Intrathalline variability of some structural and physical parameters in the lichen genus *Lasallia*

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The intrathalline variability of several physical and anatomical parameters of two lichens, *Lasallia hispanica* and *Lasallia pustulata* within the family Umbilicariaceae, was studied. In each thallus three zones or concentric rings were considered: the central zone, which includes the umbilicus, the intermediate zone, and the marginal zone. The study focussed on the thickness of the thallus and its layers, the increase of surface area and volume with hydration, the sample densities in dry and wet states, several stereological parameters (especially the volume and surface density of both symbionts related to each layer and to the thallus as a whole), and the chlorophyll content. Only slight differences were revealed between the two species, but significant intrathalline variation was observed. A marked decrease in the total chlorophyll content coincided with the thickening of the thallus from the periphery to the centre. The chlorophyll content of individual algal cells, however, presented an inverse gradient. The results suggest that the main role of the dense central zone would be as a water-holding zone while the active growth in the intermediate zone could counteract the continuous erosion of the marginal zone of these umbilicate lichens.

Key words: *Lasallia*, intrathalline variability, mycobiont, photobiont, stereology, thallus density.

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Les auteurs ont étudié la variabilité intrathallienne de plusieurs paramètres physiques et anatomiques chez deux lichens, le *Lasallia hispanica* et le *Lasallia pustulata* de la famille des Umbilicariaceae. Dans chaque thalle, on observe trois zones : la zone centrale, la zone intermédiaire et la zone marginale. Les auteurs s'intéressent surtout à l'épaisseur du thalle et à ses couches, à l'augmentation de la surface et du volume en cours d'hydratation, à la densité des thalles à l'état sec et à l'état humide, à plusieurs paramètres stéréologiques (surtout la densité en volume et en surface des deux symbiontes en relation avec chaque couche et avec le thalle dans son ensemble), ainsi qu'au contenu en chlorophylle. On n'observe que de faibles différences entre les deux espèces, mais par contre les variations intrathalliennes sont importantes. Une forte diminution du contenu total en chlorophylle coïncide avec l'épaississement du thalle, de la périphérie vers le centre. Cependant, le contenu en chlorophylle des cellules d'algue individuelles montre un gradient inverse. Les résultats suggèrent que la zone centrale dense constituerait une région pour la rétention de l'eau alors que la zone intermédiaire de croissance active pourrait contrebalancer l'érosion continue de la zone marginale, chez ces lichens ombiliqués.

Mots clés : *Lasallia*, variabilité intrathalline, mycobionte, photobionte, stéréologie, densité du thalle.

[Traduit par la rédaction]

Introduction

Lichens have thalli of relatively simple pseudotissues that are loosely integrated with one another. In the study of lichen thallus an interesting question arises: how many functional zones can be distinguished within one single thallus and how are these zones organized? It is normally assumed in foliose lichens that growth consists of a marginal and centrifugal expansion of rings or stripes of active tissue, generally reduced to a few millimetres of the margin of the thallus (Hale 1970, 1973, 1983; Jahns 1973; Hill 1981). In some cases the oldest parts of the thallus can remain active throughout its whole life (Hale 1983), but in other cases, as proved in *Parmelia conspersa* (Armstrong 1979), the central zones do not influence appreciably the radial growth of the lichen and the marginal lobes grow at the same rate, either separately or together, constituting the lichen (Armstrong 1984).

The measure of the photosynthetic rates at different parts of the thallus is an indirect evaluation of the growth pattern and revealed significantly higher values in the youngest apical zones of fruticose lichens (Moser and Nash 1978; Nash et al. 1980) and at the lobe edges of *Parmelia separata* (Nash et al. 1980). However, the results obtained by Larson (1983) and Larson and Carey (1986), estimated from photosynthetic activity measures, showed a patchlike pattern of growth in several thalli of Umbilicariaceae. Investigation of intrathalline gradients may provide a reliable basis from which previously observed physiological and morphogenetic phenomena could be, at least partially, explained.

This study attempts to assess the structural and physical intrathalline variability in two species of *Lasallia* to compare the data from each zone with the theoretical pattern of growth and to better understand how an umbilicate lichen thallus is organized. The anatomy of each thallus zone was studied in detail with the aid of stereology, a useful tool for quantifications of microscopic images (Gundersen et al. 1988). Intrathalline variations in thallus density and water-holding capacity were examined to obtain a general idea of the physical implications of anatomical changes that take place along thallus radii.

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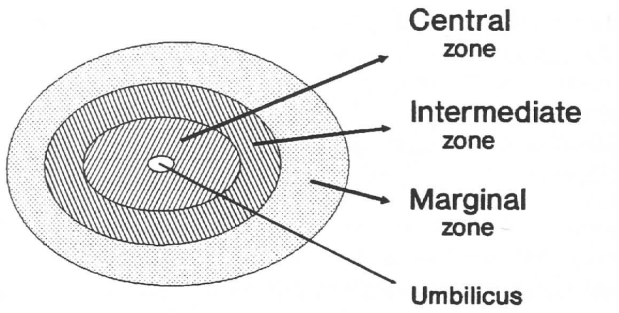


FIG. 1. Sketch of an umbilicate lichen showing the three different zones considered in the study.

The genus *Lasallia* was selected for such a study because its umbilicate growth form allows clear differentiation of individuals and correlation of the results with possible radial gradients, and because there have been previous studies of its general anatomy (Sancho and Crespo 1989; Sancho and Balaguer 1989) and ecophysiology (Sancho and Kappen 1989).

Materials and methods

Collection and preparation of samples

Thalli of *Lasallia hispanica* (Frey) Sancho & Crespo and *Lasallia pustulata* (L.) Merat were collected at El Escorial (1130 m, Madrid, Spain) in January 1990. The collected specimens were of medium size (between 4 and 9 cm in diameter). This size was considered to be sufficiently representative of the range of dimensions normally found in the field and was selected to avoid the effects of thallus size in our study, according to the suggestions made by Larson (1984) for lichens of the family Umbilicariaceae. In each specimen three zones were distinguished (Fig. 1): the central zone (C) within which is the umbilicus or holdfast, the marginal or peripheral zone (M), and the intermediate zone (I). The limits between zones were established along radii at the one-third and two-thirds points.

Two sets of material (called series 1 and 2), each consisting of 10 thalli per species, were collected. The lichens were sectioned with scissors into the three aforementioned zones. The 60 samples of series 1 were used for measurements concerning thickness, surface area, water-retention capacity, and density. The cut samples were able to absorb more water; for this reason five specimens of each species were kept whole and used as controls for the water-relations measurements. The 60 samples of series 2 were used in the stereological study and to obtain the chlorophyll data.

Thickness, surface area, volume, maximum water-retention capacity, and sample density

From each sample of series 1, two fragments of roughly 5 mm^2 were obtained. Sections 5 μm thick were obtained from one fragment with a Slee MTC cryostat; the sections were stained with lactophenol cotton blue and examined with a Zeiss Axiophot light microscope. The other fragment was sputter coated with gold and examined with a Zeiss DSM-960 scanning electron microscope (SEM). The thickness of the samples of both groups of fragments was measured in 10 different places along each transverse section and the mean value was calculated. The surface area of the remaining portion of each sample of series 1 was measured with a semiautomatic image analyzer (MOP-Videoplan, Kontron Instruments). Its oven-dry and hydrated weights were recorded to estimate its maximum water-retention capacity (prior to the weighing, samples were fully saturated and shaken to remove adherent water). The surface area of fully hydrated samples was also measured. We are certain that the thallus surface area was slightly underestimated here because of the limited extent of flat surfaces with the lichens used. In all cases the surface area was measured five times, and the average value was used for calculations. With these data the volume of dry samples (average thickness obtained with SEM multiplied by surface area of dry samples) and the volume of fully hydrated samples (average thickness obtained with cryostat multiplied by surface area of hydrated samples) were calculated. With the volume and weight data the sample density was estimated, both in dry and fully hydrated states. To estimate the real surface area of pustules the surface area of their projection on a plane parallel to thallus surface was recorded. The pustules were considered as perfect hemispheres so that their projection would correspond with that of a circumference of radius r (surface area of the projection = πr^2). Taking into account that the surface area of a hemisphere is $2\pi r^2$, we considered the theoretical value of the real surface area of pustules to be double the value of its projection.

