Water distribution, size and wall thickness in *Lycoperdon pyriforme* spores

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Laser diffractometry was used to obtain the distribution of refractive index in individual *Lycoperdon pyriforme* spores in water. This allowed the determination of the water content and density of whole spores and their components. These properties of spores were found to be very variable but protoplasts had a higher water content (51% on average) than wall (24%). The mean density of the spores was 1·20 g/ml. The wall was 0·34 μm thick on average and constituted 64% of the dry mass of the spores in addition to being very dry and dense. The presence of such a wall may play a role in the constitutive dormancy of Gasteromycete spores by providing a barrier to chemical activators.

Due to the disparity between the water contents of the wall and the protoplast the total water content of spores may be a poor indication of their physiological state.

Key words: Basidiospore structure, Dormancy, *Lycoperdon*, Spore density, Water content.

As in all living cells, water plays an important role in every developmental stage of fungal spores. The maturation of conidia is accompanied by a rise in the average refractive index, presumably caused by water expulsion, and a reverse process occurs during germination (Barer, 1956; Joseph, 1983). Humidity is reported to affect the longevity and heat resistance of conidia (Fahey, Mikolajczyk & Brody, 1978) as well as the ability of fungal spores to infect host plants (Harrison, 1984). In the case of bacterial spores, dehydration is certainly a major factor in the maintenance of dormancy and heat resistance (see e.g. Ulanowski, Ludlow & Waites, 1987; Marquis *et al.*, 1985). The ability of spores to germinate may be affected by wall permeability (Sussman, 1966). There is a need for studies of water relations and spore structure, especially in spores formed in aqueous surroundings. Fungal spores can reduce their water content to well below that of the surrounding environment, including their parent mycelial cells, but the mechanisms involved are not understood (Hawker & Madelin, 1976). Fungi can exist in extreme environmental conditions, particularly of humidity and temperature. Of considerable practical as well as fundamental interest is the resistance to desiccation and possible relationships between spore structure and temperature and humidity requirements of fungi, especially xerotolerant and thermophilic ones (Van Laere, 1986; Hill & Lacey, 1983; Sussman, 1966). Although the mean water content of spores can be measured gravimetrically fungal spores have distinct walls which often form a substantial part of the spores or are hydrophobic. There are no grounds for assuming that the water contents of the wall and of the cytoplasm are the same. Therefore, there is a need for an accurate method of obtaining the water distribution as well as content in spores.

Laser diffractometry provides information about the size, refractive index and structure of small particles (from a fraction of a micrometre to about 10 μm in size). The light scattered from a specimen is recorded as a function of angle and the resulting experimental 'pattern' (see Fig. 1) is compared with computed predictions based on light-scattering theory. Simple particle shapes (in practice almost always a sphere) and structures are assumed. Depending on the model used the mean refractive index or its distribution can be obtained in addition to the size of the particle. For biological materials the refractive index can be readily converted into properties such as solid or water content and density. In this way the state of intact living cells can be determined without affecting their metabolism, or, alternatively, during various treatments (with no delay between the treatment and the measurement). In principle, transient processes, e.g. germination, or the effects of external factors (such as chemical agents) can be observed, with monitoring of the water content, cell size and wall thickness.

The possibility of using laser diffractometry to obtain the refractive index of living cells was demonstrated by Wyatt (1972) for single bacteria and bacterial spores in air and for aqueous suspensions of vegetative bacterial cells with narrow size distributions. However, airborne cells are dehydrated and shrunken, are not in an environment in which development can take place and cannot be subjected to chemical treatments,

e.g. with germinants. Suspending cells in water solves these problems. On the other hand diverse populations of cells (such as most spores) cannot be investigated as suspensions because their polydispersity obscures internal detail. These difficulties were overcome in studies of single conidia suspended in water (Ludlow & Kaye, 1978) where mean value of the refractive index was measured and bacterial spores (Ulanowski, Ludlow & Waites, 1987) where separate values for the protoplast and the integument were obtained.

The puffball, *Lycoperdon pyriforme* Schaeff., produces an abundance of spherical basidiospores about 3·5 μm diameter and with sparse surface ornamentation consisting of scattered, low conical spines (Fig. 2). They are, therefore, ideally suited for modelling using the coated sphere approximation of the internal structure. In this study laser diffractometry measurements were carried out on single spores suspended in water and the results analysed to yield the overall size, wall thickness and the refractive indices of the protoplast and the wall.

