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MORPHOGENESIS OF AERIAL SCLEROTIA OF *COPRINUS LAGOPUS*

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SUMMARY

Aerial sclerotia originated from intercalary cells of aerial hyphae. Initially only a single cell was involved. Repeated branching from the initiation point formed the sclerotium initial which was a more or less spherical mass of undifferentiated, radially arranged cells. Accumulation of glycogen in cells towards the centre of the initial marked the commencement of maturation. Cells of the central (medullary) region went through a differentiation process which involved first a heavy accumulation of glycogen; the glycogen was then mobilized, its reduction in concentration being exactly correlated with the formation of a thick, hyaline, secondary wall. As the medulla developed, differentiation of localized areas of cells just within the margin of the initial occurred to form the protective rind layer. Wall thickening in rind cells was not preceded or accompanied by glycogen accumulation. Strains which failed to produce sclerotia behaved as stable variants, sclerotium non-production segregating as a single gene. Four such genes were recognized and some linkage information obtained.

INTRODUCTION

The Basidiomycete fungus *Coprinus lagopus* (= *C. cinereus*) produces two anatomically distinct types of sclerotium; a loosely organized form which arises in the submerged mycelium and a compact highly organized structure that is developed in the aerial mycelium (Waters, Butler and Moore, 1975). The aerial sclerotium is approximately 100–250 μm in diameter and consists of three basic tissue types arranged concentrically. The outermost layer of thin-walled dead hyphal cells is only loosely attached to the second layer, the rind of the main sclerotial body. The central region (medulla), totally enclosed by the rind, mostly contains thick-walled cells with a scattered minority of thin-walled cells.

The sclerotia of *Coprinus* have been reported to arise in a way which would represent a totally unique form of sclerotium initiation (Volz and Niederpruem, 1970). It was suggested that sclerotia were formed from already differentiated hyphal cells that can be readily found in the submerged mycelium. Our observations of sclerotium morphogenesis can not support these interpretations. Volz and Niederpruem (1970) used strains of *C. lagopus* closely related to those utilized by ourselves but it appears that they failed to distinguish between the two types of sclerotia and confused specialized cells of the submerged mycelium that are involved in glycogen storage (Madelin, 1960) with the sclerotial initials of the aerial mycelium.

This paper describes our light- and electron-microscope observations on the morphogenesis of aerial sclerotia and includes some preliminary data on the genetic control of the process.

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MATERIALS AND METHODS

The organism used was the Hymenomycete *Coprinus lagopus sensu* Lewis (= *C. cinereus* (Schaeff. ex Fr.) S. F. Gray). For the microscopic examination of the actively growing mycelium a culture slide in the form of a 1-mm wide trough constructed from two 22 × 50 mm coverslips was used. Other culture, analytical and microscopic techniques have been described previously (Waters *et al.*, 1975). Two monokaryotic wild type isolates (code numbered H1 and ZBw601) were used for following morphogenetic changes. A further forty-seven independently isolated wild types (Day, 1963) were examined for their ability to produce aerial sclerotia. Strains which failed to form sclerotia were examined genetically. Methods of genetic analysis closely followed those described by Lewis (1961) and made use of auxotrophically marked tester strains (which were first shown to be able to produce sclerotia) related to those described by Day and Anderson (1961) and Moore (1967). Progeny were inoculated individually to 9-cm Petri dishes of complete medium and then, to score sclerotium production or non-production, incubated for a total period of 65 days (10 days at 37° C followed by 55 days at 26° C).

RESULTS

Sclerotium morphogenesis

The earliest morphogenetic stages were followed by phase contrast microscopy of colonies grown in culture slides. Sclerotia originated from intercalary hyphal cells and initially only a single cell was involved. This cell was usually a member of an isolated hypha, though occasionally the parental hypha was one of a group of two or three parallel hyphae. The hypha and the cells of the hypha upon which sclerotium initials were formed were quite unremarkable.

Sclerotial inception began with the formation of lateral branches from the parental hyphal cell (Plate 1, No. 1) and repeated branching from this centre formed the sclerotium initial (Plate 1, Nos. 2–4). Plate 1, No. 5 illustrates the gross morphology of the initial and its radial symmetry. Further branches contributed to the developing structure and were of two sorts. The majority were less than 200 μm long and were restricted to the main body of the sclerotium (Plate 1, No. 4). The second type were less frequent and considerably longer (up to several mm). They passed from the initial into the surrounding mycelium and might therefore serve a conducting and/or anchoring function (Plate 1, Nos. 4 and 5).

