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STRUCTURE OF AERIAL AND SUBMERGED SCLEROTIA OF *COPRINUS LAGOPUS*

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SUMMARY

Sclerotia were formed in both aerial and submerged parts of the mycelium. In addition a layer of cells with pigmented thick walls (called brown matting) which differentiated at the air/agar interface was interpreted as an aspect of sclerotial behaviour since it was regularly formed by strains which produced submerged sclerotia and was composed of cells of similar structure to those of the outermost layer of the submerged sclerotium. Apart from producing sclerotia and oidiospores the cells of the aerial mycelium remained undifferentiated. In contrast, cells of the submerged mycelium, though initially indistinguishable from those of aerial hyphae, became individually differentiated within about 5 days of growth producing two further novel cell types; inflated cells containing glycogen, and hyaline thick-walled cells. Submerged sclerotia were pale brown in colour, irregularly shaped and about 0.5–1.0 mm in diameter. The only structure which differentiated the submerged sclerotium from the submerged mycelium was the outer rind, a layer of cells with thick, pigmented walls. The central (medulla) region contained the same cell types in the same frequencies as the general submerged mycelium. In sharp contrast the aerial sclerotia were highly organized structures composed of distinct and compact tissues. Mature aerial sclerotia were dark brown to black spheroidal structures up to 0.5 mm in diameter. An outer layer of dead and moribund hyphae surrounded the main body of the sclerotium which was bilayered with an outer rind and inner medulla. The rind was multilayered and consisted of small cells with thick pigmented walls; intercellular spaces were cuticularized. The medulla was a closely packed tissue composed predominantly of hyaline thick-walled cells of the same type as were encountered in the submerged mycelium.

INTRODUCTION

Sclerotia have been recorded as occurring in *Coprinus lagopus* (= *C. cinereus*) by Lewis (1961) and Volz and Niederpruem (1970). Lewis mentions the presence of sclerotia on plate-grown colonies while Volz and Niederpruem describe some features of their structure. The present paper expands these observations and describes the ultrastructural features of the mature sclerotium. Since sclerotia arise from a parent mycelium the ultrastructural features of vegetative hyphae are also considered. Some aspects of vegetative hyphal structure have already been reported (Marchant, Peat and Banbury, 1966; Geisy and Day, 1965; Casselton, Lewis and Marchant, 1971).

MATERIALS AND METHODS

The organism used was the Basidiomycete *Coprinus lagopus sensu* Lewis (= *C. cinereus* (Schaeff. ex Fr.) S. F. Gray). Two monokaryotic wild-type isolates, H1 (mating type

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A_5B_5) and ZBw601 (mating type $A_{40}B_{40}$) were selected from the collection maintained in this laboratory since they regularly produced sclerotia within 14 days of inoculation. Other wild types in the collection were found to take up to 7 weeks to produce sclerotia.

Colonies were usually grown on a complex complete medium (CCM) solidified with 1.5% (w/v) agar (Moore, 1966) and contained in 9 cm Petri dishes. Incubation was at 37° C without illumination.

For light microscopy sclerotia were either fixed overnight in acetic-alcohol (1 : 4) or in buffered acrolein (10% v/v in 70 mM phosphate buffer, pH 6.9). Acetic-alcohol fixed material was embedded in 54° C m.p. wax using a chloroform substitution technique (Peacock, 1969) and sectioned at 6–8 μm. Material fixed in acrolein was dehydrated through an ethanol series, embedded in a glycolmethacrylate mixture (Feder and O'Brien, 1968) and 1 μm sections cut using dry glass knives on an ultratome. These sections were treated with dimedone before staining (Feder and O'Brien, 1968). Sections were stained by the periodic acid-Schiff (PAS) reaction, reagents being prepared according to de Tomasi (Pearse, 1968). Micrographs were made using Ilford 35 mm PAN F or HP 4 film and a Reichert 'Zetopan' or Nikon photomicroscope.

For electron microscopy sclerotia were fixed in 6% glutaraldehyde buffered to pH 6.9 with 70 mM phosphate buffer. After 30 min an equal volume of buffered osmium tetroxide was added and fixation continued for a further 90 min. The final concentration of OsO_4 was 0.5%. Sucrose (1 mM) and $MgSO_4$ (0.01 mM) were also added to the buffering solution (Roth, Pihlaja and Shigenaka, 1970). Fixed material was dehydrated through an ethanol series and embedded in a resin mixture based on that of Spurr (1969).

Sections were cut with glass knives and mounted on uncoated grids, stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined in an AEI EM 6B electron microscope.

Material for scanning electron microscopy was prepared by growing mycelium on membrane filters on the surface of CCM medium. Pieces of membrane carrying both parent mycelium and sclerotia were stripped from the medium and dried *in vacuo*. Fixation prior to desiccation did not improve the preparations. Specimens fixed to studs with 'UHU' glue were coated with either silver or a gold-palladium (40/60) alloy. Observations were made using a Cambridge S4 stereoscan.