MATERIALS AND METHODS

Light scattering

Sporophores of *L. pyriforme* collected in Hertfordshire were refrigerated for five months, the spores extracted, suspended in water and purified by repeated resuspension and centrifugation. A surfactant (Tween 80, 0·005 %) was added to all but the final suspension in order to facilitate dispersal. All water was distilled and filtered through 0·2 and 0·03 μm pore size Sartorius filters. The spores were kept in water for at least 16 h prior to measurements.

Differential light-scattering patterns were obtained using a fast-scanning laser diffractometer (Ludlow & Kaye, 1978; Ulanowski, 1988). An argon–ion laser operating at the wavelength of 0·5145 μm was used as a light source. The laser beam had a power of 100 mW and was focused to a diameter of about 60 μm at the centre of a cylindrical scattering cuvette filled with filtered water. Dilute spore suspensions were delivered into the cuvette using a simple laminar flow device (Ulanowski, 1988).

Best-fit theoretical patterns were found by comparing each experimental pattern with about 40000 theoretical light scattering patterns computed from the Lorenz-Mie theory of light scattering on coated spheres (see e.g. Bohren & Huffman, 1983). An algorithm was used which ensured that no potential solutions were ignored and no subjective bias was introduced (Ulanowski, 1988).

The coated sphere model of spore structure used for calculating theoretical light scattering patterns is characterized by four independent parameters: the inner radius and refractive index and the outer radius and refractive index (Fig. 3A). The refractive index is constant within each of the two separate regions.

Water content and density calculations

The refractive index n was converted into the solid content c_s, expressed as weight of dry solids per volume of wet material, using the formula (Barer & Joseph, 1954; Ross, 1967):

$$n = n_w + c_s \alpha_s,$$

where n_w is the refractive index of water (1·336 at 0·5145 μm wavelength) and α_s is the specific refraction increment. The water content c_w (g/ml) was then calculated as:

$$c_w = D_w(1 - c_s \cdot \bar{v}_{app}),$$

where D_w is the density of water and $\bar{v}_{app}$ is the apparent partial specific volume of the solids. The density D of the wet material is:

$$D = c_w + c_s.$$

The unknown parameters were estimated from the chemical composition of spore components. The specific refraction increments were 0·172 and 0·152 ml/g, and the apparent partial specific volumes were 0·82 and 0·70 ml/g for the protoplast and the wall, respectively (Ulanowski, 1988).

RESULTS AND DISCUSSION

Theoretical patterns were fitted to 13 experimental light-scattering patterns (Fig. 1) and the best-fit solutions are shown on Fig. 3B. Overall, the results demonstrated the presence of an outer layer with a refractive index higher than in the interior – the reverse of the situation found in bacterial spores (Ulanowski *et al.*, 1987). However, a high degree of variability was apparent.

The thickness of the outer layer, 0·34 μm on average, suggested that it corresponded to the spore wall – consistently with results of electron microscopic observations of spores of *L. pyriforme* (Fig. 2) and the closely related *Langermannia*

Fig. 1. Differential light-scattering pattern obtained from a single, dormant basidiospore of *Lycoperdon pyriforme* in water (continuous line). The best-fit theoretical pattern (broken line) corresponds to the inner radius of 1·45 μm, the outer radius of 1·70 μm, and 1·40 and 1·52 as the respective refractive indices.