The maturation of the initial could be followed only in sectioned material. The first stage in maturation was manifested in cells towards the centre of the initial. Most of these increased in diameter and in all there was a concomitant increase in the content of periodic acid-Schiff (PAS) reacting material. This accumulation was followed by thickening of the walls and as wall-thickening proceeded so the amount of PAS staining of individual cells was reduced, eventually to zero. Sclerotia of this intermediate stage of development had the overall structure shown in Plate 2, No. 2, the central region of the medulla containing fully differentiated thick-walled cells as well as thin-walled cells and other cells in various stages of differentiation. The process of medullary differentiation proceeded centrifugally, gradually invading the outer part of the immature sclerotium which until this time was still composed of essentially hyphal cells in their original radial configuration.

As the medulla continued to develop, the rind also began to appear, first as localized

areas of pigmentation just within the margin of the immature sclerotium. Cells in these regions formed the thickened and pigmented walls typical of rind cells. This wall-thickening was not accompanied by any increase in cell diameter, nor, more significantly, by any prior increase in the amount of PAS positive material detectable within the differentiating cells. Rind development continued as the walls of undifferentiated cells between the already differentiated areas began to thicken and become pigmented. The pigmented cuticular material filling some of the intercellular spaces was deposited during this period of rind development. Once a complete rind layer had been established the rind increased in thickness centripetally. Thus at this stage of development the main sclerotium body was made up of three layers (Plate 2, No. 1) the rind, the intermediate layer and the medulla. A fourth, external, layer composed of cells which lay outside the newly formed rind was also present (Waters *et al.*, 1975). The intermediate layer was composed of cells destined to form either rind cells or medullary cells in the final stages of maturation. Whilst the intermediate layer was present vestiges of the original radial symmetry still remained. But once these cells had differentiated the radial arrangement was maintained only in the rind layer. The final stages of sclerotium maturation were marked by the exudation of a fluid droplet. This soon disappeared leaving the mature sclerotium adhering to the parent mycelium but without any organized point of attachment.

Morphogenesis of the secondary wall

The first detectable change in the immature sclerotium as it entered upon maturation was an increase in the amount of PAS positive material in the centrally located cells, a change which was correlated with an increase in the numbers of rosette-like bodies seen in electron micrographs. The ultrastructural characteristics of the rosette-like bodies were similar in all respects to stored polysaccharides, particularly glycogen, found in many different cell types from many different organisms. Three methods widely used in animal histology for the detection of polysaccharides and glycogen were employed here; the PAS reaction, Best's carmine (BC) technique, and Gomori's silver method (GSM). These are considered to be the most reliable, sensitive and convenient methods available (Kugler, 1966; Pearse, 1968). When applied to sections of sclerotia PAS and GSM gave positive reactions in both cytoplasm and cell wall of medullary cells. On the other hand BC gave positive staining only in medullary cytoplasm. In all instances the staining of cytoplasm was particulate whereas that of the wall was diffuse. Enzyme digestion techniques using α-(endo)-amylase on serial frozen sections were also employed. In all cases cytoplasmic staining was abolished by the enzyme treatment. However, the PAS reaction continued to stain the walls of medullary cells even after enzyme treatment. It is concluded, therefore, that medullary cells accumulate a glycogen-like polysaccharide in their cytoplasm; for simplicity this will be referred to as glycogen in the following discussion. It is also clear that the thickened walls of medullary cells contain concentrations of a material which responds to some polysaccharide staining techniques but which is not susceptible to digestion by amylase.

Thin-walled hyphal cells which contained accumulations of glycogen were always associated with hyphal aggregations in which thick-walled cells were later produced. Fig. 1 summarizes the changes in the amount of glycogen present in the form of rosettes in medullary cells of maturing aerial sclerotia. The percentage of the medullary cell sectional area occupied by glycogen rosettes increased from about 2.5% to 20% (Plate 3). This increase was not accompanied by any increase in cell wall thickness although the