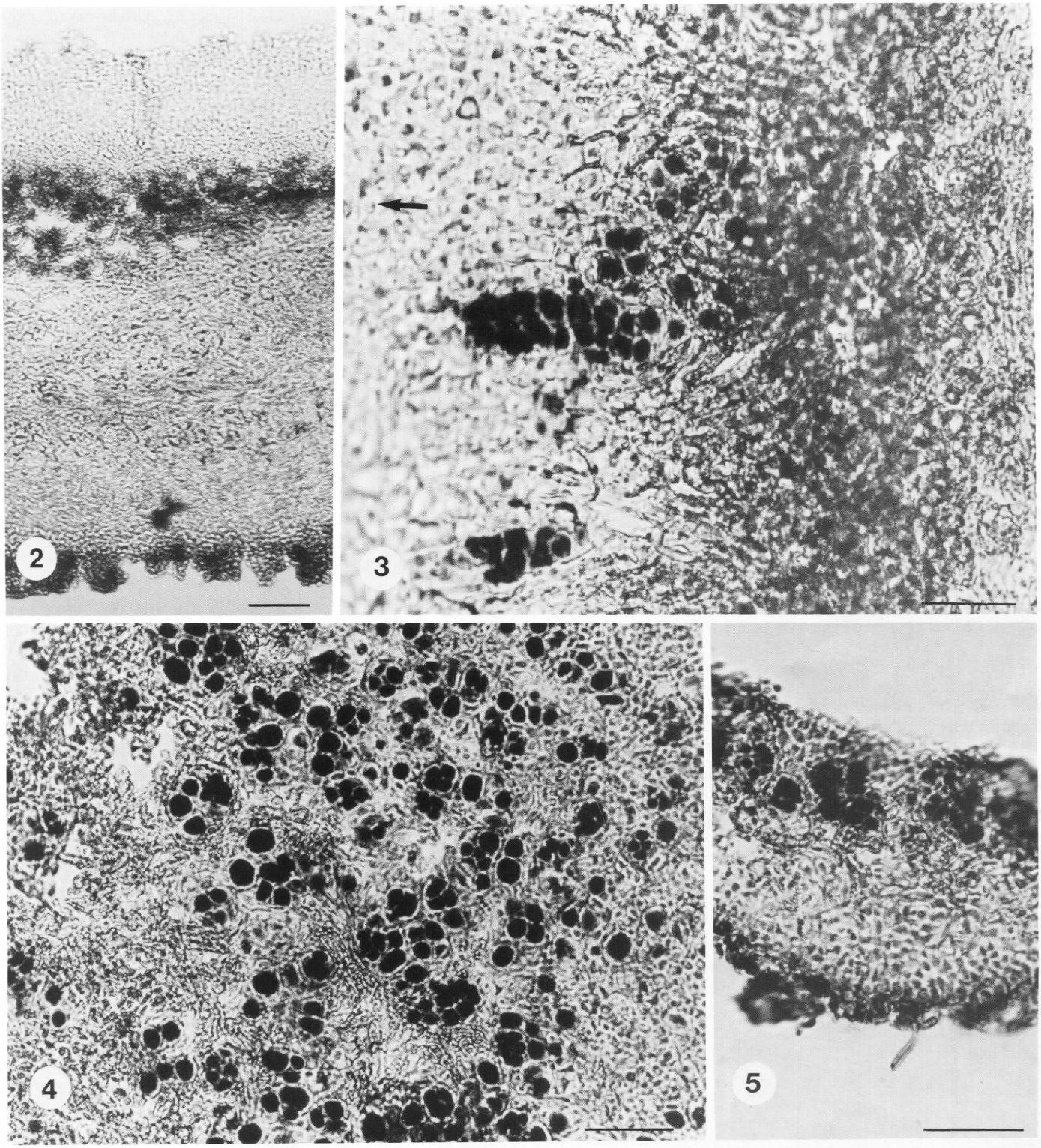
TABLE 1. Thickness of thallus layers (μm) of *L. hispanica* and *L. pustulata* at the three different zones from samples dehydrated and embedded in Spurr's resin and from fresh and hydrated samples sectioned with a cryostat

Layer	Dehydrated	Hydrated	Increase (%)
	Lasallia hispanica		
Central zone			
Upper cortex	102.3 (0.19)	106.8 (0.16)	4
Algal layer	42.4 (0.06)	49.6 (0.09)	17
Upper medulla	99.2 (0.30)	108.5 (0.09)	11
Lower medulla	104.2 (0.16)	138.8 (0.14)	33
Lower cortex	58.1 (0.33)	59.5 (0.12)	2
Intermediate zone			
Upper cortex	82.2 (0.27)	119.5 (0.16)	45
Algal layer	39.4 (0.08)	65.9 (0.10)	67
Upper medulla	50.5 (0.28)	116.2 (0.13)	132
Lower medulla	83.3 (0.24)	174.1 (0.22)	110
Lower cortex	45.1 (0.12)	50.7 (0.08)	11
Marginal zone			
Upper cortex	30.0 (0.60)	82.5 (0.12)	175
Algal layer	28.1 (0.21)	64.6 (0.15)	130
Medulla	97.0 (0.31)	212.3 (0.15)	119
Lower cortex	32.8 (0.28)	51.2 (0.14)	56
	Lasallia pustulata		
Central zone			
Upper cortex	128.5 (0.15)	128.9 (0.14)	1
Algal layer	41.9 (0.18)	64.5 (0.27)	54
Upper medulla	97.0 (0.13)	219.1 (0.29)	126
Lower medulla	97.5 (0.32)	191.1 (0.27)	97
Lower cortex	44.3 (0.22)	46.9 (0.10)	6
Intermediate zone			
Upper cortex	70.4 (0.38)	100.6 (0.11)	43
Algal layer	40.9 (0.15)	70.0 (0.16)	71
Upper medulla	41.4 (0.22)	162.3 (0.18)	292
Lower medulla	82.7 (0.22)	150.5 (0.14)	82
Lower cortex	52.2 (0.23)	61.8 (0.06)	18
Marginal zone			
Upper cortex	33.0 (0.05)	70.5 (0.15)	114
Algal layer	20.1 (0.40)	54.5 (0.09)	171
Medulla	57.2 (0.40)	189.6 (0.19)	231
Lower cortex	16.7 (0.07)	41.6 (0.19)	149

NOTE: In each case the increase in thickness and the coefficient of variation (in parentheses) are indicated (n = 10).

Stereological parameters

From series 2, three thalli of each species were chosen at random for the stereological study. From each thallus, three fragments of 4 mm^2 (belonging to the central, intermediate, and marginal zones) were taken. These fragments were embedded in Spurr's resin (Spurr 1969) as in Ascaso et al. (1986). Sections 0.8 μm thick were obtained from these embedded samples, stained with methylene blue, and



FIGS. 2–5. Light micrographs of cryostat sections of *L. pustulata* stained with lactophenol cotton blue (5 μm thick). Fig. 2. Cross section of the central zone. In this case, no algal cells exist between the medulla and the upper cortex. Scale bar = 40 μm. Fig. 3. Algal layer and medulla of the central zone. The columnar arrangement and the scarcity of the algal cells should be noted. The arrow points to thallus upper surface. Scale bar = 20 μm. Fig. 4. Algal layer of the intermediate zone. Scale bar = 20 μm. Fig. 5. Cross section of the marginal zone. Scale bar = 40 μm.

examined with light microscopy. Twelve micrographs from each of the samples, each with a final magnification of ×1375, were taken by means of a systematic sampling of photographic fields of the sections. Spurr's resin was used to obtain sections as thin as possible, a very crucial aspect in stereology (Cruz-Orive 1990), since no significant alterations are induced by this resin (Ascaso and Valladares 1991).

The main stereological parameters used in the three-dimensional quantitative description of the symbionts were the volume density (Vv, the volume of the structure related to the containing volume,

expressed as a percentage or as $\mu m^3/\mu m^3$) and the surface density (Sv, surface area of the structure in relation to the reference volume, $\mu m^2/\mu m^3$). Both were calculated according to the following formulae from Gundersen et al. (1988):

$$[1] \quad Vv = \frac{\Sigma\ PS}{\Sigma\ PR}$$

$$[2] \quad Sv = \frac{2M\ \Sigma\ I}{TLL\ \Sigma\ PR}$$

where PS is the number of points of the grid test system hitting the structure of interest, PR is the number of points hitting the reference, M is the magnification, I is the number of intersections with the test lines, and TLL is the test line length.

The basic stereological parameters (such as Vv and Sv) should be accompanied by a subindex that reflects the structure of interest and the reference structure. For example, $Vv_{cell,\ algal\ layer}$ represents the proportion of the algal layer volume occupied by the cell (or cells). The measurements of Vv and Sv were made over transverse-section micrographs, which were "vertical sections" sensu Baddeley et al. (1986), with a cycloid test system (Cruz-Orive and Weibel 1990). Another stereological parameter used in this study was Vs, which represents the volume of a certain structure per thallus surface. Vs is obtained through multiplying the volume density (Vv) by the thickness of the thalli. We have calculated the Vs values for the cells of both symbionts in relation to the thallus surface ($\mu m^3/\mu m^2$).

Chlorophyll measurements

Fragments of 20 mg were taken from each of the 60 samples of series 2 for chlorophyll measurements. For the extraction of chlorophyll *a* and *b*, dimethyl sulphoxide (DMSO) was used as in Barnes et al. (1992). DMSO was reported to successfully extract chlorophyll from lichens (Ronen and Galun 1984; Manrique et al. 1989; Harrison et al. 1989; Boonpragob and Nash 1991; Balanguer and Manrique 1991). From the stereological data, which allowed the estimation of the volume of photobiont per thallus weight, the chlorophyll content of the algal cells (expressed as μg Chl/mm^3 algal cell) was calculated.

Results

Microscopic observations

Light microscopy revealed notable differences among the three zones considered within each specimen (central, intermediate, and marginal). These differences concerned both the total thallus thickness and the arrangement of the fungal hyphae within each layer, as well as the amount and distribution of the photobiont cells within the algal layer (Figs. 2–14). The central zone was generally the thickest (Table 1), with a very dense medulla and cortex and with a discontinuous algal layer often with no or few algal cells (Figs. 2 and 3, respectively). The intermediate zone was varied, with an algal layer that could be of great thickness (Fig. 4). The marginal zone was generally thin (Fig. 5; Table 1), with layers of sparse cells and a more or less continuous algal layer. The thallus anatomy was quite similar in the two species studied, with the same pattern of variation within the zones considered.