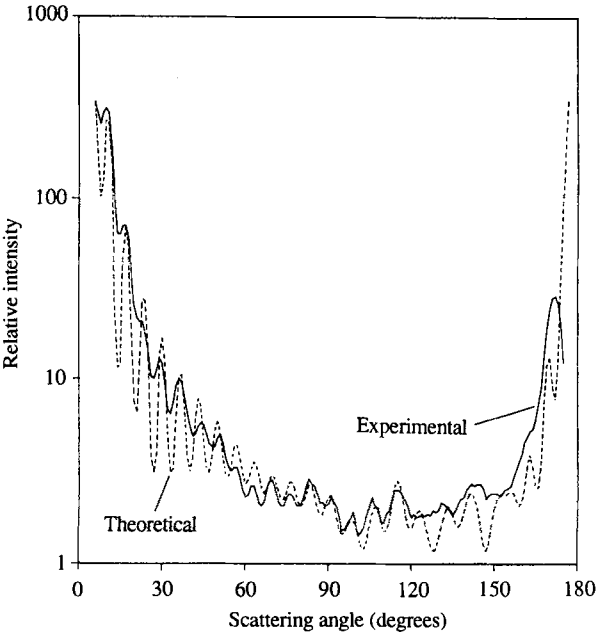
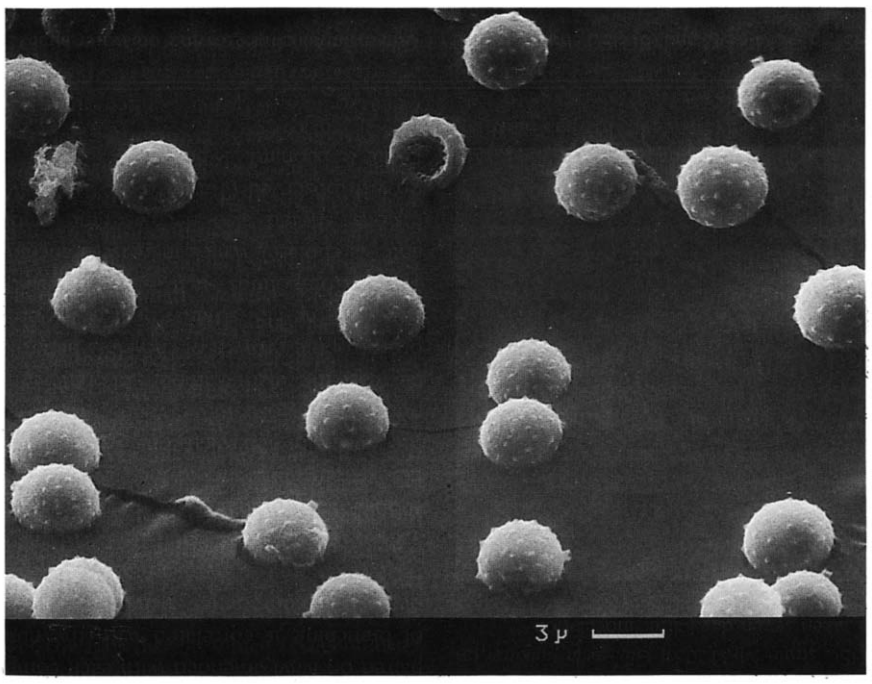


Fig. 2. Scanning electron micrograph of *Lycoperdon pyriforme* basidiospores.



gigantea (Batsch) Rostk., where walls were often seen to be invaginated or broken. The mean values of the sizes and refractive indices as well as the calculated water contents and densities are given in Table 1.

Three of the spores could be regarded as anomalous. They corresponded to a homogeneous sphere, to a nearly homogeneous sphere and to a particle with very low refractive index and very thin outer layer, respectively. Because of an uncertainty in the value of the wall thickness (due to the similarity of the refractive indices) they were excluded from some calculations, as indicated in Table 1.

The mean value of the water content of the protoplasts, 51%, was much lower than in vegetative cells, where it is generally 85–90% (Cochrane 1958), reflecting the fact that the spores were dormant. However, it was still considerably higher than the water content of whole spores which was 37%, due to a contribution to the total from the very dry wall. The disparity between the protoplast water content and the total water content questions the significance of the latter as an indicator of the physiological state of fungal spores.

Published data offer little information which could be used for a direct comparison of water contents of basidiospores. Values reported for conidia range from 6 to 88%, reflecting different growth conditions, spore age, measurement techniques and conditions (especially humidity) as well as interspecific differences (Hawker & Madelin, 1976). The water content at 100% relative humidity is larger (65–70%; Griffin, 1981; Fahey *et al.*, 1978; Marquis *et al.*, 1985) and has more relevance to measurements carried out in water.

In immersion refractometry measurements the values of the 'mean' refractive index of fungal spores vary between about 1·42–1·43 (Joseph, 1983) and 1·44 (Barer, 1956) for various conidia and 1·47 for basidiospores (*Tricholomopsis rutilans*, Agaricales) (Knecht, 1975). In the present study the mean

Fig. 3. A, Coated sphere model of the structure of spores; B, best-fit solutions for the light-scattering patterns from 13 individual, dormant basidiospores of *Lycoperdon pyriforme*. Each line represents four parameters of the best theoretical solution for one spore: the left-hand end corresponds to the radius and refractive index of the protoplast, the right-hand end to the outer radius and the refractive index of the wall.