RESULTS

The two strains studied differed in their sclerotial behaviour. H1 produced only aerial sclerotia whereas ZBw601 produced sclerotia on both the aerial and submerged parts of the colony. Neither strain produced sclerotia until the agar surface of plate cultures had been completely covered with mycelium for 4–5 days. This usually required a total of 10 days incubation for H1 and 9 days for ZBw601. During development of aerial sclerotia only two stages were distinguishable to the unaided eye: *immature* sclerotia appeared as small unpigmented spheroids, these becoming pigmented as the *mature* sclerotium developed. Aerial sclerotia of ZBw601 were always associated with localized areas of pigmented mycelium (brown matting) on the agar surface which appeared 1 or 2 days before immature sclerotia were seen. H1 very rarely produced brown matting, sclerotia of this strain arose in otherwise undifferentiated regions of aerial mycelium. The submerged sclerotia of ZBw601 appeared after 8–10 days incubation and were not related to particular areas of the parent mycelium.

Colony structure

Colonies of ZBw601 were composed of three distinct regions; aerial mycelium, brown matting and submerged mycelium. Cells of the aerial mycelium represented the simplest condition, upon which secondary changes were imposed. Vegetative hyphal cells were separated by the characteristic dolipore septa (Geisy and Day, 1965) (Plate 1, No. 1). A membraneous structure that may represent an aspect of lomasomal activity was generally associated with the dolipore septum. Lomasomal elaborations were of two types; they were usually composed of numerous vesiculate structures (Plate 1, No. 2) but were sometimes made of flattened sacs (Plate 1, No. 3). Golgi dictyosomes were never observed. Electron-dense rosette-shaped bodies *c.* 300 nm in diameter were present in most cells in the peripheral regions of the cytoplasm (Plate 1, No. 1). Each rosette was composed of smaller (27–30 nm) subunits. The size and structure of the rosettes are characteristic of polysaccharide reserve material (Bracker, 1967; Kugler, 1966).

The brown matting formed at the air/agar interface consisted of a single layer of cells in which just one cell type predominated (Plate 2, No. 1). A particular feature of these cells was the pigmented wall thickening (200–300 nm thick) which was almost entirely restricted to the outer (i.e. air-exposed) wall. The inner wall always remained unthickened while the lateral walls showed a gradient of thickening from their outer edges. Cells of the brown matting arose as lateral branches from both aerial and submerged hyphae; differentiation occurred when the branches contacted the agar surface. Dolipore septa were not observed.

In colonies which had been incubated for 2 or 3 days the aerial and submerged mycelia were morphologically indistinguishable. However, the submerged mycelium in mature colonies had hyphae quite different from those of the aerial mycelium. The first indication of any change was the formation of lateral branches by otherwise unexceptional vegetative hyphae. These branches gave rise to irregularly inflated cells with intensely PAS positive contents (Plate 1, No. 5), a feature which correlated with the large number of polysaccharide rosettes observed in electron micrographs of similar cells. These cells undoubtedly correspond to the glycogen-containing inflated cells reported by Madelin (1960). The second differentiated cell type in submerged mycelium began to appear at the same time as the inflated cells and was characterized by its extremely thick cell wall (1–3 μm) which was faintly PAS positive. This type arose from intercalary hyphal cells and it was possible to find single thick-walled cells adjacent to unaltered hyphal cells. The process of secondary differentiation was continuous and older hyphae (5–6 days) were composed mainly of thick-walled cells. During differentiation the cytoplasm of these cells was PAS positive but this progressively disappeared as the cell matured. Unaltered hyphal cells could also be found in the submerged mycelium though their frequency reduced as the colony aged. Dimensions of the various cell types are summarized in Table 1.

The mature aerial sclerotium

Mature aerial sclerotia were dark brown to black, more or less spherical and variable in size although most were in the range 100–250 μm in diameter. Plate 1, No. 6 illustrates the gross structure; three tissue layers were apparent—the outer diffuse layer, the rind and the medulla. The outermost diffuse layer (Plate 2, No. 3) was composed of apparently dead hyphal cells whose cytoplasm was reduced to membrane fragments and vesiculate structures (Plate 2, No. 2). Many had crenulate cell walls (Plate 3, No. 1) which may

Table 1. *Dimensions of cells comprising the mature vegetative colony of* Coprinus

Region of the colony	Types of cell present	Cell dimensions (μm) Length	Breadth	Wall thickness
Aerial mycelium	Hyphal cells	50–200	2–3	0.09–0.15
Brown matting	Pigmented thick-walled cells	4–9	3–4	0.09–0.30
Submerged mycelium	Inflated cells	15–20	7–10	0.09–0.15
	Hyaline thick-walled cells	55–90	4–7	1.0–3.0

indicate they were damaged during preparation for sectioning. This outer layer, though only loosely attached and often sloughed off during fixation, was always present in mature aerial sclerotia and is therefore regarded as an integral part of their structure.