The upper part of the medulla at the intermediate zone was loosely arachnoidal (Fig. 6). Under this upper medulla, a densely prosoplectenchymatic layer integrated by long hyphae arranged mainly along the centrifugal axis was observed (Fig. 7). Both upper and lower cortices (Figs. 8 and 9) were paraplectenchymatic.

The algal layer at the central zone had large algal cells arranged in distant, sparse clusters (Fig. 10). The medulla maintained the structure observed at the intermediate zone, but its prosoplectenchymatic portion was more dense, lacking most of the free spaces among bunches of hyphae (Fig. 11). The arachnoidal portion of the medulla (Fig. 12) was not always present within the central zone. On the contrary, the marginal zone showed an algal layer of medium-size algae, frequently arranged in dense clusters (Fig. 13), a very thin upper cortex of 3–6 cells thick, and a very loose medulla, almost entirely composed of the arachnoidal band (Fig. 14).

The pustulated areas showed basically the same internal structure as the nonpustulated areas with the exception of the prosoplectenchymatic part of the medulla and the lower cortex that were not present in these areas, as already noted in a previous study (Sancho and Balaguer 1989).

The observations with SEM allowed us to further distinguish the structure of the medulla from the three different zones of the thallus considered. The dense arachnoidal section of the medulla within the central zones, with hyphae enlarged by the accumulation of crystallized lichen substances (Fig. 15), contrasted with that of the intermediate zone (Fig. 16) and marginal zone (Fig. 17). The hyphae connecting the algal layer with the lower medulla within the last two zones were thin and left many free spaces. Within the central zone the groups of hyphae of the prosoplectenchymatic medulla left little space free, and the fungal cells were so closely packed and fused that it was very difficult to distinguish the limits among them (Figs. 18 and 19). This degree of packing was not evident within the intermediate zone (Figs. 20 and 21).

Influence of water on thickness, surface area, volume, and density

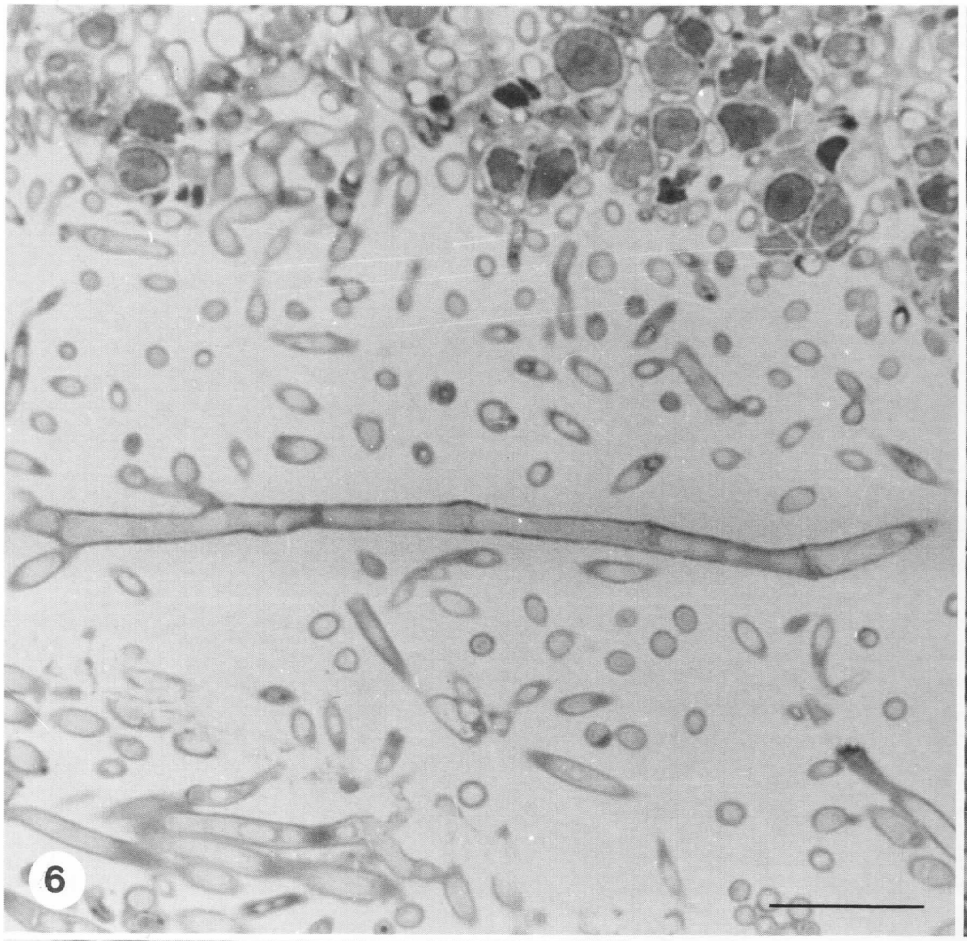
The measurement of total thallus thickness was strongly influenced by the technique used to obtain transverse sections of the thallus (Fig. 22). The greater differences were found in *L. pustulata*, in which the average thickness of the hydrated samples was almost double that of the dry samples (Table 1).

The thickness of the upper cortex, algal layer, and lower

FIGS. 6–9. Light micrographs of sections of *L. hispanica* and *L. pustulata* stained with methylene blue embedded in Spurr's resin (0.8 μm thick). Fig. 6. Algal layer and upper medulla of the intermediate zone in *L. hispanica*. Scale bar = 20 μm. Fig. 7. Lower medulla of the intermediate zone in *L. pustulata*. Scale bar = 20 μm. Fig. 8. Upper cortex of the intermediate zone in *L. pustulata*. Scale bar = 10 μm. Fig. 9. Lower cortex of the intermediate zone in *L. pustulata*. Scale bar = 20 μm.

FIGS. 10–14. Light micrographs of sections of *L. hispanica* and *L. pustulata* stained with methylene blue and embedded in Spurr's resin (0.8 μm thick). Fig. 10. Algal layer of the central zone in *L. hispanica*. Fig. 11. Lower medulla of the central zone in *L. pustulata*. Fig. 12. Upper medulla of the central zone in *L. hispanica*. Fig. 13. Upper cortex, algal layer, and medulla of the marginal zone of *L. pustulata*. Fig. 14. Lower medulla and lower cortex of the marginal zone in *L. hispanica*. Scale bars = 20 μm.

