* and *Umbilicaria spodochoa* has been investigated by applying stereological techniques to quantify cellular organelles and structures. The stereological parameters calculated were volume density (Vv) and surface density (Sv). To obtain these parameters for each cellular structure, a systematic sampling of photographic fields of the algal layer for each sample was utilized. Significant differences between the two lichen species with respect to both symbionts were observed. The cellular structures of the photobiont that differed the most between the two lichen species were the chloroplast, which differed by 15% in the Vv values and 25% in the Sv values, the pyrenoid (as a whole and in the diameter of pyrenoglobuli) and the mitochondria. With respect to the mycobiont, the vacuolar apparatus was of great importance in characterizing the two species, as was to a lesser extent the protoplast volume occupied by concentric bodies.

Introduction

Ultrastructural studies of lichen symbionts have traditionally been approached from a descriptive and qualitative point of view (Jacobs & Ahmadjian 1971; Galun *et al.* 1971; Peveling 1973, 1976; Peveling & Galun 1976). Currently, more is known about the ultrastructure of the photobiont than that of the mycobiont, although some studies have considered both symbionts (Boissière 1982; Honegger 1982*a*) giving emphasis at times to certain aspects of their interrelationships (Honegger 1984, 1985, 1986; Lallemand *et al.* 1986). The overall ultrastructure of the mycobiont has been given little attention (Boissière 1979); generally, ultrastructural studies have concentrated on specific aspects, such as concentric bodies (Griffiths & Greenwood 1972; Galun *et al.* 1974) or ascus structure (e.g. Letrouit-Galinou 1973; Honegger 1982*b,c*). Included among these studies are the first descriptive studies of the ultrastructure of lichens of the family Umbilicariaceae (Ascaso & Galvan 1976; Peveling 1977; Scott & Larson 1984).

In quantifying cellular organelles and structures in lichens one currently faces not only a paucity of preliminary studies that might give a general idea of some basic stereological parameters (such as volume density, Vv, and surface density, Sv), but also the necessity of fine-tuning sampling and measurement methods. Ultrastructural studies of lichens in the family Umbilicariaceae carried out by Ascaso *et al.* (1985, 1986), Scott & Larson (1986) and Eversman & Sigal (1987) represent a first step in quantification, although limited to two-

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dimensional morphometric determinations. The use of stereological techniques to study lichen ultrastructure, allowing three-dimensional estimation of quantitative data, began with a study of the photobiont of two lichens in the family Umbilicariaceae (Ascaso & Valladares 1991).

The present work reports new ultrastructural data on *Lasallia hispanica* and *Umbilicaria spodochoa* obtained by applying stereological techniques to quantify cellular organelles and structures. Additionally, some methodological aspects of quantification from micrographs are discussed. This investigation represents a continuation of the study of the photobiont of these lichens (Ascaso & Valladares 1991), and the initiation of quantitative ultrastructural studies of the mycobiont in the family Umbilicariaceae.

Quantitative studies can make possible a new and significant advance in the understanding of the variability of organelles and cellular structures, which is necessary for comparisons between distinct species, distinct microhabitats, distinct seasons or different treatments. The necessity of fine-tuning quantification methods for cellular structures of the symbionts was pointed out by Eversman & Sigal (1984, 1987) upon evaluating cellular damage caused by pollution using morphometric techniques. Stereology is a very useful technique with great potential, and stereological studies applied to the algal layer are of particular importance since they touch upon diverse ultrastructural aspects and contribute information that complements data from eco-physiological, taxonomic or air pollution studies.

Materials and Methods

Thalli of *Lasallia hispanica* (Frey) Sancho & Crespo and *Umbilicaria spodochoa* DC. were collected moist at El Escorial (Madrid, Spain) in April 1990. The material of *U. spodochoa* used is represented by an undescribed variety given the provisional epithet *carpetana* by L. G. Sancho (personal communication). Three samples from each of four different thalli of both lichen species were taken from the zone intermediate between the umbilicus and the margin. These samples were fixed in glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in ethanol solutions and embedded in Spurr's resin for transmission electron microscopy (Ascaso *et al.* 1986). The ultrathin sections obtained were stained with Reynolds lead citrate.

Two series of micrographs of the algal layer sections of each of the eight thalli were taken. One series (ten micrographs of each thallus), with a final magnification of $\times 3450$ has been done to measure parameters concerning the symbiont cells as a whole. The second one (35 micrographs of each thallus), with a final magnification of $\times 18\,500$, was used to measure intracellular structures. In all cases the micrographs were taken by systematic sampling of photographic fields of the algal layer.

The stereological parameters volume density (V_v , the volume of the structure related to the containing volume, expressed as a percentage or as $\mu\text{m}^3/\mu\text{m}^3$) and surface density (S_v , surface area of the structure in relation to the reference volume, $\mu\text{m}^2/\mu\text{m}^3$) were calculated according to the following formulae (Gundersen *et al.* 1988):

$$V_v = \frac{\Sigma \text{ points hitting structure}}{\Sigma \text{ points hitting reference}}$$

$$S_v = \frac{2 (\text{magnification}) \Sigma \text{ intersections with test lines}}{\text{Test line length} \cdot \Sigma \text{ points hitting reference}}$$

The basic stereological parameters (such as V_v and S_v) should be accompanied by a subindex that reflects the structure of interest and the reference structure. For example, $V_{v, \text{pyrenoid, chloroplast}}$ represents the proportion of chloroplast volume occupied by the pyrenoid.