cytoplasmic contents did undergo some reorganization: the rosettes were no longer peripherally located but became distributed throughout the cytoplasm and the few large vacuoles were replaced by a larger number of smaller vacuoles some of which had electron dense contents (Plate 3, No. 1). Once the cellular concentration of glycogen reached 20% of the sectional area the number of rosettes fell and wall thickening could be detected (Plate 4, No. 1). At this stage the walls were about 0.25 μm thick and the glycogen rosette concentration about 10%. The cytoplasm was markedly more electron dense and elaborations of the plasmalemma were sometimes observed in these cells. The elaborations were of the vesiculate lomasomal type (Plate 4, No. 2) previously described in aerial hyphal cells (Waters *et al.*, 1975). In cells with walls about 0.5 μm thick, glycogen could be detected by PAS staining despite the fact that the characteristic rosette structure was now absent from electronmicrographs (Plate 4, No. 3). Although rosettes were not detectable the cells were filled with their 27–30 nm diameter subunits, the so-called alpha particles, and the cytoplasm was strongly electron dense in consequence. Elaborations of the plasmalemma were no longer apparent. The mature cells were usually between 3 and 7 μm wide and had walls between 0.5 and 3.0 μm thick. In such cells the cytoplasm was severely restricted and it was not unusual for a cell to have a lumen only 0.25 μm in diameter. Although so restricted, the electron dense cytoplasm still retained its integrity.

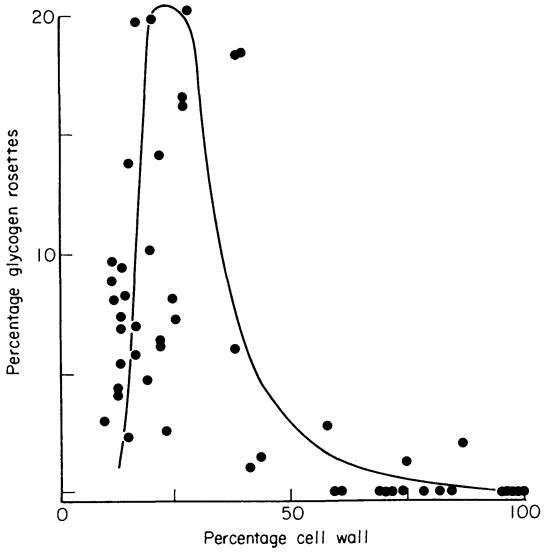


Fig. 1. Relationship between the number of glycogen rosettes in the cytoplasm and the thickness of the cell wall in differentiating medullary cells. The data for the figure were obtained from electron micrographs; the area of the section occupied by glycogen rosettes on the one hand and by the cell wall on the other hand were each expressed as a percentage of the total area of the cell.

Whilst the production of the thick-walled medullary cells described above involved a complex pathway of changes in cellular organization, no such pattern was evident in the formation of thick walls of rind cells. The changes were entirely restricted to the pigmented secondary thickening of the cell wall. PAS staining of these cells did not indicate the presence of glycogen although scattered rosettes were detectable by electron microscopy.

Genetic analysis of sclerotium formation

Among the set of independently isolated wild-type monokaryons initially examined five were found to be unable to produce sclerotia. These had the identification numbers H9, A3, B1, 2H1 and L1. Complementation tests were carried out by making dikaryons between the strains and assessing their ability to form sclerotia. Formation of sclerotia by the dikaryon indicates complementation between two independent genetic defects of the parental monokaryons. A full set of tests was possible between H9, B1, 2H1 and L1; A3 failed to dikaryotize with the others and is therefore excluded from this discussion. All of the dikaryons produced sclerotia. Similarly, all dikaryons made between H9, B1, 2H1 and L1 and the sclerotium-producing strain ZBw601 also produced sclerotia. Dikaryons made between H9 and some of its progeny of compatible mating type (i.e. homoallelic dikaryons which must, by definition, be non-complementing) failed to produce sclerotia. The conclusion is drawn, therefore, that each of the non-sclerotiating strains represents a recessive 'mutation' in a different gene. The genes have been given the symbol *scl* (*scl*erotium) and are individually identified as follows: *scl*-1, strain H9 and its derivatives; *scl*-2, strain B1; *scl*-3, strain 2H1; *scl*-4, strain L1. Linkage tests were carried out by crossing different *scl* strains with one another and with known auxotrophic markers; the data obtained are summarized in Table 1. Strain L1 was unique in that although it regularly formed vigorous dikaryons none of these formed fertile sporophores. Characteristically L1 dikaryons produced sporophore initials which successfully developed into primordia but then aborted, to be replaced by a new crop of initials which behaved similarly. Consequently no linkage data was obtained for *scl*-4. The behaviour, though, may represent a pleiotropic expression of the *scl*-4 defect and therefore indicate a close relationship between sclerotium and sporophore morphogenesis. The data of Table 1 are sufficient to show that (*a*) the inability to produce sclerotia acts as a genetic character which segregates in each case as a single gene; (*b*) *scl*-1 and *scl*-2 are linked and are members of linkage group III; (*c*) *scl*-3 is distantly linked to linkage group IV.