FIGS. 15–21. SEM micrographs of *L. hispanica* and *L. pustulata*. Fig. 15. Upper medulla of the central zone in *L. pustulata*. Scale bar = 10 μm. Fig. 16. Upper medulla of the intermediate zone of *L. hispanica*. Scale bar = 10 μm. Fig. 17. Upper medulla of the marginal zone of *L. hispanica*. Scale bar = 10 μm. Fig. 18. Lower medulla of the central zone of *L. pustulata*. Scale bar = 10 μm. Fig. 19. Detail of Fig. 18. Scale bar = 2 μm. Fig. 20. Lower medulla of the intermediate zone of *L. hispanica*. Scale bar = 10 μm. Fig. 21. Detail of Fig. 20. Scale bar = 2 μm.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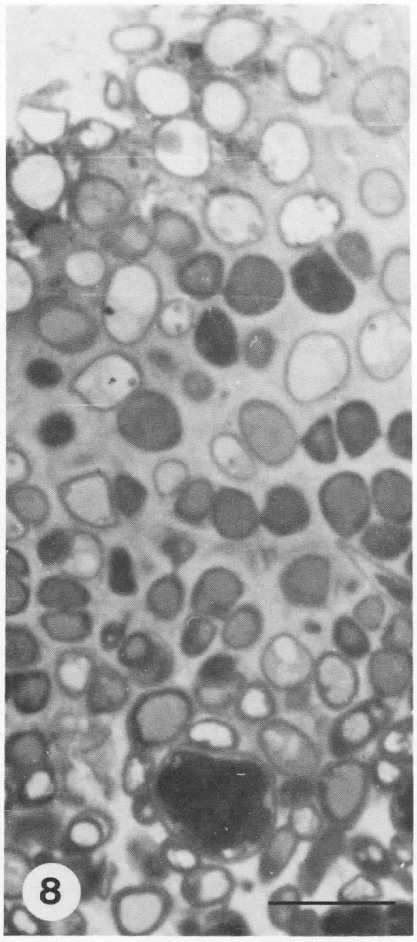
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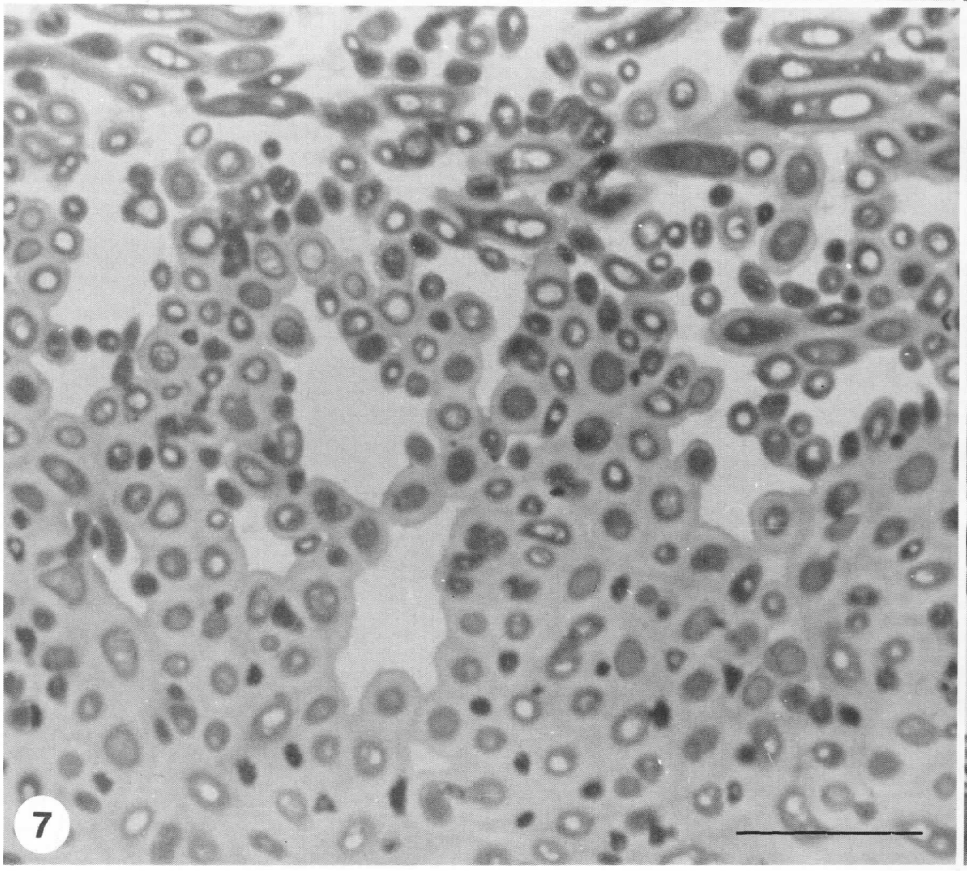
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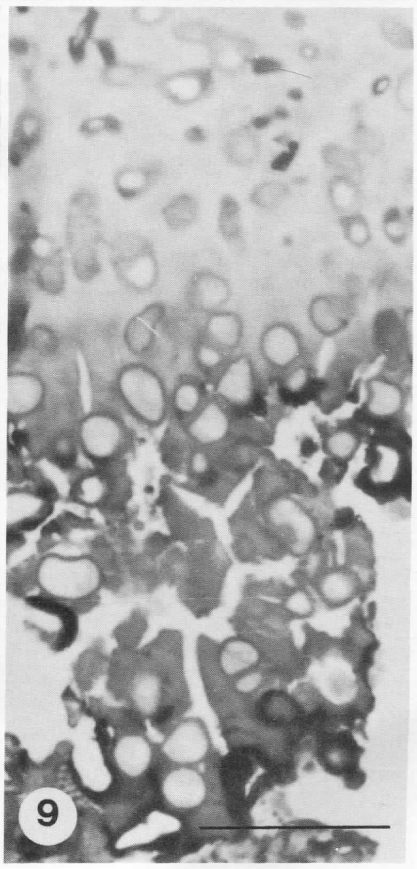
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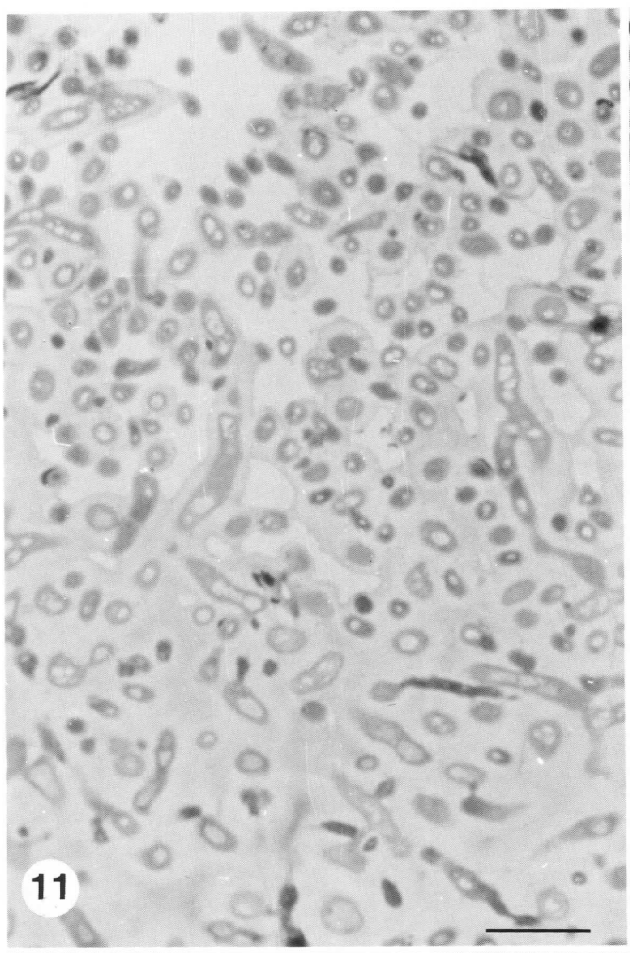
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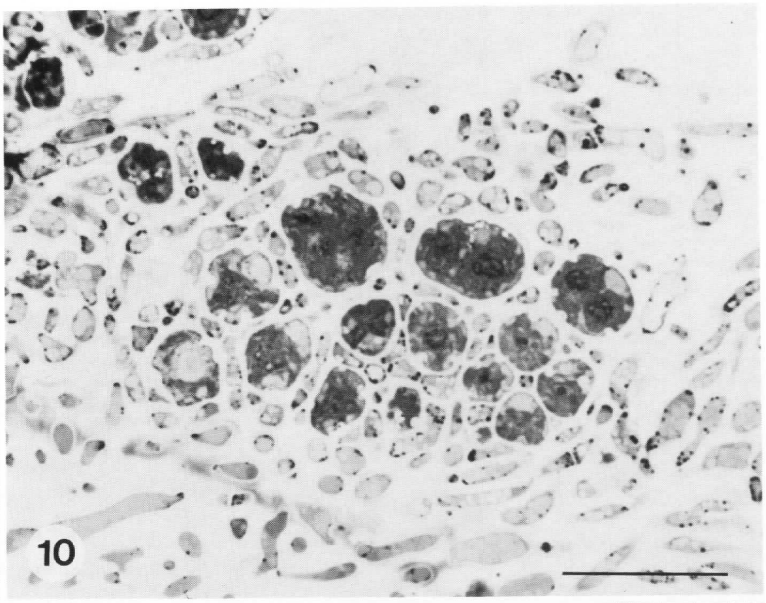
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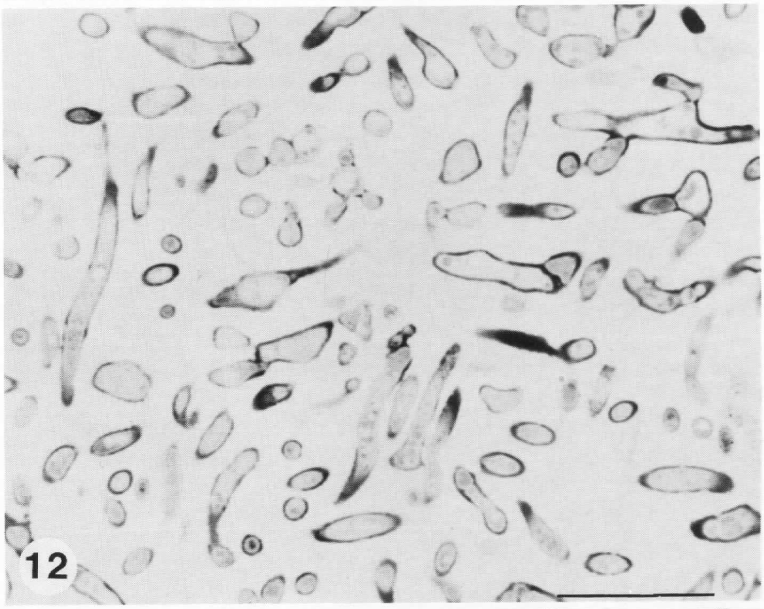
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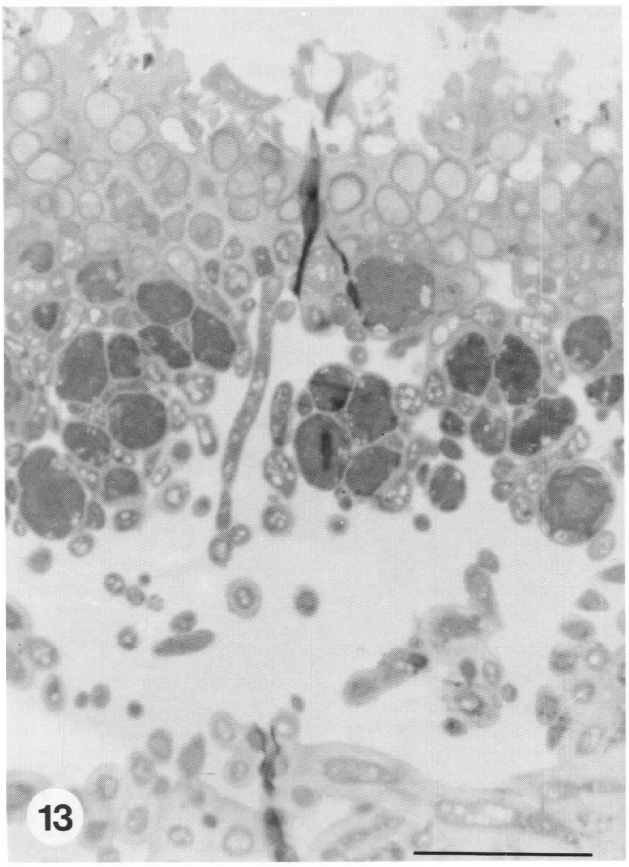
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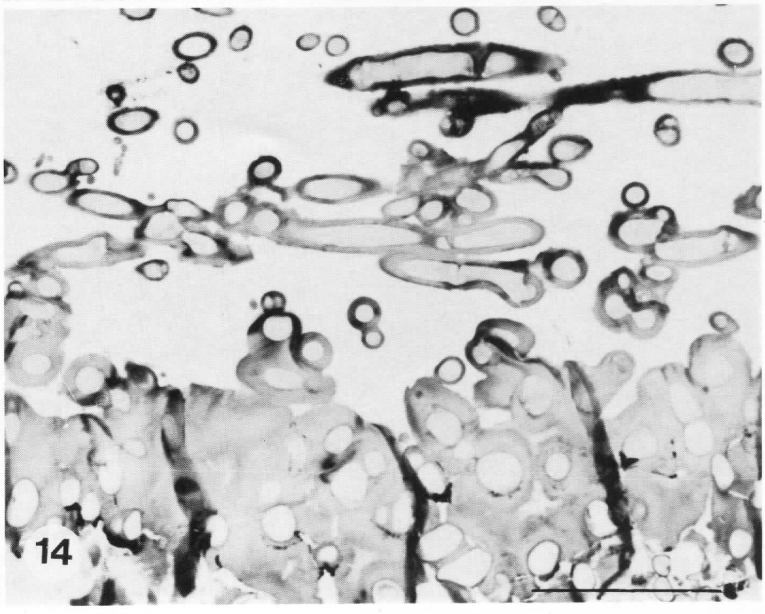
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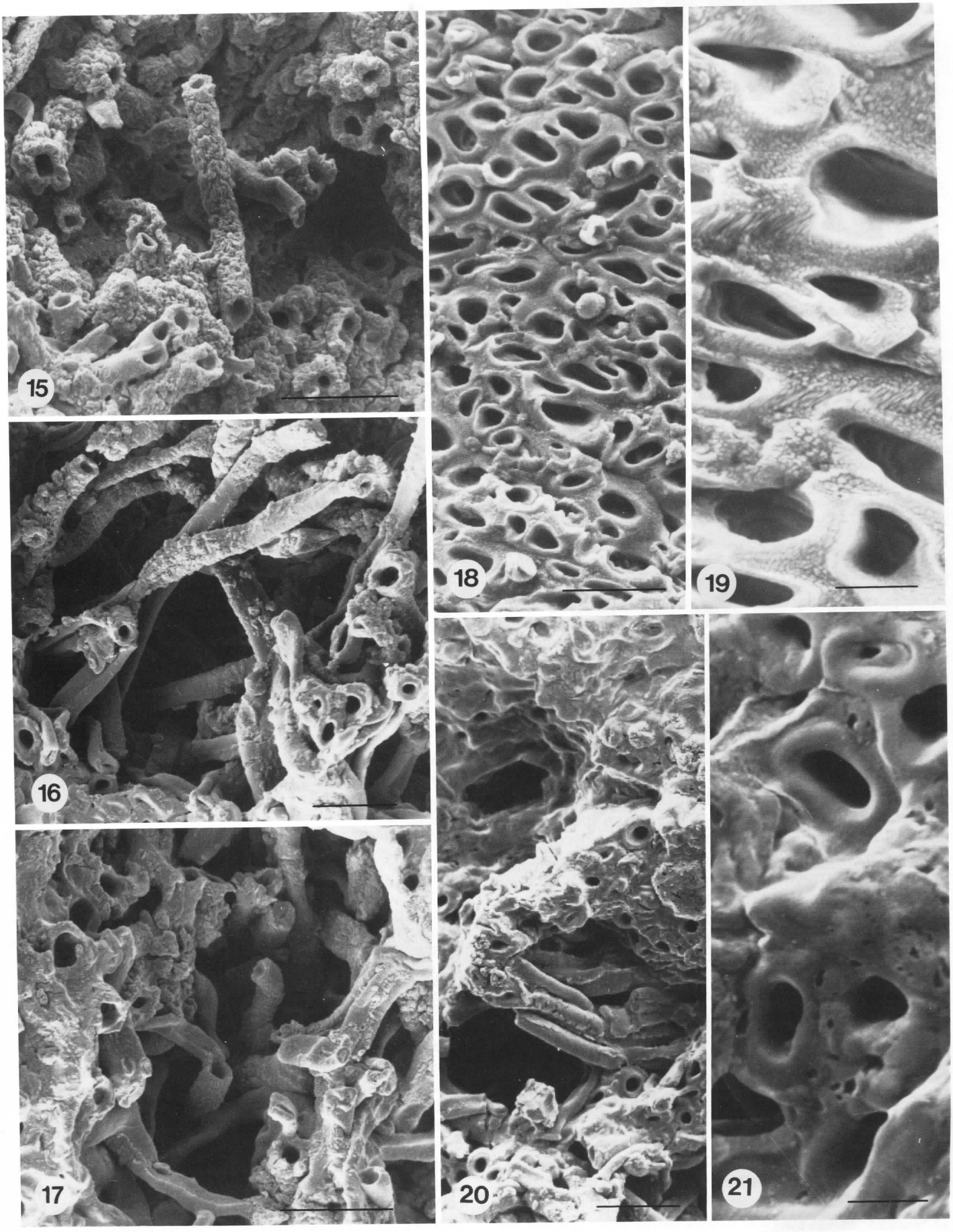
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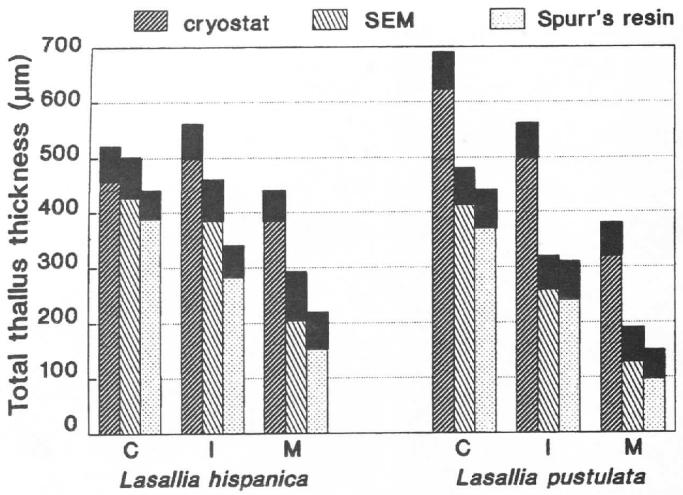