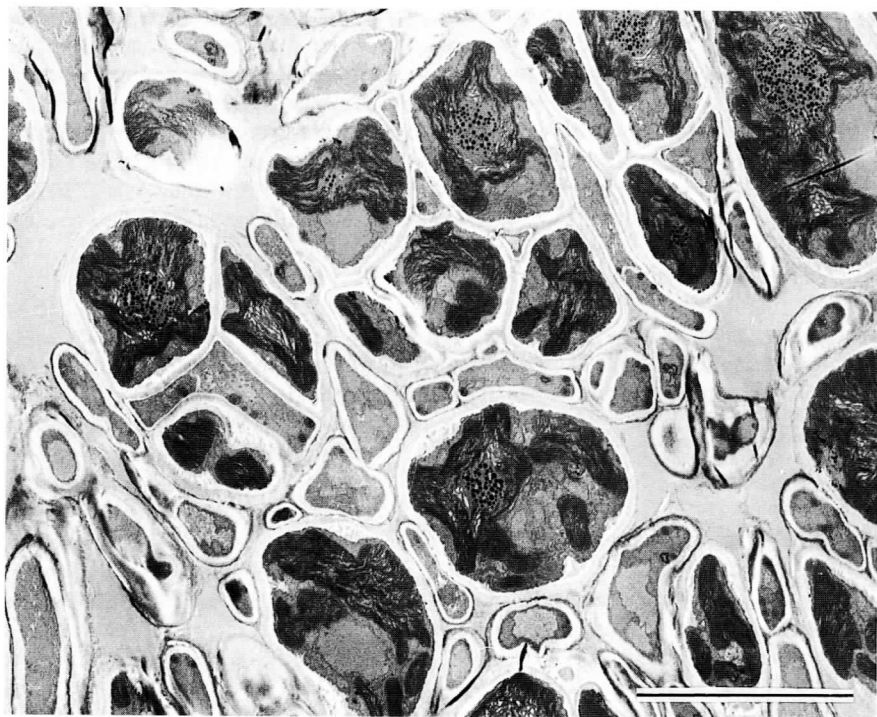


FIG. 1. Transmission electron micrograph of the algal layer of *Lasallia hispanica*. Scale = 10 μm .

The measurements of V_v and S_v were made over transverse section micrographs of the algal layer, which were 'vertical sections' *sensu* Baddeley *et al.* (1986) with a cycloid test system (Cruz-Orive & Weibel 1990).

The measurements of the diameter of the pyrenoglobuli were made with a semiautomatic image analyser (MOP-Videoplan, Kontron). The data are represented by the mean and the coefficient of variation (that is, the standard deviation divided by the mean).

A principal component analysis (Seal 1964) was applied to the V_v values of the intracellular structures. A one-way analysis of variance (LSD method) has been made to study interspecific differences in the diameter of 2436 pyrenoglobuli distributed among the eight thalli studied. Previously the homogeneity of variances (Cochran's C test = 0.234, $p = 5.3 \times 10^{-14}$; Hartley's test = 4.07), the randomness of data and the normal distribution of this variable were tested.

Results

Photobiont

Figure 1 shows the algal layer of *Lasallia hispanica* and Fig. 2 contains some of the most interesting ultrastructural aspects of the photobiont (*Trebouxia* sp.) cells of both lichens.

Within the photobiont, the protoplast comprises approximately 70% of the total cellular volume in both lichen species (Table 1), which implies that nearly one-third of the volume of photobiont cells is occupied by the cell wall. The surface density of the protoplast with respect to the cell is slightly greater in *L. hispanica* ($0.74 \mu\text{m}^2/\mu\text{m}^3$) than in *Umbilicaria spodochoa* ($0.69 \mu\text{m}^2/\mu\text{m}^3$).

TABLE 1. *Stereological parameter values of some intracellular structures and organelles of the photobionts studied*

	Photobiont of	
	<i>Lasallia hispanica</i> *	<i>Umbilicaria spodochoa</i>
Protoplasm		
Vv-cell‡	68.9 (0.06)	71.7 (0.08)
Sv-cell	0.74 (0.11)	0.69 (0.20)
Chloroplast		
Vv-protoplast	71.3 (0.03)	85.9 (0.02)
Sv-protoplast	1.21 (0.22)	0.96 (0.22)
Stroma and thylakoid membranes		
Vv-protoplast	63.5 (0.07)	79.7 (0.04)
Nucleus		
Vv-protoplast	4.2 (0.14)	0.7 (0.71)
Sv-protoplast	0.08 (0.21)	0.02 (0.35)
Mitochondria		
Vv-protoplast	3.5 (0.20)	1.9 (0.26)
Sv-protoplast	0.18 (0.15)	0.08 (0.27)
Pyrenoid		
Vv-protoplast	9.0 (0.15)	6.0 (0.26)
Vv-chloroplast	12.5 (0.20)	6.9 (0.30)
Sv-chloroplast	0.19 (0.12)	0.10 (0.23)
Pyrenoglobuli		
Vv-pyrenoid	31.9 (0.11)	26.5 (0.19)
Lipidic storage bodies		
Vv-protoplast	0.8 (1.00)	2.0 (0.05)
Multivesicular complexes		
Vv-protoplast	1.7 (0.35)	1.2 (0.33)
Dense bodies		
Vv-protoplast	2.0 (0.60)	0.2 (0.50)
Starch		
Vv-protoplast	0.4 (0.75)	0.7 (0.57)
Vv-chloroplast	0.6 (0.69)	0.8 (0.50)

*Mean with coefficient of variation in parenthesis.

‡Vv = percentage of the volume of the structure stated; Sv = surface area in relation to the volume of the structure stated ($\mu\text{m}^2/\mu\text{m}^3$).