Table 1. *Results of crosses carried out to test for linkage between* scl *genes and known auxotrophic markers*

Genes involved	Linkage group of auxotrophic marker	Total Progeny	Total Recombinants	% recombination
scl-1 × *scl*-2	–	84	24	28.6*
scl-2 × *scl*-3	–	112	54	48.2
scl-1 × *ad*-5	II	142	57	40.1
scl-1 × *me*-8	V	147	79	53.7
scl-2 × *ad*-5	II	131	64	48.9
scl-2 × *pdx*	III	99	26	26.2*
scl-2 × *me*-8	V	125	52	41.6
scl-3 × *ad*-5	II	86	45	52.3
scl-3 × *pdx*	III	64	30	46.9
scl-3 × *me*-8	V	64	38	59.4
scl-3 × *nic*-4	IV	89	25	28.1*
scl-3 × *ad*-1	IV	74	24	32.4*
scl-3 × *me*-9	IV	108	33	30.6*

* Significant deviations from random segregation indicating linkage.

Discussion

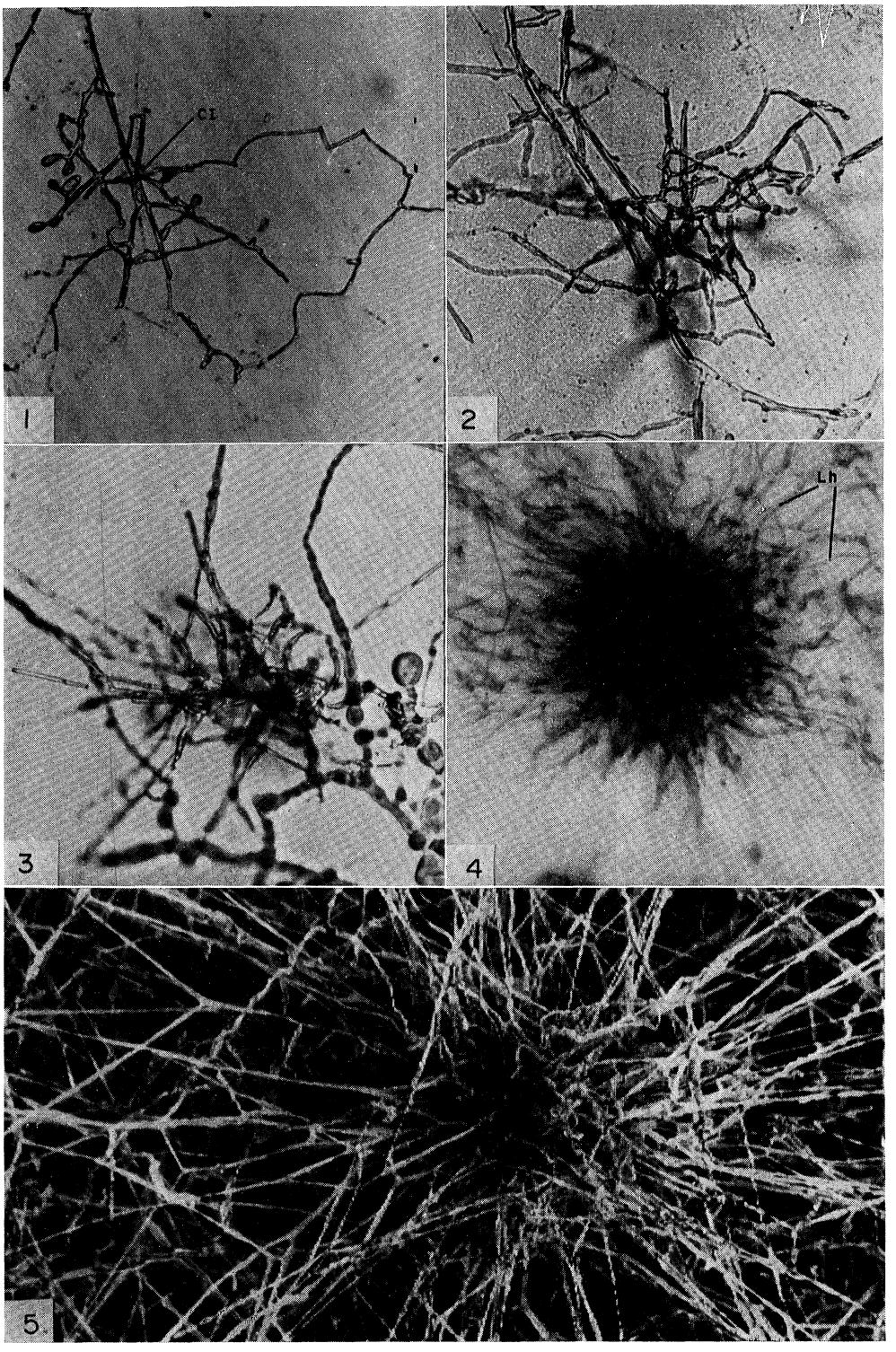
The first macroscopic indication of sclerotium formation in *Coprinus lagopus* was the appearance of more or less spherical tufts of unpigmented hyphae. These hyphal tufts have often been recorded in studies of sclerotium morphogenesis (Neal, Webster and Gunn, 1934; Townsend and Willetts, 1954; Raper and Fennell, 1965). Indeed, where