FIG. 22. Mean total thallus thickness of the central (C), intermediate (I), and marginal (M) zones of *L. hispanica* and *L. pustulata* from hydrated samples (cryostat sections) and dry samples (embedded in Spurr's resin or air dried and examined with a scanning electron microscope). The black portion of the bars indicates the confidence interval for the mean (ANOVA, $p < 0.05$, $n = 10$).

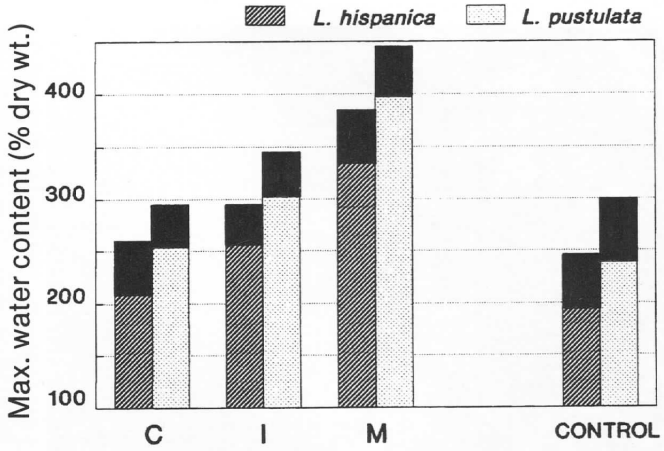

FIG. 23. Mean maximum water content, expressed as a percentage of the dry weight of the thallus, of the central (C), intermediate (I), and marginal (M) zones and uncut thalli (control) of *L. hispanica* and *L. pustulata*. The black portion of the bars indicates the confidence interval for the mean (ANOVA, $p < 0.05$, $n = 10$; controls $n = 5$).