The chloroplast occupies a lesser percentage of protoplast volume in *L. hispanica* (71.3%) than in *U. spodochoa* (85.9%). Nonetheless, the surface density of the chloroplast with respect to the protoplast is greater in *L. hispanica* than in *U. spodochoa* (1.21 as compared to 0.96 $\mu\text{m}^2/\mu\text{m}^3$). This indicates that

differences in form or size exist between the photobiont chloroplasts of the two lichens. Considering the form of this structure in the micrographs (Figs 1,2A), we believe that in the photobiont of *L. hispanica* the chloroplast is more lobed and perhaps somewhat smaller than that of the photobiont of *U. spodochoa*. The differences in relative volume between the chloroplasts of the two photobionts are more pronounced if the volume occupied by the pyrenoid and by starch is not taken into consideration. The stroma and thylakoid membranes occupy almost 80% of the protoplast volume in *U. spodochoa* but hardly 63% in *L. hispanica*.

The volume of protoplast occupied by the photobiont nucleus (Fig. 2B) varies greatly between the two species studied as well as among thalli of the same species. The Vv and Sv values for the nucleus with respect to the protoplast are generally greater in *L. hispanica* than in *U. spodochoa* although in *U. spodochoa* the values fluctuate significantly around the average. However, it is important to consider that these differences may indicate different phases in the cellular cycle of the photobiont.

Mitochondria (Fig. 2A) occupy 3.5% of the photobiont protoplast of *L. hispanica* but only 1.9% of that of *U. spodochoa*.

The *Trebouxia* cells of both lichens studied have an 'impressa' type of pyrenoid (*sensu* Friedl 1989a,b). The pyrenoid (Fig. 2C) occupies a greater percentage of the protoplast volume in *L. hispanica* (9.0%) than in *U. spodochoa* (6.0%). This difference becomes more extreme if the chloroplast, within which the pyrenoid is located, is taken as the reference volume, since in *L. hispanica* the volume occupied by the chloroplast is smaller than in *U. spodochoa*, as mentioned above. Thus, in the photobiont of *L. hispanica* the pyrenoid represents 12.5% of the chloroplast volume, while in that of *U. spodochoa* the pyrenoid represents only 6.9% of chloroplast volume. We have observed that in approximately one out of every twenty sections of algal cells in *U. spodochoa*, nearly always coinciding with equatorial sections of very large cells, the pyrenoid appears as two or sometimes three more-or-less circular masses, rather than as a single mass (Fig. 2D). This observation may indicate the presence of a lobed pyrenoid or more than one pyrenoid in these cells, which could be an initial phase of cell division.

The $Sv_{\text{pyrenoid, chloroplast}}$ ranges from $0.01 \mu\text{m}^2/\mu\text{m}^3$ in *U. spodochoa* to $0.19 \mu\text{m}^2/\mu\text{m}^3$ in *L. hispanica*. Pyrenoglobuli occupy more of the pyrenoid volume in *L. hispanica* (31.9%) than in *U. spodochoa* (26.5%). Additionally, the pyrenoglobuli of *L. hispanica* appear to be larger, judging by the larger average diameter observed in this species. The variation in diameter of pyrenoglobuli between different thalli is significantly greater than the variation within thalli ($F=358.6$, $p<0.001$). Representing the average value with 99% confidence intervals (Fig. 3), we see that the four thalli of *L. hispanica* show pyrenoglobuli with average diameters clearly greater (average value for the four thalli = 97 nm, coefficient of variation = 0.24) than those of *U. spodochoa* (average value for the four thalli = 62 nm, coefficient of variation = 0.22).

The lipid-like storage bodies (often termed lipidic storage bodies, Fig. 2F) show great fluctuations in abundance at both the interthallus and intrathallus levels. In the specimens analysed they occupy, on average, a greater percentage of the photobiont protoplast in *U. spodochoa*. Similar values and fluctuations