The multilayered rind totally surrounded the central medulla (Plate 1, No. 6). A surface view showed a reticulate pattern of junctions between closely packed cells (Plate 3, No. 2). Individual rind cells were much shorter than vegetative hyphal cells (5–10 × 2–4 μm) and their walls were uniformly thickened (200–300 nm) and heavily pigmented. The walls of rind cells were bilayered (Waters, Butler and Moore, 1972) though this feature was often obscured by extracellular accretions of a pigmented material (Plate 3, No. 2). This cuticular substance was most frequently observed on the outer sclerotial surface but also occurred in some of the intercellular spaces deep within the rind. Most rind cells contained living contents (Plate 3, No. 3). The cytoplasm was PAS negative. Dolipore septa were not observed, nor was any other form of pore.

In the fully mature medulla thick-walled cells predominated with the less frequent thin-walled cells randomly interspersed among them. The thin-walled cells differed in few respects from the basic hyphal level of organization. The thick-walled cells were similar to the cells with thick hyaline walls found in the submerged mycelium though, at 10–20 μm long, considerably shorter (Plate 4, Nos. 1, 2 and 3). Dolipore septa were a regular feature of the transverse walls between pairs of thin-walled cells (Plate 4, No. 4). They were also observed in transverse walls separating thick- from thin-walled cells indicating that even cells with completely different structures could be in intimate cytoplasmic contact (Plate 5, No. 2).

Submerged sclerotia

The gross structure of the submerged sclerotium with its rind and medulla is illustrated in Plate 5, No. 3. This type of sclerotium was less regular in shape, usually larger (0.5–1.0 mm) and paler than the typical aerial sclerotium. The rind layer was invariably one cell thick and composed of cells with pigmented and unevenly thickened walls similar to the cells of the brown matting. No living cytoplasm was identifiable in electron-micrographs of the submerged sclerotial rind cells examined. The medulla of submerged sclerotia was loosely organized, the cells being separated by large spaces filled with medium. Three cell types were recognized, each was identical in size, structure and frequency to its counterpart in the submerged mycelium.

DISCUSSION

Set against the background of the various sclerotial types described in the literature the aerial sclerotium of *Coprinus lagopus* is quite simple in its organization. The thickened walls and absence of dolipore septa or other pores in rind cells, together with the occlu-

sion of intercellular spaces with cuticular material (Remsberg, 1940; Corner, 1950; Townsend and Willetts, 1954) and the pigmentation (Chet, Henis and Mitchell, 1967) all suggest that the function of the rind is to protect the medulla during conditions adverse to normal hyphal growth. The extreme outer layer of dead unmodified hyphal cells does not appear to serve any function but rather to represent the surplus material of the sclerotial initial. Such cells have been observed before (Brefeld, 1877; Remsberg, 1940) although in some fungi these outer cells are greatly modified and possess species-specific morphologies (Neal, Webster and Gunn, 1934; Remsberg, 1940).

In some fungi the sclerotium medulla is divided into distinct zones (Townsend and Willetts, 1954; Chet, Henis and Kislev, 1969) but this seems to be less common than the rather more amorphous arrangement described here for *C. lagopus*. It has been suggested (Volz and Niederpruem, 1970) that the sclerotial medulla of *C. lagopus* consists entirely of thin-walled cells. This interpretation cannot be supported by our observations; in all the *mature* aerial and submerged sclerotia examined thick-walled medullary cells were always present, and in the former type of sclerotium were always the most numerous.

The wall of young hyphal cells seen in section (Plate 1, No. 4) resembled most closely that of *Schizophyllum commune* (Hunsley and Burnett, 1970). This wall forms the basis for the secondary wall thickening which occurs in rind and medullary cells. Very little published information is available about the fine structure and composition of thick-walled cells in the fungi. A number of thickened walls have been illustrated (e.g. Scurti and Converso, 1965; Chet *et al.*, 1969; Heintz and Niederpruem, 1971) but as far as we are aware the macrofibrillar hyaline thick wall described for *Coprinus* medullary cells (Waters *et al.*, 1972) is unique.