such hyphal tufts do not occur a completely different type of sclerotial structure results —as in polyporaceous parasites of trees (Campbell and Munson, 1936)—which is very like the submerged sclerotium of *Coprinus* (Waters *et al.*, 1975). A general conclusion from this and other investigations is that the hyphal tufts which represent immature sclerotia are formed by a process of repeated branching and increased septation. In *Sclerotium rolfsii* (Townsend and Willetts, 1954) the period of branching is also accompanied by hyphal fusions; such fusions were not observed at this stage of development in *Coprinus lagopus*. The immature sclerotia of many fungi consist essentially of vegetative hyphal cells in a more or less random arrangement (Townsend and Willetts, 1954); but in *C. stercorarius* (Brefeld, 1877), *Sclerotium cepivorum* and *Coprinus lagopus* there is clear evidence that the hyphal elements of an initial all radiate from a central point. In fact our observations on *C. lagopus* indicate that a single hyphal cell initiates the entire process. The close comparison with early events in sporophore morphogenesis (Matthews and Niederpruem, 1972, 1973) is striking and, like the behaviour of *scl*-4 described above, suggests a close relationship between the early stages of these two outwardly dissimilar developmental processes.

The pattern of development described here is in no way similar to that described by Volz and Niederpruem (1970). These authors describe isolated bulbous cells which increase in number to form aggregations, some of which become sclerotia when peripheral cells differentiate into a rind layer. Although Volz and Niederpruem (1970) clearly state that sclerotia can be formed within the aerial mycelium as well as in the submerged mycelium, there is no evidence that they recognized the profound differences in sclerotium structure which are so clearly apparent between these different locations.

The most remarkable intracellular changes observed during sclerotium maturation centred on the relationship between glycogen accumulation and the secondary cell wall in medullary cells. An increase in the amount of glycogen in these cells would not be surprising. Such accumulations have been noted in ageing vegetative hyphal cells of both submerged and aerial mycelium (Madelin, 1960; Marchant, Peat and Banbury, 1967) and Madelin's study indicated that in the dikaryon such reserves were mobilized to support development of the sporophore. It would not be unexpected, therefore, for the cells of a perennating structure like a sclerotium to accumulate polysaccharide reserves to be utilized when the structure eventually germinates. The disappearance of the 'reserve polysaccharide' and the exact correlation of this disappearance with the synthesis of an extremely thick and unusually structured secondary cell wall is, though, an unexpected aspect of sclerotium maturation. Although numerous differentiating medullary cells were observed no specialized cell organelles which could be directly implicated in this unusual sequence of events were seen.

The very unusual structure of the secondary wall suggests it might serve some special function. A protective function is unlikely in view of the fact that the mature medulla is totally enclosed by a multilayered rind which has cuticularized intercellular spaces (Waters *et al.*, 1975). That the secondary wall itself serves as a long-term polysaccharide storage organ is suggested by the events which accompany its formation and by the fact that the mature medulla consists almost entirely of living cells with these secondarily thickened walls. The implication of our observations is that in the sclerotium of *Coprinus* glycogen serves only a transitory storage function; the long-term storage of polysaccharide being the role of the macrofibrils of the secondary wall of medullary cells. Investigations of sclerotium germination and the role of glycogen metabolism in sclerotium formation are underway.