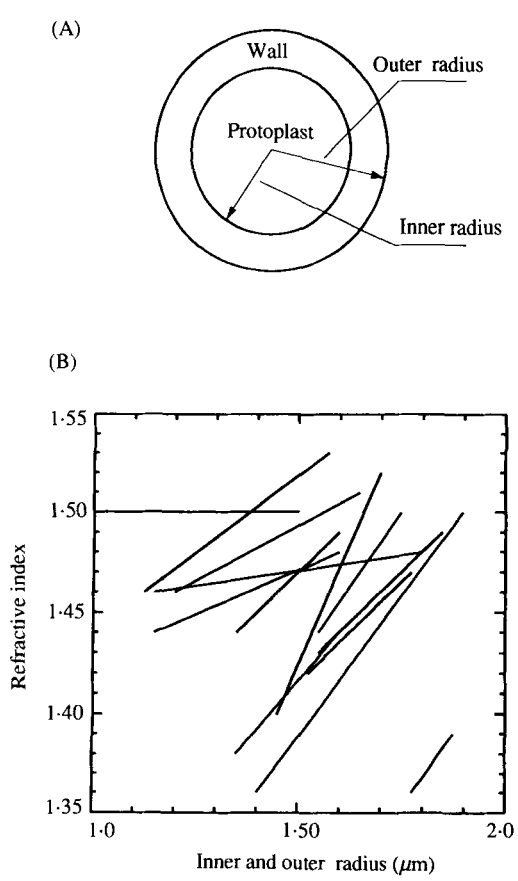


Table 1. Distribution of refractive index, water and density within 13 basidiospores of *Lycoperdon pyriforme* (standard deviation given in parentheses). Water content and density are calculated from the refractive index

Region of spore	Radius (μm)	Refractive index (−)	Water content (g/ml)	Water content (%)*	Density (g/ml)
Protoplast	1·37(±0·16)†	1·43(±0·04)	0·57	51	1·11
Wall	0·34(±0·11)†‡	1·49(±0·04)	0·31	24	1·29
Whole spore	1·71(±0·13)	—	0·44	37	1·20

* On a fresh-weight basis.

† Three anomalous spores were excluded (see text).

‡ Thickness.

refractive index was about 1·46. Previously published results of laser diffractometry measurements show the mean refractive index (obtained on the basis of the homogeneous sphere model) of 1·46 for *Penicillium chrysogenum* and 1·50 for *Aspergillus niger* conidia (Ludlow & Kaye, 1978).

Fungal spores are reported to have densities commonly around 1·1–1·2 g/ml (Hawker & Madelin, 1976; Gregory, 1973). For spores of the closely related *Langermannia gigantea* immersed in a 55% sucrose solution the density was 1·26 g/ml (Gregory & Henden, 1976). In pure water the density would be slightly lower due to slight swelling of the spores, making it close to the value of 1·20 g/ml calculated in this study. This value reflects the low water content of the spores.

Gasteromycete spores germinate with great difficulty under laboratory conditions and may require chemical activators (Wilson & Beneke, 1966) and wall permeability could be a contributing factor, as is thought to be the case with spores of other fungi (Sussman, 1966). The spores under study here were permeable to water (in spite of being hydrophobic), as evidenced by slight swelling during optical microscopy examinations, also reported elsewhere (Gregory & Henden, 1976). However, the wall constituted as much as 64% of the dry mass of the spores on average and was very dense and dry. Such a wall might be a barrier to molecules larger than those of water and it might prevent penetration of germination activators. Moreover the wall of *L. pyriforme* appears to restrict the swelling of the spores thus limiting the protoplast water content to a level (51% on average) which is probably barely sufficient for germination to take place.

This investigation demonstrated the ability of laser diffractometry to provide information about the structure and the physiological state of fungal spores in aqueous surroundings, particularly about the water content and distribution. An advantage of this method is the ability to study cells in their natural state, unaltered by preparation procedures. The fact that measurements on single cells can be made allows study of population heterogeneity and use of very small samples. The main limitation is at present the shape of cells.

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