The cells constituting the aerial sclerotium were markedly different from those of the aerial mycelium in which the structures developed; conversely the submerged sclerotium possessed a medulla which contained cells of the same sorts and in the same proportions as occurred in the submerged mycelium and a rind comprised of cells very like those of the brown matting layer that overlaid the submerged mycelium. Observations of sclerotium morphogenesis show that aerial sclerotia arise by development from differentiated sclerotial initials but submerged sclerotia appear when regions of the submerged mycelium are isolated by formation of a rind layer. It is clear that the aerial and submerged sclerotia of *Coprinus* are not homologous but represent two quite different aspects of sclerotial behaviour in this organism. The fact that some strains (like H1) produce only a single type of sclerotium while others (like ZBw601) produce both lends further support to this suggestion. The mature aerial sclerotium described here does not differ from the concept of the sclerotium as a fully differentiated fungal structure (Butler, 1966) whereas the submerged sclerotia are closely analogous to the polyporus condition (Campbell and Munson, 1939) and to *Armillaria mellea* (Campbell, 1934) where the body of the sclerotium is a region of unexceptional mycelium that is delimited by a layer or layers of differentiated cells.

Both sclerotium types are resistant structures that are developed towards the end of the vegetative life of the colony. Aerial sclerotia are clearly highly organized propagules specifically evolved for this purpose. The status of submerged sclerotia is less certain as it is only the rind layer which differentiates them from the submerged mycelium in which they are found. The close similarity in structure between rind cells of the submerged sclerotia and cells of the brown matting suggests that submerged sclerotia may be an expression of an attempt by the colony to separate off the exposed aerial mycelium and

enclose the whole of the rest of the colony within a protective layer of cells. On this interpretation submerged sclerotia are small regions where the enclosure has been completed, whereas an area of brown matting represents an isolated plate of an incomplete rind around the whole colony.

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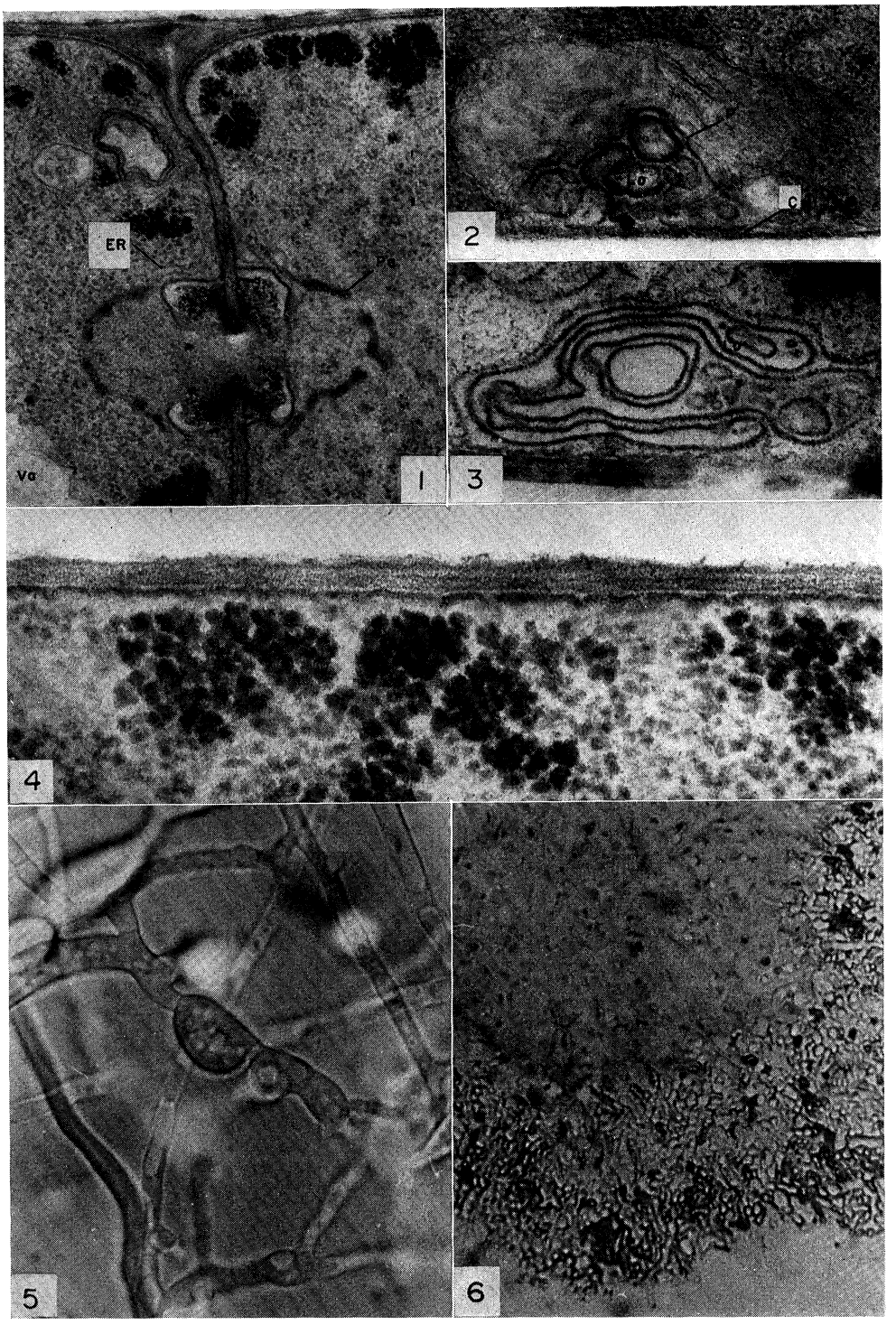