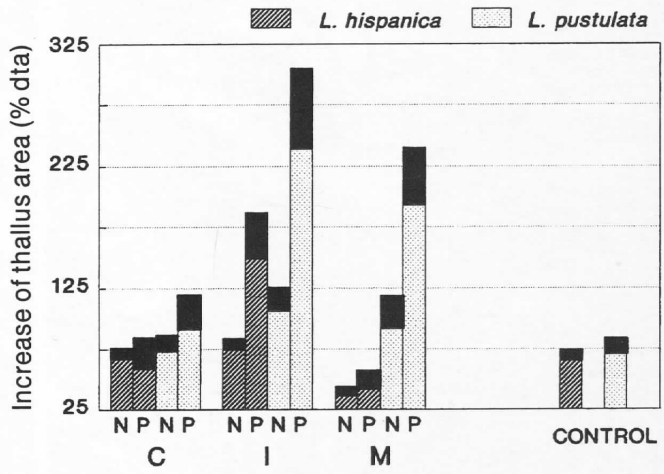

FIG. 24. Mean increase of thallus surface area with hydration, expressed as a percentage of the dry thallus area (dta), of the central (C), intermediate (I), and marginal (M) zones and uncut thalli (control) of *L. hispanica* and *L. pustulata*. In this case the pustulate (P) and the nonpustulate (N) zones of the samples were distinguished. The black portion of the bars indicates the confidence interval for the mean (ANOVA, $p < 0.05$, $n = 10$; controls $n = 5$).

cortex showed a similar pattern in the two species studied, with larger increases due to hydration in the marginal zones. However, the total thallus thickness increased more with hydration in *L. pustulata* than in *L. hispanica*, mainly as a consequence of the remarkable increase in thickness with hydration of the arachnoidal part of the medulla of *L. pustulata*.

The increase of thickness with hydration clearly mirrors the maximum water content of the different zones and species studied (Fig. 23). *Lassallia pustulata* managed to take up more water than *L. hispanica* in both the one-piece thalli (controls) and the cut samples. The central zone increased its weight at maximum hydration between 200 and 300%, the intermediate zone between 250 and 350%, and the marginal zone between 350 and 450%. The central zone, and to a lesser extent the intermediate zone, retained the water for a longer period of time than the marginal zone.

The thallus surface area was also modified by water uptake differently between zones (Fig. 24). The central zone was least modified by hydration, but in this case the intermediate and not the marginal zone was most affected by water, especially the pustulated regions of this zone, which were able to increase in surface area up to three times with hydration.

The increase of the thallus volume due to hydration (Fig. 25) presented a pattern similar to that of the thallus surface area but with a wider range of variation (while the increase in area oscillated between 50 and 300%, the increase in volume oscillated between 100 and 800%) and maximum values in the intermediate zone only in *L. hispanica*.

Thallus density at each zone is shown in Table 2. In the dry samples, the central zone was always the most dense, and density decreased toward the thallus margin. However, the density of the hydrated samples was lowest at the intermediate zone because of the large increase in its surface area with water uptake.

Three-dimensional quantitative features of the mycobiont

The stereological data of the mycobiont of both species are shown in Table 3. The protoplasm of the hycobiont cells ($Vv_{protoplasm,\ reference\ layer}$, the volume of the protoplasm of the fungal cells in relation to the volume of the reference layer) represented roughly half of the total volume of both cortices but only one-third of the total volume of the algal layer and the medulla. However, this stereological parameter did not show significant changes among zones, and the same was also true for $Vv_{cell,\ total}$ and $Vs_{cell,\ thallus}$. Considering the volume density of the cells of each layer in relation to the thallus as a whole ($Vv_{cell,\ total}$) and in relation to its surface ($Vs_{cell,\ thallus}$), it can be appreciated that the upper cortex and the medulla (especially its dense lower part) are the layers that provided the most mycobiont volume to the lichen. It is remarkable that the upper medulla, with a thickness between one-fourth and one-sixth of the total thallus thickness, represented only 9–11% of the total thallus protoplasmic volume. The gradual decrease of $VS_{cell,\ thallus}$ from the centre to the margin mirrors the progressive narrowing of the thallus and all its layers.

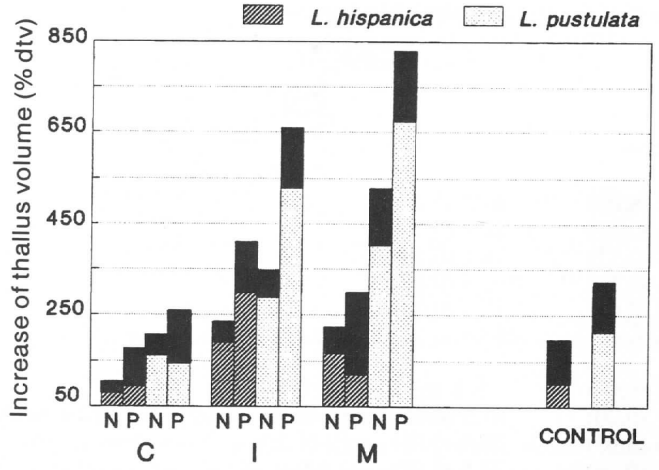

FIG. 25. Mean increase of thallus volume with hydration, expressed as a percentage of the dry thallus volume (dtv), of the central (C), intermediate (I), and marginal (M) zones and uncut thalli (control) of *L. hispanica* and *L. pustulata*. In this case the pustulate (P) and nonpustulate (N) zones of the samples were distinguished. The black portion of the bars indicates the confidence interval for the mean (ANOVA, $p < 0.05$, $n = 10$; controls $n = 5$).

TABLE 2. Pustulate surface area (PSA, expressed as a percentage of the thallus surface area), density of dry (DD) and wet (DW) samples, and increase in density (DI) due to hydration in the three zones of *L. hispanica* and *L. pustulata*

Zone	PA (%)	DD ($g \cdot cm^{-3}$)	DW ($g \cdot cm^{-3}$)	DI (%)
Lasallia hispanica				
Central zone	54.4 (0.10)	1.81 (0.05)	2.86 (0.06)	58
Intermediate zone	41.0 (0.09)	1.34 (0.05)	1.65 (0.05)	23
Marginal zone	47.3 (0.10)	1.24 (0.04)	1.71 (0.05)	38
Lasallia pustulata				
Central zone	53.2 (0.09)	1.80 (0.06)	2.32 (0.06)	29
Intermediate zone	46.7 (0.09)	1.78 (0.05)	1.85 (0.05)	4
Maraginal zone	31.9 (0.10)	1.62 (0.05)	1.97 (0.05)	22

NOTE: The coefficient of variation is indicated in parentheses ($n = 10$).

The ratio of surface area to volume (obtained as Sv/Vv) of the mycobiont cells reflected the distinct morphology of the hyphae of each layer, although they did not show any clearly interpretable intrathallinen or interspecific variations. This ratio was lower than that for the cells of the upper cortex and the algal layer and greater than that for the long medullar cells. The increase in the ratio observed in the upper cortex of the central zone of *L. pustulata* should be due mainly to the existence of old or dead cells that decrease in volume because of loss of protoplasm.

Three-dimensional quantitative features of the photobiont and chlorophyll data

The photobiont occupied between one-fifth and one-third of the algal layer volume (Table 4; $Vv_{cell,\ algal\ layer}$). Although a slight increase of $Vv_{cell,\ algal\ layer}$ towards the margins could be noted (especially in *L. hispanica*), the increasing importance of the photobiont towards the periphery becomes more evident when it is considered in relation not to the algal layer but to the whole thallus. Taking together the volume of both symbionts without considering intercellular spaces (Fig. 26), a clear

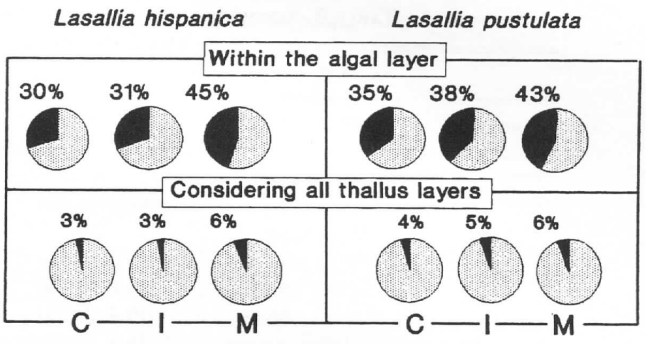

FIG. 26. Volume of the photobiont cells (black zone) versus volume of the mycobiont cells (shaded zone) without considering intercellular spaces in the central (C), intermediate (I), and marginal (M) zones of *L. hispanica* and *L. pustulata* within the algal layer and considering all the layers of the thallus.

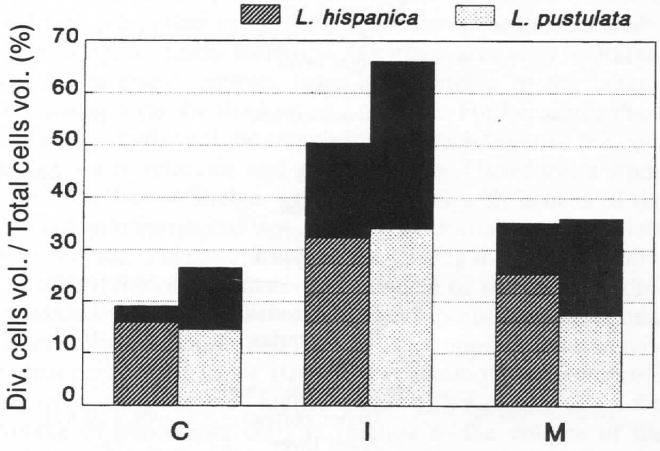

FIG. 27. Mean percentage of the volume of the photobiont cells represented by dividing cells into the central (C), intermediate (I), and marginal (M) zones of *L. hispanica* and *L. pustulata*. The black portion of the bars indicates the confidence interval for the mean (ANOVA, $p < 0.01$, $n = 3$).

increase of the volumetric importance of the photobiont towards the margins could be seen.