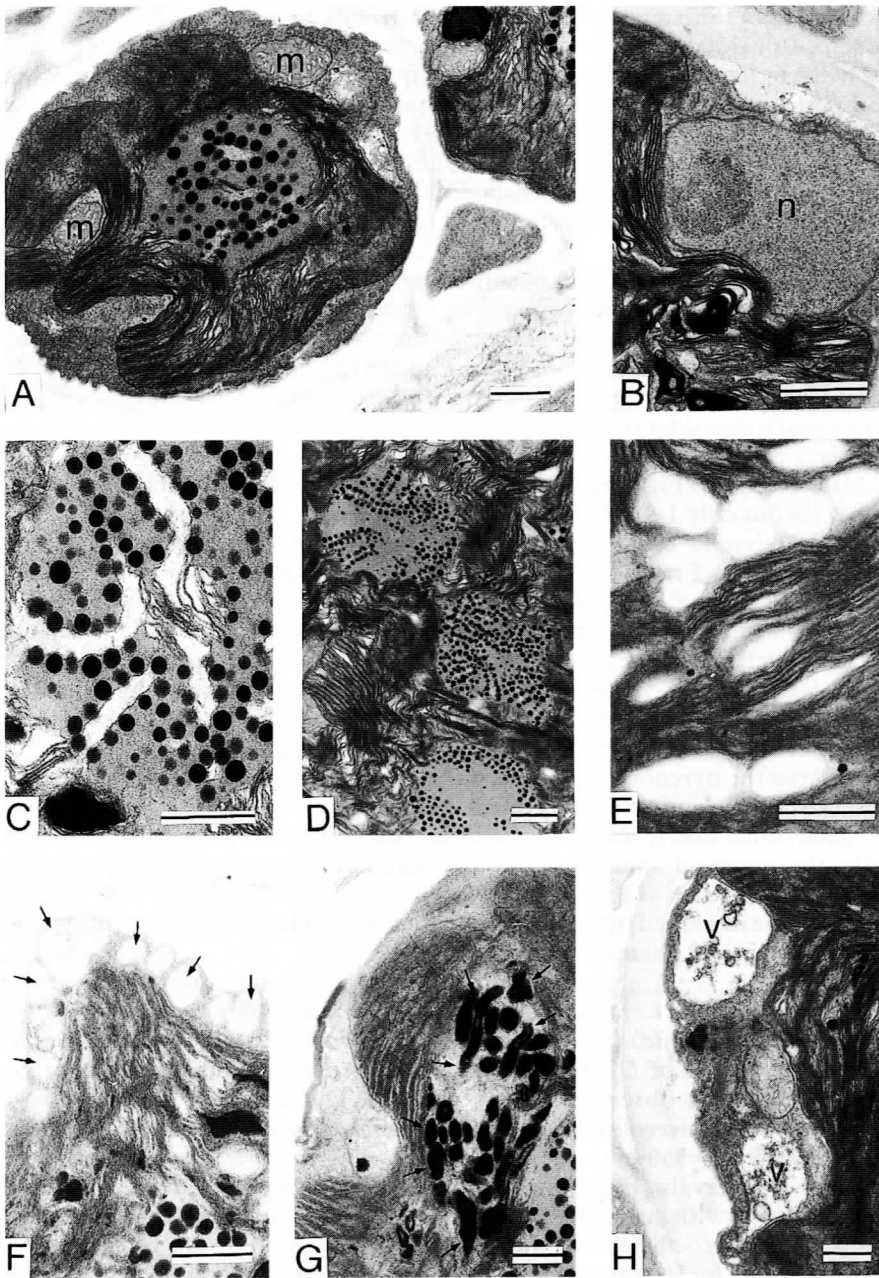


FIG 2. Transmission electron micrographs of the photobiont of *Lasallia hispanica* (A, B, C, F and G) and *Umbilicaria spodochooa* (D, E and H), photobiont cell (m=mitochondria). B, Nucleus (n). C, Pyrenoid. D, Pyrenoid. E, Starch. F, Lipidic storage bodies. G, Dense bodies. H, Multivesicular complexes (v). Scale = 0.5 μ m.

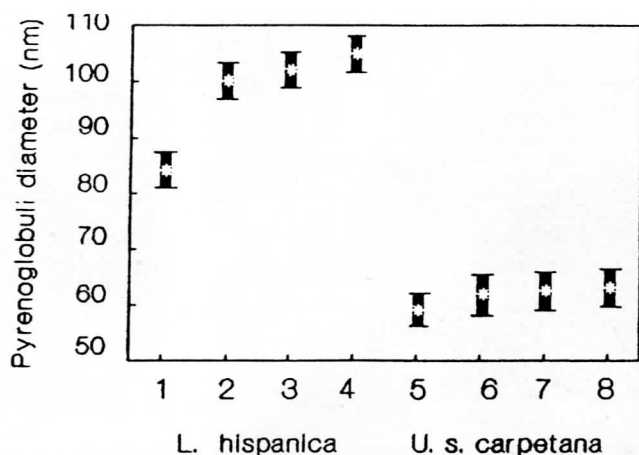


FIG 3. Mean diameter of pyrenoglobuli in photobiont cells of *Lasallia hispanica* (thalli 1–4) and *Umbilicaria spodochoa* (thalli 5–8) with the 99% confidence intervals (LSD method).

were obtained with the 'dense bodies' (also called myelin-like bodies), electron-dense structures of unknown nature (Fig. 2G). Multivesicular complexes (Fig. 2H) occupy between 1% and 2% of the algal cell protoplast. Starch (Fig. 2E) occupies, on average, between 0.4 and 0.7% of the protoplast volume in the photobionts of both lichens. 'Proteinaceous bodies' (*sensu* Ascaso & Valladares 1991) were not observed.

Mycobiont

Figure 4 shows some ultrastructural aspects of the mycobiont. In the hyphae of the algal layer in both lichens, the protoplasm represents between 44% and 47% of the total cell volume (Table 2), which implies that the cell wall occupies more than half of the volume of the cell.

The vacuolar apparatus was the principal component of the mycobiont cells analysed, where it occupied between one fifth (in *U. spodochoa*) and one third (in *L. hispanica*) of the protoplast volume, in some cases reaching much higher values. We categorized at least three different vacuole types according to their morphology. The most common type of vacuole had heterogeneous granular contents (Fig. 4A); in *U. spodochoa* most of the vacuoles observed were of this type. The other two types had homogeneous contents, but with contents of different shape; in one case the content was irregular (Fig. 4B) and in the other case the content was round, with a more or less circular border (Fig. 4C). This type of round vacuole with homogeneous contents was characteristic of *L. hispanica*. The value of $SV_{\text{vacuoles, protoplast}}$ varies between $0.52 \mu\text{m}^2/\mu\text{m}^3$ in *U. spodochoa* and $0.76 \mu\text{m}^2/\mu\text{m}^3$ in *L. hispanica*.

The nucleus (Fig. 4A) occupies 4.4% of the protoplast of the mycobiont in *L. hispanica* and 6.3% in *U. spodochoa*.