H. WATERS, R. D. BUTLER AND D. MOORE—*STRUCTURE OF* COPRINUS *SCLEROTIA*

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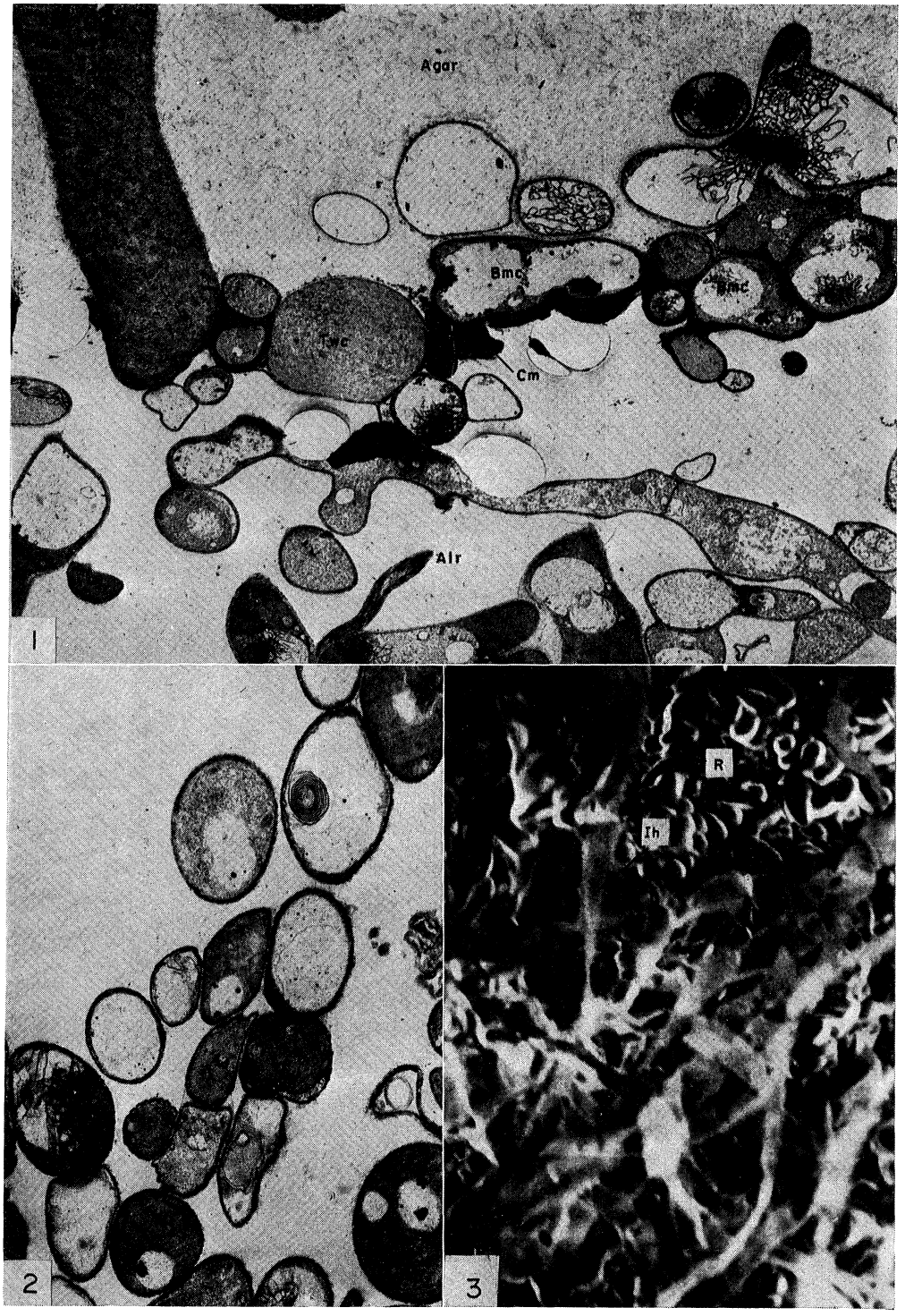