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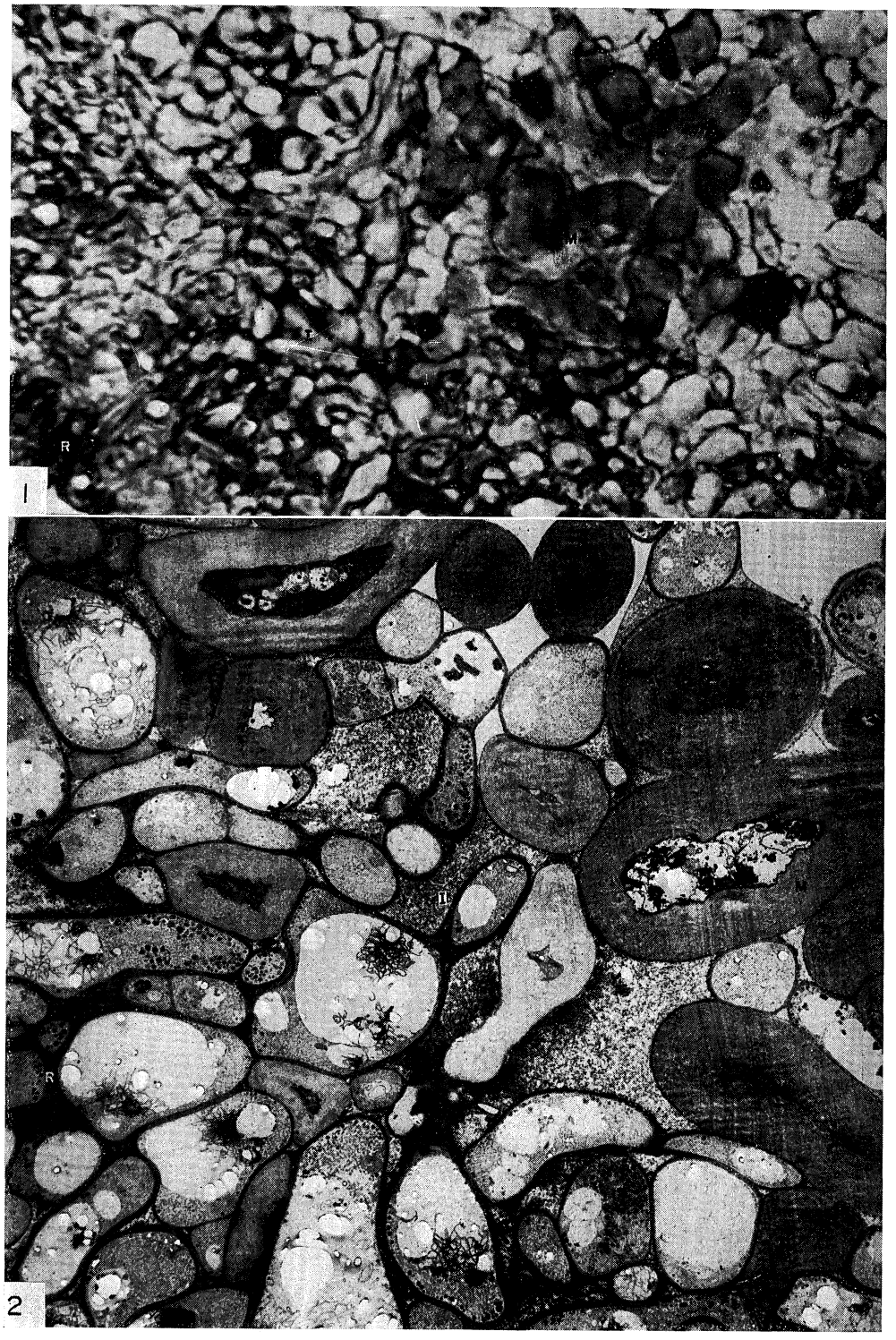