Mitochondria (Fig. 4A and 4D) are present in similar proportions in the mycobiont within the algal layer of both lichens studied, occupying around 3.6% of the protoplast and with a surface density of about $0.21 \mu\text{m}^2/\mu\text{m}^3$

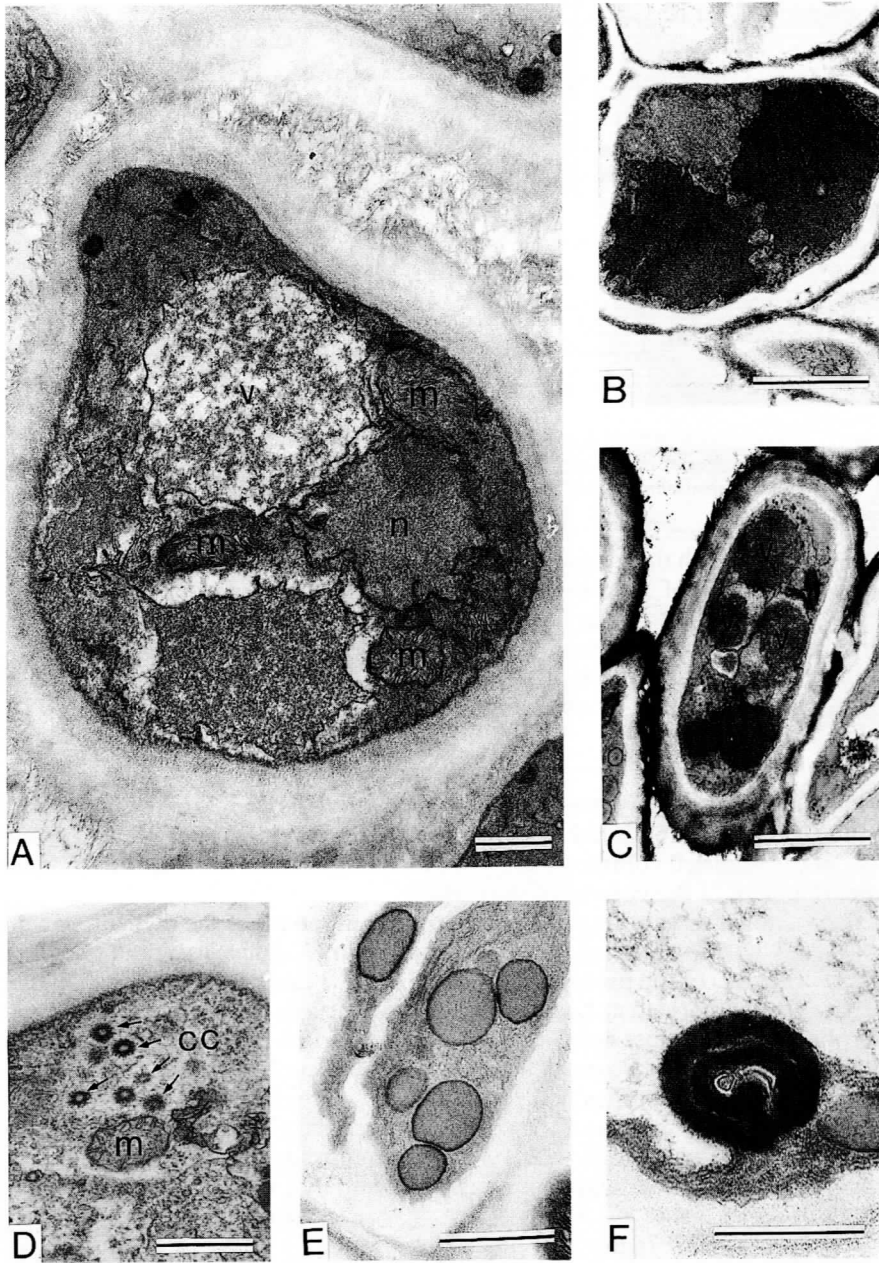


FIG 4. Transmission electron micrographs of the mycobiont of *Lasallia hispanica* (B, C, E and F) and *Umbilicaria spodochoea* (A and D). A, Mycobiont cell with heterogeneous contents (m=mitochondria, n=nucleus, v=vacuole). B, Vacuoles with homogeneous contents. C, Round vacuoles with homogeneous contents. D, Concentric bodies (cc) and mitochondrion (m). E, Lipidic storage bodies. F, Dense body. Scale=0.5 μ m.

TABLE 2. Stereological parameter values of some intracellular structures and organelles of the mycobionts studied

	Mycobiont of	
	<i>Lasallia hispanica</i> *	<i>Umbilicaria spodochoa</i>
Protoplast		
Vv-cell‡	47.3 (0.08)	44.3 (0.06)
Sv-cell	1.56 (0.18)	1.48 (0.14)
Vacuoles		
Vv-protoplast	31.1 (0.28)	17.1 (0.18)
Sv-protoplast	0.76 (0.26)	0.52 (0.18)
Nucleus		
Vv-protoplast	4.4 (0.23)	6.3 (0.21)
Sv-protoplast	0.12 (0.17)	0.14 (0.25)
Mitochondria		
Vv-protoplast	3.7 (0.19)	3.5 (0.17)
Sv-protoplast	0.22 (0.19)	0.20 (0.29)
Lipidic storage bodies		
Vv-protoplast	2.5 (0.24)	4.6 (0.26)
Concentric bodies		
Vv-protoplast	1.6 (0.19)	2.3 (0.09)
Dense bodies		
Vv-protoplast	0.7 (0.57)	0.1 (1.00)

*Mean with coefficient of variation in parenthesis.

‡Vv = Percentage of the volume of the structure stated; Sv = Surface area in relation to the volume of the structure stated ($\mu\text{m}^2/\mu\text{m}^3$).

with respect to the protoplast. Lipidic bodies (Fig. 4E) are considerably more abundant in fungal cells within the algal layer of *U. spodochoa* than in those of *L. hispanica*. The zone of lesser electron density in which the concentric bodies are located (Fig. 4D) occupy a percentage of the protoplast volume between 1.6% in *L. hispanica* and 2.3% in *U. spodochoa*.