The percentage of the volume of the whole algal cells represented by the dividing algal cells presented the highest values in the intermediate zones (Fig. 27).

The volume of photobiont per thallus surface unit ($Vs_{cell,\ thallus}$) decreased towards the marginal zone, as occurred with the mycobiont, because of the thinning of all layers from the center to the periphery.

The ratio of surface area to volume of the algae of both lichens fluctuated between 0.5 and 0.6 and reflected the large volume of their spheroidal shape in contrast with the irregular or long cells of the mycobiont.

The chlorophyll parameters (Table 5) showed significant differences within the thallus in the two species. The total chlorophyll content increased progressively and significantly towards the thallus margins, in clear agreement with the stereological parameters already mentioned. However, the chlorophyll per photobiont volume presented a different intrathalline pattern, with a decrease towards the thallus margin in *L. hispanica*. The ratio of chlorophyll *a* to *b* showed the highest values at the intermediate zone in the two species.

TABLE 3. Stereology of the mycobiont within each of the thallus layers of *L. hispanica* and *L. pustulata* at the three different zones

Layer	$Vv_{prot, ref}$	$Vv_{cell, total}$	$Vs_{cell, thallus}$	Sv/Vv
		Lasallia hispanica		
Central zone				
Upper cortex	54.1 (0.54)	23.7 (0.02)	96.2 (0.03)	0.7
Algal layer	—	5.3 (0.05)	21.6 (0.05)	0.7
Upper medulla	17.6 (0.26)	9.4 (0.17)	38.2 (0.18)	1.6
Lower medulla	40.0 (0.06)	15.4 (0.08)	62.5 (0.09)	1.3
Lower cortex	36.4 (0.05)	11.7 (0.07)	47.5 (0.08)	1.5
Total	38.7	65.5	266.0	1.1
Intermediate zone				
Upper cortex	47.0 (0.32)	26.2 (0.04)	78.8 (0.5)	0.9
Algal lalyer	—	6.1 (0.20)	81.5 (0.20)	0.9
Upper medulla	25.7 (0.21)	7.0 (0.03)	20.9 (0.03)	1.2
Lower medulla	43.1 (0.06)	25.8 (0.08)	77.5 (0.08)	0.9
Lower cortex	55.0 (0.13)	13.7 (0.13)	41.3 (0.13)	0.6
Total	43.5	78.9	237.0	0.9
Marginal zone				
Upper cortex	53.8 (0.39)	14.9 (0.06)	27.9 (0.06)	0.9
Algal layer	—	5.2 (0.46)	9.9 (0.46)	1.0
Medulla	25.4 (0.56)	14.4 (0.20)	84.2 (0.20)	1.2
Lower cortex	52.8 (0.46)	16.1 (0.17)	29.3 (0.17)	0.8
Total	36.1	67.0	125.9	1.0
		Lasallia pustulata		
Central zone				
Upper cortex	49.9 (0.50)	20.5 (0.32)	84.0 (0.32)	1.1
Algal layer	—	5.3 (0.08)	21.5 (0.08)	0.9
Upper medulla	26.3 (0.30)	11.2 (0.10)	46.0 (0.10)	1.5
Lower medulla	35.5 (0.37)	18.8 (0.21)	76.9 (0.21)	1.4
Lower cortex	47.2 (0.32)	9.1 (0.07)	37.2 (0.07)	1.1
Total	40.7	64.9	265.6	1.2
Intermediate zone				
Upper cortex	49.7 (0.41)	19.2 (0.08)	55.1 (0.08)	0.7
Algal layer	—	6.3 (0.19)	18.1 (0.19)	0.7
Upper medulla	26.4 (0.35)	7.3 (0.02)	20.9 (0.02)	1.5
Lower medulla	35.2 (0.39)	21.9 (0.07)	63.0 (0.07)	1.4
Lower cortex	47.5 (0.36)	14.2 (0.13)	40.9 (0.13)	0.9
Total	41.0	68.9	198.0	1.0
Marginal zone				
Upper cortex	52.8 (0.03)	25.9 (0.06)	33.0 (0.06)	0.9
Algal layer	—	6.1 (0.15)	7.7 (0.15)	0.9
Medulla	35.3 (0.08)	15.9 (0.10)	40.0 (0.10)	1.3
Lower cortex	46.7 (0.04)	11.0 (0.02)	13.9 (0.02)	0.8
Total	41.8	74.5	94.6	1.0

NOTE: The stereological parameters are $Vv_{prot, ref}$ (volume of the protoplasm of the fungal cells in relation to the volume of the reference layer, as %), $Vv_{cell, total}$ (volume of the fungal cells in relation to the volume of all thallus layers, as %), $Vs_{cell, thallus}$ (volume of the fungal cells in relation to the thallus surface area, in $\mu m^3/\mu m^2$), and Sv/Vv (ratio of the surface area of the plasmalemma to the volume of the protoplasm for the fungal cells of each layer, except the algal layer, in which this ratio was calculated relative to the volume of the whole fungal cell including the wall). The coefficient of variation is indicated in parentheses ($n = 3$).

TABLE 4. Stereology of the photobiont of *L. hispanica* and *L. pustulata* at the three different zones

	L. hispanica			*L. pustulata*		
Parameter	Central zone	Intermediate zone	Marginal zone	Central zone	Intermediate zone	Marginal zone
$Vv_{cell, algal layer}$	21.6 (0.03)	20.8 (0.27)	29.1 (0.18)	28.3 (0.34)	27.4 (0.11)	29.3 (0.52)
$Vs_{cell, thallus}$	9.2 (0.03)	8.2 (0.25)	8.2 (0.18)	11.0 (0.07)	11.2 (0.20)	5.9 (0.19)
Sv/Vv	0.5	0.6	0.5	0.6	0.6	0.6

NOTE: The stereological parameters are $Vv_{cell, algal layer}$, volume of algal cells in relation to the volume of the algal layer (in %), $Vs_{cell, thallus}$, volume of algal cells in relation to the thallus surface area (in $\mu m^3/\mu m^2$), and Sv/Vv, ratio of the surface area of the algal plasmalemma to the volume of the algal protoplasm. The coefficient of variation is indicated in parentheses ($n = 3$).

TABLE 5. Chlorophyll data for the three zones of *L. hispanica* and *L. pustulata*

Parameter	*L. hispanica*			*L. pustulata*		
	Central zone	Intermediate zone	Marginal zone	Central zone	Intermediate zone	Marginal zone
Chlorophyll concn.	1.69 (0.04)	1.76 (0.04)	2.67 (0.05)	1.95 (0.04)	2.47 (0.04)	3.31 (0.05)
Chlorophyll content of algal cells	159.6 (0.14)	91.80 (0.14)	84.8 (0.14)	109.3 (0.14)	118.7 (0.14)	106.9 (0.14)
Chlorophyll *a* to *b*	1.86 (0.03)	2.66 (0.03)	1.60 (0.03)	1.67 (0.03)	2.60 (0.03)	2.07 (0.03)

NOTE: The parameters are chlorophyll concentration (expressed as μg chlorophyll/mg oven-dry weight of thallus sample), chlorophyll content of the algal cells (expressed as μg Chl/mm^3 algal cell), and the ratio of chlorophyll *a* to *b*. The coefficient of variation is indicated in parentheses (n = 10).

Summarized description of the three zones

The central zone is the thickest and most dense zone. Despite being the zone least able to take up water (although it can be retained for a long period of time) this zone greatly increased in density at maximum hydration because of its low increase in volume. This was the zone poorest in chlorophyll content because of the small percentage of the thallus volume occupied by the photobiont. Its algal cells, however, were the richest in chlorophyll.

The intermediate zone presented intermediate values only for certain parameters (maximum water content, chlorophyll content, and volume density of the photobiont). It is the zone within which the pustules increased the most in surface area with hydration. A great increase in volume with hydration was also noted, although with respect to this parameter the intermediate zone did not differ significantly from the marginal zone. This fact can explain the low density at maximum hydration despite the absorption of more water than in the central zone. The intermediate zone had the highest chlorophyll *a* content.

The marginal zone is able to absorb the largest amount of water in relation to its dry weight but at the same time loses water the fastest. The density of this zone increased somewhat with hydration, since its weight increased proportionately more than its volume with water uptake. This was the zone richest in total chlorophylls and the one in which the photobiont represented the largest percentage of the sample volume. However, its algal cells had the least chlorophyll content per algal volume.

Discussion

If the density of the thallus is obtained from thallus dry weight, the values of the area and thickness must also be obtained from dry material, otherwise an intermediate value between the dry and the wet sample could be calculated. The density values provided by Snelgar et al. (1981*a*) for *Sticta latifrons* and *Pseudocyphellaria amphisticta* are between one-third and one-seventh of those obtained here for samples of the two *Lasallia* species. This difference may be due to a different thallus anatomy (the lichens mentioned possess a loose and rather hollow medulla that represents more than half of the total thallus thickness) but also to the use of the hydrated thickness together with dry weight. Density at maximum hydration may be a very important parameter in physiological studies of poikilohydric organisms, such as lichens, which modify their volume with passive uptake of water.