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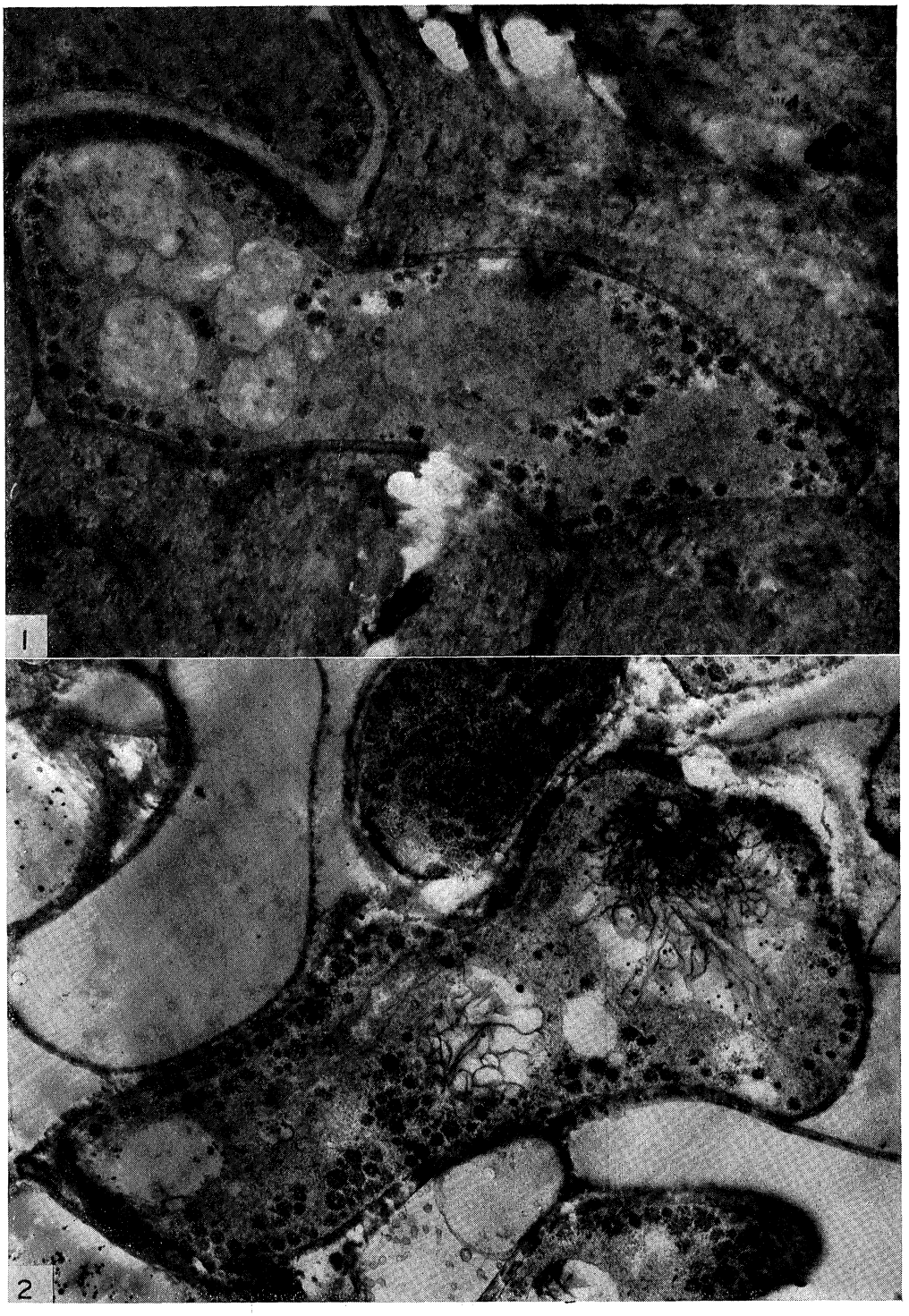