'Dense bodies' (Fig. 4F) are few within the mycobiont cells, occupying between 0.1 and 0.7% of the protoplast volume.

Table 3 shows the surface-to-volume ratios of the main intracellular structures and organelles in both symbionts of the two lichens studied. The structures are in order of decreasing size for each symbiont. In the two symbionts and for both species the surface-to-volume ratio increases with decreasing size of the structure. On average, the cell sections as well as their organelles are smaller for the mycobiont, which explains the higher surface/volume ratio compared to the equivalent structures in the photobiont. The table shows some interesting irregularities that, moreover, reflect the discussion above. Thus, the photobiont chloroplast of *L. hispanica* has a surface/volume ratio greater than that of the pyrenoid, a structure of undoubtedly

TABLE 3. Surface/volume ratio of the cells and of the main intracellular structures in both symbionts of the two lichens studied

Surface/volume ratio	<i>Lasallia hispanica</i>	<i>Umbilicaria spodochoa</i>
PHOTOBIONT		
Cell	0.9	0.7
Protoplasm	1.1	1.0
Chloroplast	1.7	1.1
Pyrenoid	1.5	1.4
Nucleus	1.9	2.8
Mitochondria	5.2	4.2
MYCOBIONT		
Cell	1.2	1.2
Protoplasm	3.3	3.3
Vacuoles	2.4	3.1
Nucleus	2.7	2.2
Mitochondria	5.9	5.7

smaller size since it is contained within the chloroplast. This fact relates to the idea presented above that the chloroplast of *L. hispanica* is more lobed than that of *U. spodochoa*. A similar situation occurs with the mycobiont protoplast in the algal layer of both species, which has a surface/volume ratio greater than that of the structures contained within the protoplast, such as vacuoles and nuclei. There are notable differences between the two species in surface/volume ratios of the nucleus and mitochondria of the photobiont and the nucleus and vacuoles of the mycobiont.

Finally, an attempt has been made to characterize and differentiate the thalli of the two lichen species studied by means of a multivariate analysis of principle components. This analysis was performed with relative volumetric abundance data (Vv) of cellular structures of both symbionts within the algal layer of both species examined. Specifically, Vv values for ten photobiont structures and seven mycobiont structures were taken. As seen in Fig. 5, the eight thalli studied are perfectly segregated according to the two components that make up the most variability (component 1 takes up 48.8% and component 2, 15.8% of variability). Component 1, which is the most important, clearly separates the two species studied, with negative values corresponding to the four thalli of *L. hispanica* and positive values to *U. spodochoa*. Especially significant in the separation of the two species are the Vv values for the stroma, thylakoid membranes, mitochondria, pyrenoid, and nucleus in the photobiont, and the vacuolar apparatus, concentric bodies and lipidic bodies in the mycobiont. It is interesting to note the greater dispersion of points corresponding to thalli of *L. hispanica*, which reflects a greater variability among thalli of this lichen, according to the variables considered.

Discussion

Our findings to date indicate that the parameters Vv and Sv are sensitive to the sampling method (Valladares & Ascaso 1990), particularly when the cells are

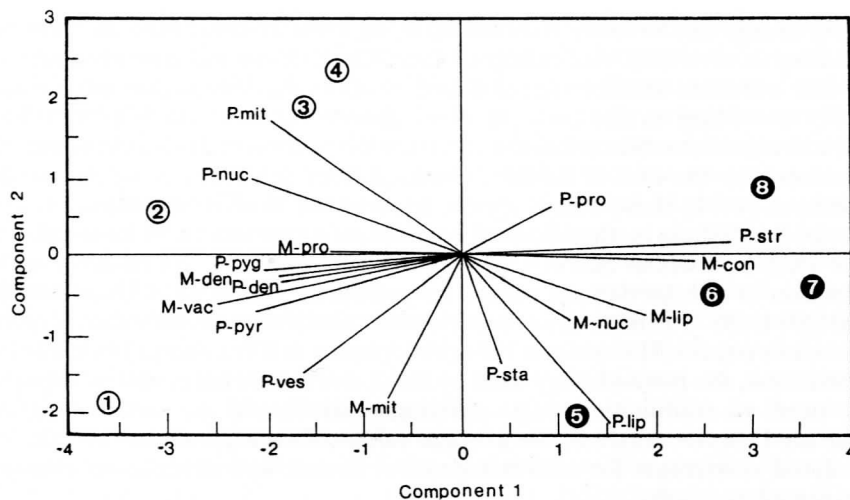


FIG 5. Principal component analysis. Representation of the weight of the variables according to the components 1 and 2 in the segregation of thalli studied. Thalli 1–4 are *Lasallia hispanica*; 5–8 are *Umbilicaria spodothroa*. The variables considered corresponding to the volume density of ten cellular structures of the photobiont (P) and seven of the mycobiont (M); see Tables 1 and 2. con = concentric bodies; den = dense bodies; lip = lipidic storage bodies; mit = mitochondria; nuc = nucleus; pro = protoplasm; pyg = pyrenoglobuli; pyr = pyrenoid; sta = starch; str = stroma and thylakoid membranes; vac = vacuolar apparatus; ves = multivesicular complexes.

irregular and are arranged in heterogeneous and anisotropic layers as in the pseudotissues of heteromerous lichens. The systematic sampling of photographic fields within these layers assures a statistically unbiased representation of all types of sizes of cells. However, if a substantial percentage of cells are incomplete due to the size of the fields, an underestimation of values for $Sv_{\text{protoplast, cell}}$ and $Sv_{\text{chloroplast, protoplast}}$ is obtained. On the other hand, if only complete cells are sampled, without underestimation of the parameters cited, a significant overestimation of $Vv_{\text{protoplast, cell}}$ and $Vv_{\text{chloroplast, protoplast}}$ values results, due to the scarcity of small sections or cellular fragments with little protoplasm that is typically obtained with this method. Therefore, we consider it necessary to take measurements from photographic fields systematically sampled at two levels of magnification: medium to low magnification to estimate volume densities and especially surface density of the wall and the cellular protoplast, and high magnification to estimate Vv and Sv of the cellular organelles.