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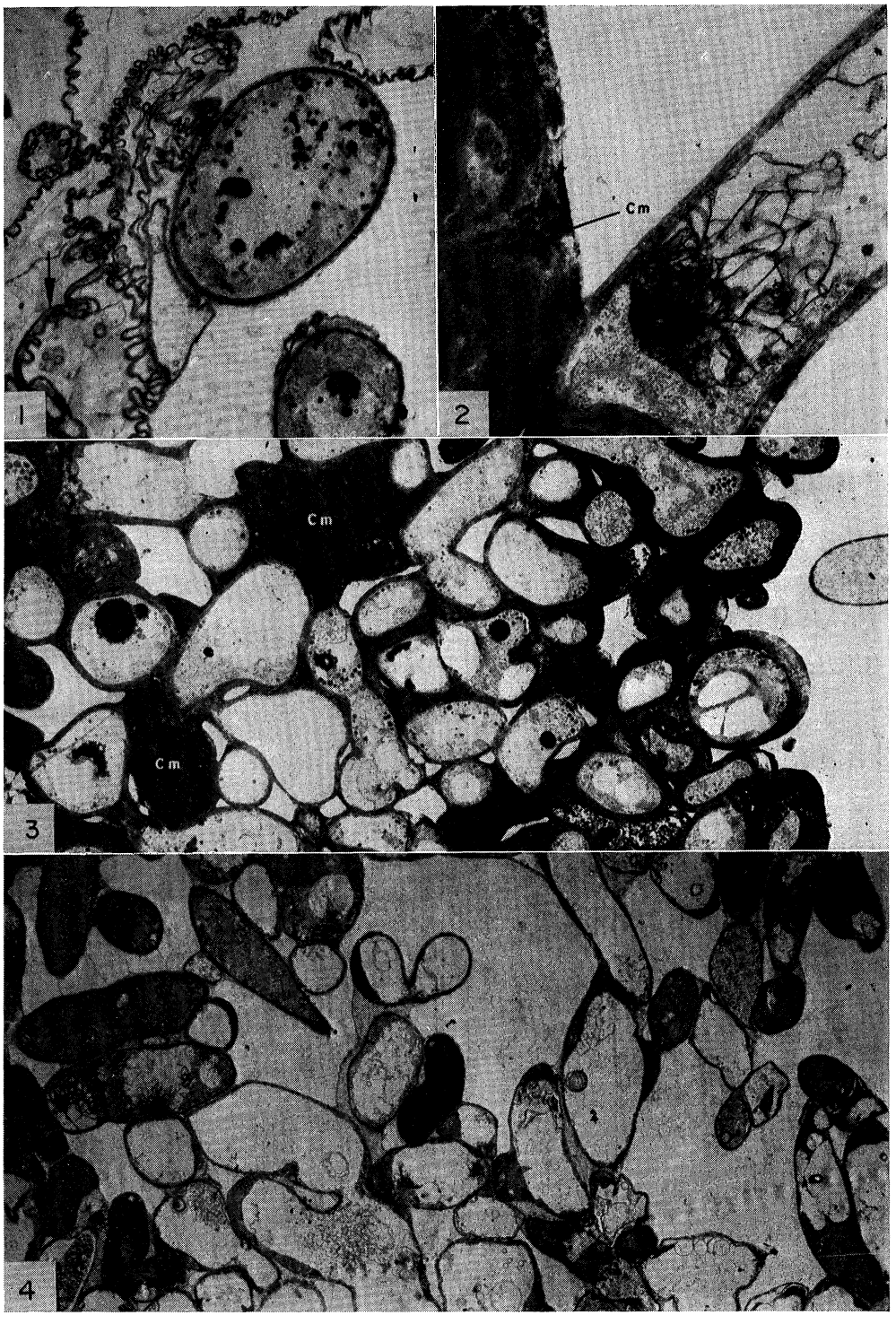