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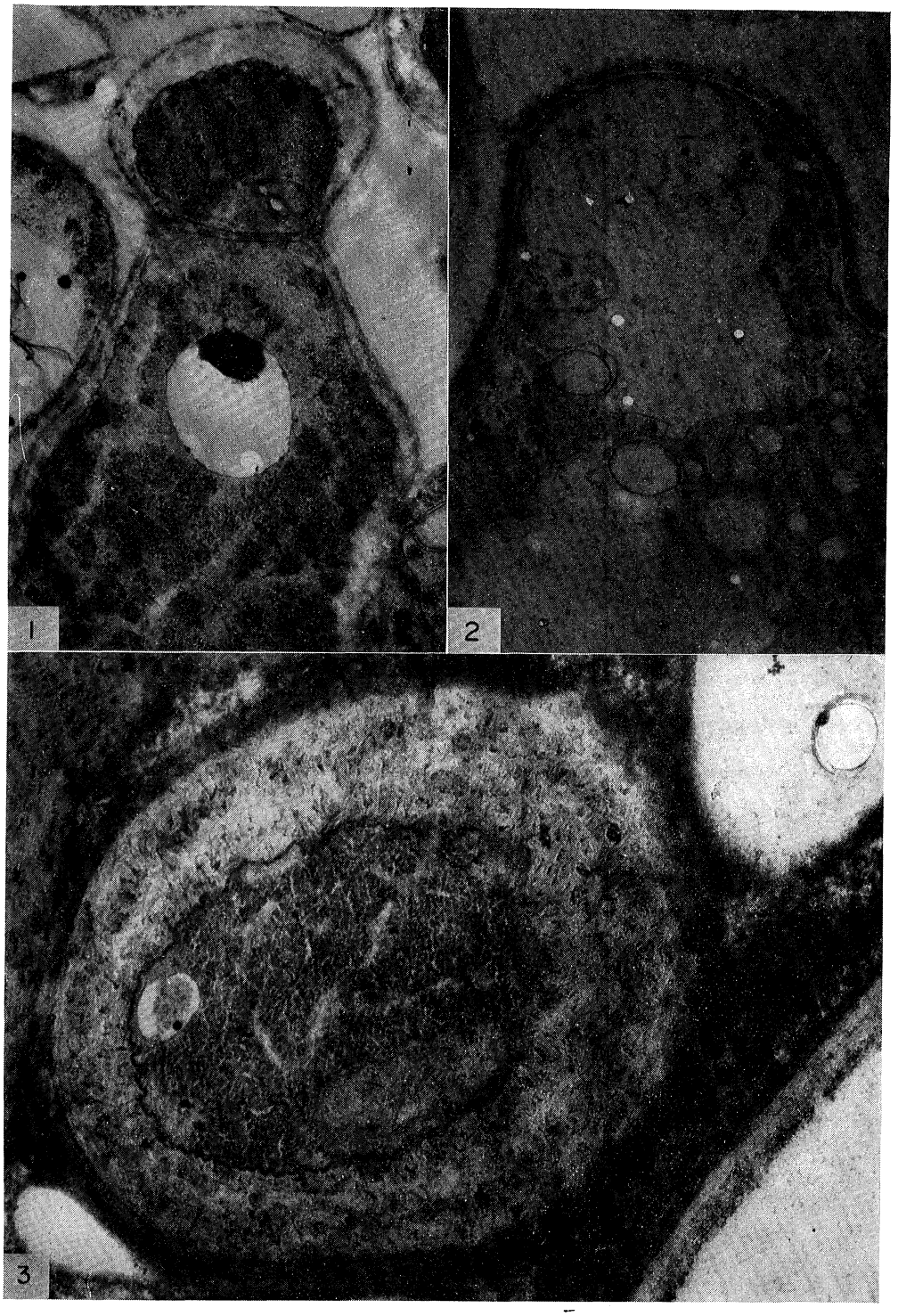

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Explanation of Plates

Plate 1

Nos. 1–5. Culture slide preparations of developing sclerotia.

No. 1. Early stages showing the centre of initiation (CI). × 200.

No. 2. Increased size of structure as branching proceeds. × 300.

No. 3. Very immature sclerotium. × 300.

No. 4. Immature sclerotium showing main body with hyphae of determinate growth and the radiating 'long hyphae' (Lh). × 300.

No. 5. Scanning electron micrograph of immature sclerotium at a stage similar to that shown in No. 4. × 800.

Plate 2

No. 1. Sclerotium at the three-layer stage showing the developing rind (R), the intermediate layer (I), and the developing medulla (M). × 1500.

No. 2. Section of sclerotium at the three-layer stage showing the rind (R), the thick-walled cells of the medulla (M) and the thin-walled cells of the intermediate layer (I). × 3750.

Plate 3

No. 1. Thin-walled cell from the medulla of a maturing sclerotium with a glycogen rosette concentration of about 2.5%. × 15,000.

No. 2. Thin-walled cells from the medulla of a maturing sclerotium. The larger cell has a glycogen rosette concentration of about 3% whereas the concentration in the smaller cell is about 20%. × 10,750.

Plate 4

No. 1. Wall thickening is becoming evident in these cells where the glycogen concentration is high but the rosette configuration is no longer apparent. × 15,000.

No. 2. Thin-walled medullary cell showing vesiculate lomasomes. × 20,250.

No. 3. Thick-walled medullary cell. Note the absence of discrete glycogen rosettes. × 40,000.