The use of test systems instead of image analysers saves a great deal of work without appreciable loss of precision and without introducing bias in the measurements (Gundersen *et al.* 1988). For this reason, the test systems are particularly appropriate for measurement of surface area, since the perimeter (used to estimate surface) is the most error-prone of measurements traced from micrographs using a digitizer tablet (Mercer *et al.* 1990). Additionally, when it is easy to obtain 'vertical sections' (sections perpendicular to a given plane), as is the case with lichens, the problem of isotropy in the estimation of surface

measurements can be easily resolved by using a test system based on cycloids (Baddeley *et al.* 1986). According to Cruz-Orive (personal communication) and our previous studies (unpublished results) the calculation of surface density according to the formula $(4/\pi) \cdot (\text{perimeter}/\text{area})$ (in Weibel 1973) produces significant bias when the structure being measured show substantial anisotropy. In the case of Sv data obtained from this formula by Ascaso & Valladares (1991) these biases appear to have led to overestimation of this parameter. All these methodological considerations are especially important in those studies in which quantification is a crucial aspect, as for example with evaluation of cellular damage caused by pollution. The work of Eversman & Sigal (1984, 1987) on this subject represented a significant advance with respect to previous papers (Holopainen 1984; Holopainen & Kärenlampi 1984, 1985). Nonetheless, the methodology used by these authors includes several aspects that need re-examination (e.g. pyrenoglobuli should be counted again using the Disector method; the average values of each parameter should be calculated as averages for each independent sample and never as an average between micrographs).

The ultrastructural study of *Trebouxia* spp. as a lichen photobiont can provide important information about the symbiosis, but several points must be kept in mind in extrapolating and generalizing results obtained in one specific lichen species. Taxonomically, the mycobiont of a heteromerous lichen is regarded as the dominant partner; not too much importance has been assigned to the alga in lichen classification (Hawksworth *et al.* 1983). Friedl (1990) has observed that in some species of *Parmelia* there is a low photobiont specificity; more than one *Trebouxia* species can serve as photobiont within a single lichen species. In the genus *Parmelia*, only one species of *Trebouxia* was found in any given thallus, but in one fully developed thallus of *Diploschistes muscorum* the presence of two different *Trebouxia* species as the photobiont was demonstrated (Friedl 1987). The photobionts of the two lichens of the family Umbilicariaceae that are studied here both belong at least to the same group within the genus *Trebouxia* ('*impressa*', Friedl 1989b). It has been demonstrated that in some cases photobionts are shared and exchanged among lichens from the same locality (Friedl 1990). These two facts make it quite probable that the two photobionts studied here belong to the same species of *Trebouxia*, although the possibility that they represent different species cannot be excluded. Additionally, whether or not they represent the same species, a significant part of the ultrastructural differences observed probably could have been induced in relation to ecophysiological optima of the lichens as a whole. For this reason, comparative study of photobionts from distinct lichen species that inhabit distinct microhabitats within the same locality reveals aspects not only related to the photobiont itself, but also to the distinct biological strategies of each lichen.

In the present study we have observed significant intrathalline variability and large differences between the two lichen species with respect to the chloroplast and other photobiont structures, such as the pyrenoid, used in the systematics of the genus *Trebouxia*. Ultrastructural differences between freshly extracted and cultured *Trebouxia* have been described, particularly with reference to the chloroplast (e.g. Laudi *et al.* 1969). In certain instances

it has been demonstrated that the size and form of the chloroplast may be modified by light conditions, the structure enlarging under conditions of darkness (Brown *et al.* 1988). The relationship between dimensions and incident radiation appears to also be reflected in the present study, where, as was found by Ascaso & Valladares (1991), *U. spodochoa*, which grows under conditions of lesser illumination, has a greater percentage of protoplast volume occupied by the chloroplast. The pyrenoid may also be modified by the physiological condition of the photobiont (Brown *et al.* 1987; Ascaso *et al.* 1988). The main alterations fundamentally affect the reticulum of tubules that traverses the pyrenoid, which is very important in *Trebouxia* taxonomy (Friedl 1989a,b), and the quantity, distribution and size of pyrenoglobuli. The pyrenoglobuli, which are also of taxonomic value, appear to decrease in number and, in some lichens, become arranged peripherally under conditions of dehydration (Ascaso & Galvan 1976; Ahmadjian 1982; Ascaso *et al.* 1986). In the photobionts of the two lichens examined in the present study, the pyrenoglobuli occupy a similar percentage of the pyrenoid volume. The smaller diameter of the pyrenoglobuli in *U. spodochoa* is offset by their greater number. These results are reminiscent of those obtained by Ascaso *et al.* (1986) for *Trebouxia* from thalli of *L. pustulata*. In that study, freshly fixed thalli showed more pyrenoglobuli per μm^2 of pyrenoid than desiccated material, but occupied the same percentage of the pyrenoid volume due to their smaller size. An interesting question is posed by the occasionally complex pyrenoid of the *U. spodochoa* photobiont, sections of which reveal several independent masses within the chloroplast that may possibly represent separate pyrenoids. In view of all this, we believe that quantitative studies of the photobiont both within the lichen and in culture, with particular attention to the chloroplast and pyrenoid, will permit a delimitation of the variability of these structures and allow an assessment of their value as taxonomic characters within the genus *Trebouxia*.