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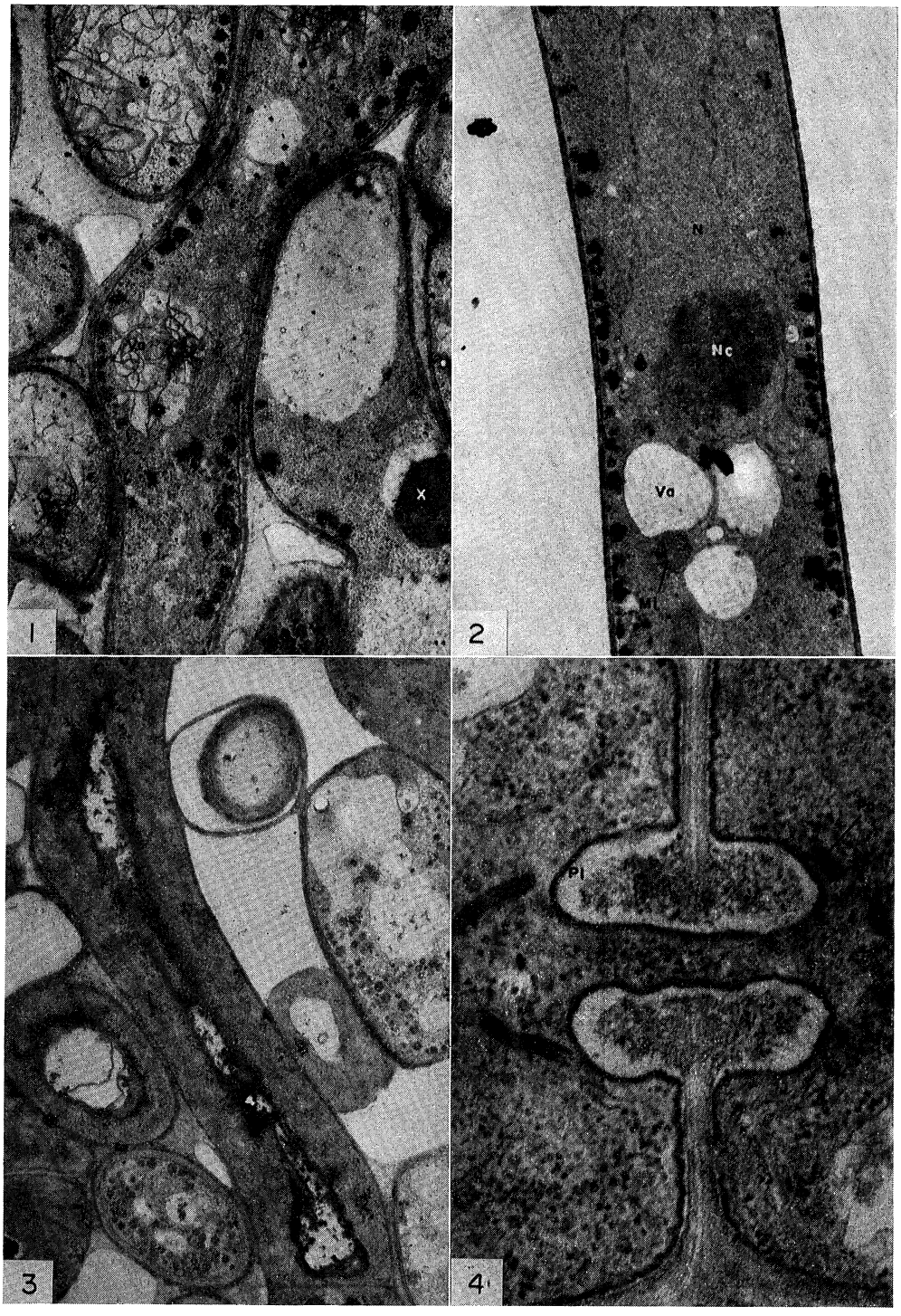

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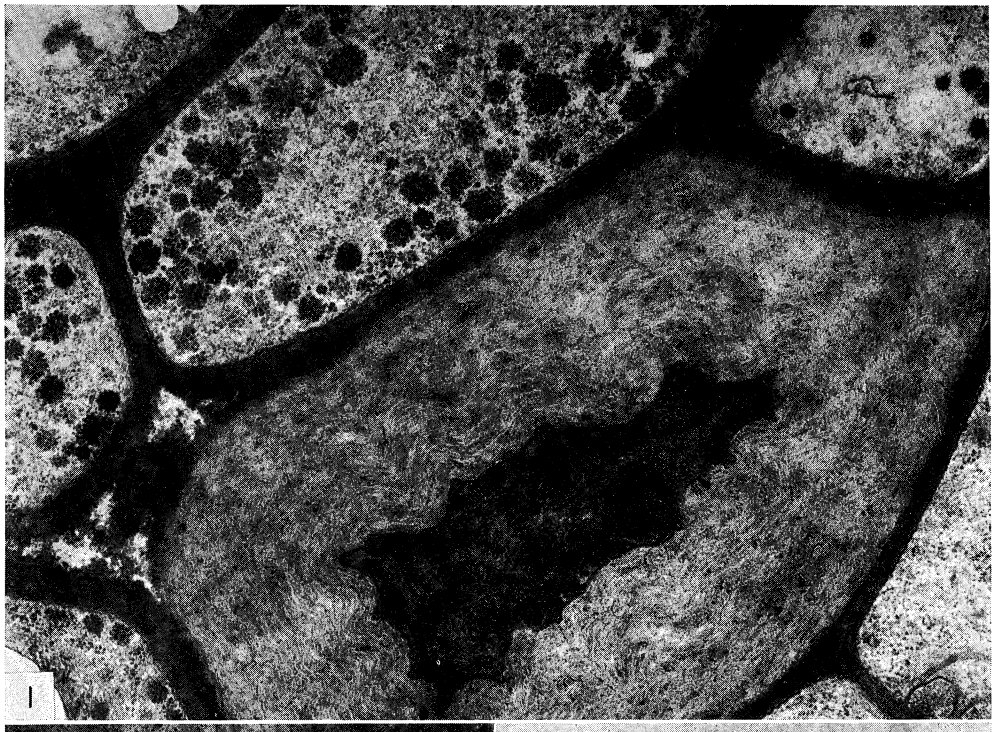
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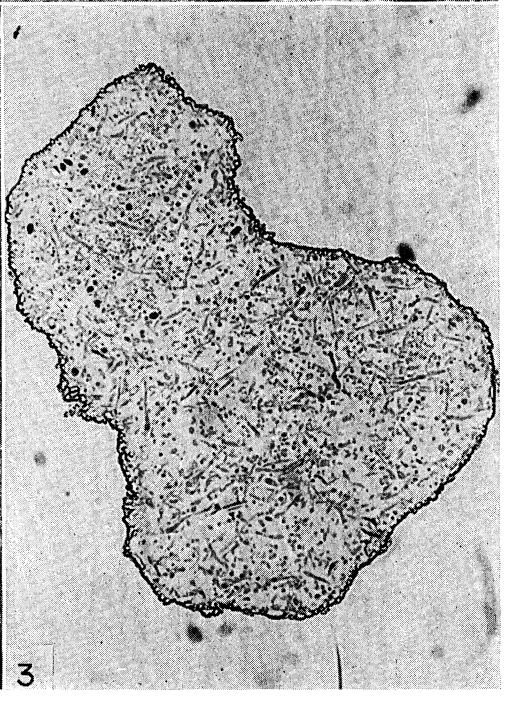
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EXPLANATION OF PLATES