The values of the ratio of surface area to weight in *Umbilicaria antarctica* obtained by Harrison et al. (1989) are similar, with appropriate transformations, to those obtained here in *Lasallia*. In agreement with the findings of Larson (1979) on other *Umbrilicaria* species, these authors found that this ratio increases with hydration and decreases in large specimens, the latter effect possibly due to an increase of thallus thickness with age. Their data agree with the results of the present study, since samples greatly increased in surface area with hydration and the heaviest samples (usually belonging to the central zone) were also the thickest and densest. Furthermore, these studies do not reveal the importance of each layer in the control of water relations and gas exchange. Therefore, a study similar to that of Snelgar (1981*a*, 1981*b*) with lichens of the family Umbilicariaceae would be highly desirable to intergrate physiological and structural data concerning this lichen family.

The scarcity of quantitative anatomical or structural studies on lichens makes the discussion and comparison of our results rather difficult. Collins and Farrar (1978) applied some stereological parameters to the study of the symbionts of *Xanthoria parietina*; they obtained higher values of $Vv_{photobiont,\ thallus}$, the volume of photobiont cells in relation to the volume of the thallus, in this lichen than those obtained here for *Lasallia* (7% of thallus volume occupied by the algal cells vs. 2–5%), whereas the values of $Vv_{mycobiont,\ thallus}$, the volume of mycobiont cells in relation to the volume of the thallus, were lower (43 vs. 65–79%). These differences correlate with differences in photosynthesis and dark respiration: the net photosynthesis of *X. parietina* oscillates between 0.34 and 0.8 mg CO_2/(g dry wt. · h), whereas that of *Lasallia* oscillates between 0.39 and 0.60 mg (CO_2/(g dry wt. · h). The dark respiration of *X. parietina* lies between −0.18 and −0.24 mg CO_2/(g dry wt. · h), whereas in *Lasallia* it lies between −0.39 and −0.45 mg CO_2/(g dry wt. · h) (Korhonen and Kallio 1987; Sancho and Kappen 1989). However, the stereological differences between *X. parietina* and *Lasallia* could be partly due to differences in the extent and method of quantification.

The main differences between the two species studied here concern the maximum water uptake and the total chlorophyll content. The values of both parameters were significantly higher in *L. pustulata*. We consider that both differences can be explained by the presence in *L. pustulata* of coralloid isidia that in addition to providing more algal cells with only a slight contribution to weight, makes possible a more efficient and greater total uptake of water, as was found by Larson (1981) in *Umbilicaria deusta*.

The chlorophyll values obtained here using DMSO are larger than those obtained previously with the same species collected in nearby localities (Sancho and Kappen 1989) but using acetone in the pigment extraction. A very similar situation was pointed out by Harrison et al. (1989) and can be con-

sidered to be due to the higher efficiency of DMSO in the extraction. Moreover, the chlorophyll *a* to *b* ratios with DMSO are higher than those usually obtained in lichens, and values greater than two or even four must be considered normal values in extractions with this compound (Barnes et al. 1992).

The lichen thallus must provide an efficient water-holding structure without limiting free CO_2 exchange (Coxson et al. 1983). The anatomical differences between the pustulate and nonpustulate zones of *Lasallia* thalli are only in the medulla and the lower cortex, since the upper cortex remains almost invariable throughout the thallus (Sancho and Balaguer 1989). The development of a thick and dense medulla on the lower part of the algal layer, although increasing the amount of capillary-held water in the thallus, will restrict CO_2 diffusion from below (Snelgar et al. 1981*a*; Coxson et al. 1983). Therefore, the gas exchange that takes place in the pustules (which lack the dense prosoplectenchymatic part of the medulla and the lower cortex) must occur at higher rates than that of the nonpustulate areas, whereas the latter areas may retain more water and for a longer period of time. The pustules in the intermediate zone are usually better developed and show the largest increase in surface area with hydration. The intermediate zone as a whole has the lowest density at maximum hydration. In addition, the intermediate zone has the largest proportion of dividing algal cells, unlike that of fruticose lichens where the highest frequency of dividing algae was at the tips (Greenhalgh and Anglesea 1979; Hill 1989). The consideration of all these facts together suggests that the intermediate zone optimizes water relations and gas diffusion, making this the zone with the most active growth. The pustules themselves might originate by strong proliferation of medullar hyphae, as was suggested by Sancho and Balaguer (1989). This would imply extensive diffuse growth in the genus *Lasallia*, as already found in the genus *Ramalina* (Sanders 1989).

The marginal zone, with its high chlorophyll content and high volumetric representation of the photobiont, could also have a high growth rate, in agreement with the marginal pattern of growth traditionally assumed for foliose lichens (Hale 1973; Armstrong 1984). Moreover, the great increase in thallus density with hydration observed at the marginal zone may not have an extremely negative effect on gas diffusion, since the maximum hydration state is not maintained for very long in this zone. Nevertheless, the growth of the margins must be affected by erosion and fragmentation. Thus, we suggest that the main role of the dense central zone would be as a water-holding zone while the growth in the intermediate zone could counteract the continuous erosion of the marginal zone.

In addition to gas-exchange properties, three principal structural or anatomical features may influence the net photosynthesis of a lichen: the number of photobiont cells and amount of photosynthetic pigments, the mass of respiring mycobiont, and the thickness and density of the upper cortex. The decreasing percentage of the algal layer volume occupied by the photobiont towards the umbilicus accords with the low number of algal cells found in the central and oldest parts of other lichens (Greenhalgh and Anglesea 1979; Anglesea et al. 1983; Hill 1985, 1989) and also with the low chlorophyll content found at the oldest zones in fruticose lichens (Kärenlampi 1970; Nash et al. 1980; Legaz et al. 1986). How is photobiont development selectively inhibited at the central parts of the *Lasallia* thallus? Smith (1976) and Hill (1985) state that the algal population must be under a control that limits its proliferation more than that of the mycobiont. Honegger (1987) suggests a possible role of lichen substances in this control.

In the studies of the lichen substances in the genus *Lasallia* some contradictory results were obtained. Culberson and Culberson (1958) and Mirando and Fahselt (1978) did not detect any chemical variation with the age, size, or zone of the thallus. Posner et al. (1990, 1991) found a heterogeneous intrathalline distribution of secondary products. Seriñá et al. (1991), by contrast, showed clear correlations between some lichen substances and the size or zone of the lichen, suggesting that the accumulation of lichen substances at the margins of thalli of *L. pustulata* could reflect a higher metabolic activity of this zone. Our study shows more total chlorophyll in margins but is not consistent with the idea of algal control by phenolics, since if lichen substances somehow control the algal growth, the photobiont would not be so scarce in the central zone in which the lichen substances seem also to be scarce (Seriñá 1991). More studies are necessary to investigate the possible role of lichen substances in controlling the algal populations.

The relation between the number of photobiont cells and the chlorophyll content has not been studied in detail, with the exception of a tentative approach using subalpine macrolichens (Strobl and Türk 1990). Nash et al. (1980) comment that it is necessary to know whether the observed intrathalline gradient in the chlorophyll content was due to variations in the number of algae, variations in the chlorophyll content per algal cell, or a combination of both. Our study reflects that both variables decreased towards the thallus center, but the photobiont volume density decreased faster, resulting in algal cells with higher chlorophyll content at the central zone.

The question concerning the relationship between net photosynthesis and the mass of respiring mycobiont was not tackled directly here, but the stereological data indicate the volumetric importance of the mycobiont in each layer and in relation to the whole thallus. As was mentioned, the volumetric importance of the mycobiont decreases towards the margins, whereas that of the photobiont increases. This fact, if demonstrated in other lichens, should help explain the maximum rates of net photosynthesis found in the youngest zones of thalli by other authors (Moser and Nash 1978; Nash et al. 1980).

Another consideration is the effect of the upper cortex on photosynthesis. The provision of a thick upper cortex, which protects the algal cells from desiccating too rapidly and from the negative effects of extremely intense solar radiation (Kershaw and MacFarlane 1980), will also create a high internal resistance to CO_2 diffusion from above. The structure and density of the upper cortex have a strong influence on some physical properties of the thallus such as its inner temperature due to radiation, a combined effect of hydration level and colour of the upper surface (Sancho et al. 1992). The overall effect of the upper cortex at the different zones in the studied species cannot be estimated with certainty, since where the upper cortex was thickest it was not very dense, and vice versa.

The existence of structural intrathalline gradients that could reflect physiological gradients is consistent with previous work that found nonrandom intrathalline distributions of lichen substances and net photosynthetic rates (Kärenlampi 1970; Mirando and Fahselt 1978; Nash et al. 1980; Legaz et al. 1986; Seriñá et al. 1991; Manrique and Lopez 1991). In lichens within the family Umbilicariaceae, however, patchlike intrathalline patterns have been found in relation to gas

exchange, thermal resistance, and isoenzymes (Larson 1983; Larson and Carey 1986). The existence of well-defined intrathalline patterns of some basic structural parameters is clear (e.g., the thickness of the thallus and its layers, the volume density of the symbionts), although many anatomical intrathalline parameters vary in a completely different way within the thallus and can become complicated with thallus age. Therefore, a complex and apparently mosaiclike distribution of physiological parameters within the thallus can be expected, despite the fact that many of them have a relatively simple structural basis.

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