In the present work, the ultrastructure of the mycobiont within the algal layer has been studied. The data should be interpreted with due consideration to variation in morphological and ultrastructural features of the hyphae according to their position in the thallus (Boissière 1982).

The concentric bodies were thought to be unique to lichenized fungi when they were first described by Peat (1968) until they were discovered in two non-lichenized ascomycetous fungi by Griffiths & Greenwood (1972). Very little is known today regarding the composition of these structures (Galun *et al.* 1974), and nothing has been reported concerning their origin and function. In our material, concentric bodies usually occur in clusters of five bodies per cell section on average. There are no data indicating the total number of these structures in the protoplasm of a given fungal cell, but in some cases the sum may approach fifty to a hundred bodies (Boissière 1979). The concentric bodies are frequently surrounded by an electron-transparent, ribosome-free area of the protoplasm called the 'matrix' (Brown & Wilson 1968) or 'halo' (Galun *et al.* 1974). In the thalli studied here the volume of the hyphal protoplasm occupied by the matrix or halo showed reasonably constant values for each species (1.6% in *L. hispanica* and 2.3% in *U. spodochoa*) with little intrathalline variability.

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The principal component of the mycobiont cell analysed was the vacuolar apparatus, occupying between one-fifth and one-third of protoplasm volume. These values, which appear to be greater than those obtained for other species of *Umbilicaria* (Scott & Larson 1984), may reflect an active metabolism and concentrated storage of metabolites. The most common type of vacuole, with heterogeneous and granular contents, resembled the v3-type vacuoles observed by Boissière (1982) in *Peltigera canina*. Vacuoles with homogeneous contents resembled the autophagic vacuoles of type LS described by Boissière (1982); however, mesosome-like bodies were not observed in the interior of these vacuoles, which may indicate that they are not the same type of structure. Vacuoles with round, dense, homogeneous contents may correspond to vacuoles of type v2 (Boissière 1982). This latter type of vacuole (Fig. 4C) may appear similar to lipidic bodies (Fig. 4E), which are also present in the hyphae of the algal layer, but the presence of a tonoplast, which occasionally is clearly visible surrounding the round contents, allows the two structures to be distinguished.

The lipidic and starch storage bodies may reflect distinct metabolic states of the cells of both symbionts. For the photobiont, it has been demonstrated that these structures fluctuate according to the ecological requirements of the species and with the seasons (Scott & Larson 1986; Fiechter & Honegger 1988). In some cases, changes in these storage bodies may occur rapidly (Brown *et al.* 1988). Modifications in the starch bodies of the photobiont as a result of thallus rehydration have been observed (Peveling 1977). The previous studies have treated only the storage bodies of the photobiont, and only three of them (Scott & Larson 1986; Eversman & Sigal 1987; Brown *et al.* 1988) have included morphometric quantifications. In the present work we report for the first time stereological data on the storage bodies of the photobiont as well as the mycobiont. We feel that comparative studies that deal with these structures should be accompanied by quantitative data, since variations in these structures generally affect parameters that are easy to measure (number and size).

Comparison of data obtained for each of the two symbionts reveals several interesting points. Nonetheless, it must be remembered that part of these differences may be slightly influenced by a certain degree of plasmolysis, which is present only in the cells of the photobiont and which in any case we believe to affect the measurements by no more than 5%. There are great differences in the proportion of the cellular volume that is occupied by the cell wall; whereas in the photobiont cells of both species the wall occupies hardly a third of cellular volume, in the mycobiont cells of the algal layer it occupies more than half. Also notable is the large surface area of the plasmalemma in relation to cellular volume of the mycobiont in both lichens studied (about $1.5 \mu\text{m}^2/\mu\text{m}^3$), more than double that of the photobiont (about $0.7 \mu\text{m}^2/\mu\text{m}^3$). The volume densities of the mitochondria and nucleus with respect to the protoplast show greater differences between the photobionts of the two lichen species studied than between photobiont and mycobiont of the same lichen. Lipidic storage bodies occupy a greater percentage of protoplast volume, in both photobiont and mycobiont cells, in *U. spodochoa*.

From these results, together with the principal components analysis (Fig. 5), we consider the stereological parameters relative to the chloroplast, pyrenoid,

and mitochondria of the photobiont to have been useful in characterizing and differentiating the two lichen species studied, and above all in confirming previously obtained results from other samples of the same species (Ascaso & Valladares 1991). The stereological parameters concerning the vacuoles and the concentric bodies have been very efficient in the characterization of the mycobionts studied. It is noteworthy that the stereological quantification of the cellular structure with the greatest volume, the chloroplast of the photobiont and the vacuolar apparatus of the mycobiont, has proved significant in the ultrastructural differentiation of the symbionts within the algal layer of the lichens studied.

The quantitative values of many cellular structures and organelles show great interhalline and intrathalline variability, and may become modified according to the lichen's physiological state, as noted above. For this reason we consider it highly desirable to undertake quantitative ultrastructural studies that focus on changes influenced by season (following the studies of Holopainen 1982; Fiechter & Honegger 1988), locality or microhabitat (developing ideas from Scott & Larson 1986; Friedl 1990), contrasting the results with those of experimental studies carried out under controlled conditions (continuing with the lines of investigation followed in Ascaso *et al.* 1985, 1986, 1988; Brown *et al.* 1988).

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