Plate 1

No. 1. Section through hyphal cells showing dolipore septum and parthenosome (Pa), associated endoplasmic reticulum (ER) and vacuole (Va). × 30,000.

No. 2. Vesiculate lomasome (Lo) of hyphal cell in the space between the plasmalemma (P) and cell wall (Cw). × 42,200.

No. 3. Laminate lomasome (Lo) contained within the plasmalemma of a hyphal cell. × 42,200.

No. 4. Section through hyphal cell showing the layered wall and the plasmalemma. × 96,000.

No. 5. Submerged mycelium possessing inflated cells with refractive contents, a thick-walled cell and unaltered hyphae (culture slide preparation, positive phase contrast. × 850.

No. 6. Radial section of aerial sclerotium showing outer investing layer, rind and medulla (PAS-stained GMA section). × 420.

Plate 2

No. 1. Section of brown matting at the air/agar interface of a mature mycelium. Note the uneven thickening of the cell walls of the brown matting cells (Bmc), the cuticular material (Cm), the undifferentiated cells of the aerial mycelium, and the thick-walled cells (Twc) of the submerged mycelium. × 3850.

No. 2. Cells of the outer investing layer of the aerial sclerotium showing membrane remnants in cells without living contents. × 3400.

No. 3. Scanning electron micrograph of the surface of an aerial sclerotium showing the rind (R) and the hyphae of the outer investing layer (Ih). × 4200.

Plate 3

No. 1. Cells of the outer investing layer with their crenulate walls and associated vesicles (V). × 14,000.

No. 2. Section through surface of an aerial sclerotium showing the outer layer of cuticular material (Cm). The projecting cell probably forms part of the investing layer. × 17,250.

No. 3. Section through the rind of an aerial sclerotium. Note the cell arrangement and the cuticular material (Cm) which fills some of the intercellular spaces. × 4800.

No. 4. Medulla of the immature aerial sclerotium showing living thin-walled cells with interspersed dead cells. × 5000.

Plate 4

No. 1. Thin-walled cells of the medulla of an aerial sclerotium. Note the crystalline inclusion (X). The web-like structures in the vacuoles (Va) are probably fixation artefacts. × 12,500.

No. 2. Longitudinal section of a vegetative hypha showing nucleus (N) with nucleolus (Nc), mitochondria (Mi) and peripheral glycogen rosettes. × 12,000.

No. 3. Longitudinal section of a thick-walled cell of the medulla of an aerial sclerotium; compare with No. 2 × 4500.

No. 4. Section through dolipore septum between two thin-walled cells of the medulla showing parthenosome (Pa) and pore lip (Pl). × 60,000.

Plate 5

No. 1. Thick-walled cell with dispersed glycogen rosettes. Adjacent thin-walled cells have an essentially hyphal organization. × 22,000.

No. 2. Adjacent thin- and thick-walled cells communicating through a dolipore septum (Ds). Note the difference in cytoplasmic density between the two cells. × 12,000.

No. 3. Section of submerged sclerotium showing its irregular shape, the single cell layer of the rind and the loosely organized medulla. Compare with Plate 1 No. 6. × 75.