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ONTOGENY OF APOTHECIA OF *SCLEROTINIA SCLEROTIORUM* (LIB.) DE BARY

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R I N G K A S A N

Pertumbuhan morfologi dan struktur dari pada "apothecia" Sclerotinia sclerotiorum (Lib.) de Bary telah dipelajari dengan berbagai cara.

Apothecia dibentuk dari sel-sel bakal yang letaknya di dalam sclerotium, terdiri dari sel-sel kecil (kira-kira bergaris tengah 0,5 - 2,5 µm), berdinding tipis, dan berkaitan satu dengan yang lainnya sehingga membentuk parenkima semu. Setelah kira-kira 20 hari, maka sel-sel bakal tersebut akan muncul kepermukaan sclerotium. Mula-mula sekali merupakan penonjolan, berwarna kuning kecoklat-coklatan, kemudian berubah menjadi bentuk batang dengan ujung yang sedikit meruncing dan pertumbuhannya biasanya mengarah ke sumber cahaya. Selanjutnya bagian ujungnya berubah menjadi bentuk cawan yang melebar (apothecia).

A B S T R A C T

The morphogenesis and structure of apothecia of Sclerotinia sclerotiorum (Lib.) de Bary were studied by a number of different techniques.

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Apothecia develop from sclerotium initials which consist of small, thin-walled, closely fitting generative hyphae cells (about 0.5 - 2.5 µm in diam.). The initials grow within the sclerotium and after about 20 days became apparent from the surface of the sclerotium as small protuberances. After emergence from the sclerotium the initial was light buff-colour, rod-shaped and slightly pointed at the tip with the direction of growth usually being towards the light source. From this, funnel-shaped top developed and its ultimate expansion into the disk-like cap (apothecia).

INTRODUCTION

There have been many morphological studies of the development of apothecia (Ezekiel, 1921; Godfrey, 1923; Drayton, 1934 and 1937; Henson and Valleau, 1940; Lane and Sproston, 1955; Bedi, 1956, 1962 and 1963; Terui and Harada, 1969a and b; Elliott, 1965; Bergquist and Lorbeer, 1972). Much of the data that is available on the morphogenesis of the apothecium of *Sclerotinia sclerotiorum* has come from general studies of the fungus or by assuming that its ascocarp develops in the same manner as those of other Discomycetes that have been studied. The report by de Bary (1887) of the formation of the apothecium of *S. sclerotiorum* from a small "centrum" within the sclerotium appears to be the first reference to early initiation of the ascocarp. Only a few workers (Björling, 1951) have referred to the centrum since this early report; most observations have been on the extra-sclerotial growth of the apothecium.

This project was undertaken to obtain morphological details of the development of the apothecium of *S. sclerotiorum* from its early initiation within the sclerotium to the formation of the mature ascocarp.

MATERIALS AND METHODS

All experiments were conducted in the laboratory of Plant Pathology, University of New South Wales.

The culture of *Sclerotinia sclerotiorum* used in this study was originally isolated from diseased french beans. It was grown in Petri dishes, each containing potato dextrose

agar medium (PDA). The inoculated agar plates were incubated at 18° - 20°C.

Several different techniques were also studied to determine the best way of producing apothecia on sclerotia. Purdy's method (1967) was found to be very effective and was used in this experiments. Also water agar was used instead of water.

Two month old sclerotia of *Sclerotinia sclerotiorum* growing on PDA were transferred to water agar in Petri dishes under aseptic conditions and incubated at 15°C under continuous light about 15 lumens/sq.ft. Apothecial initials were apparent on the surfaces of the sclerotia after about three weeks and matured about 10 to 15 days later. When initials were formed in the medium, the stipes sometimes continued to grow into the agar for several days and then became darkly pigmented. The pigment often accumulated in the medium around the stipe and growth of the latter ceased. However, most stipes were able to grow through the medium until they reached the surface and normal discs then developed above the substrate.

Apothecia at different stages of development were collected, measured and fixed in FAA (Formalin, 5; acetic acid, 5; 50% alcohol, 90 v/v) for storage.

Thin sections were cut of sclerotia in which apothecial initials were developing and the sections were examined with light and transmission electron microscopes to determine the type of tissues from which the primordia had developed. Thin and thick serial sections of matured sclerotia were also examined to determine the distribution of the nests, and some nests were dissected out from the sclerotial tissue to determine whether there is any structure resembling archegonia, trichogyne, phialides or microconidia in the initials or in the adjacent sclerotial hyphae.

For microscopic examination material was either frozen and sectioned in a freeze-microtome or embedded in paraffin or in araldite and permanent preparations made. Dehydration was by means of a tertiary butyl alcohol series (Johansen, 1940). Longitudinal sections 12 µm thick were cut, deparaffinized in an alcohol series, stained with iron-alum-haematoxylin or Alcian blue and Orange G and mounted in Canada balsam.

RESULTS

Apothecial initiation

The first external evidence that apothecia were developing, was the appearance of black, shining, raised areas, about 1/2 mm. in diameter, on the surfaces of the sclerotia. These

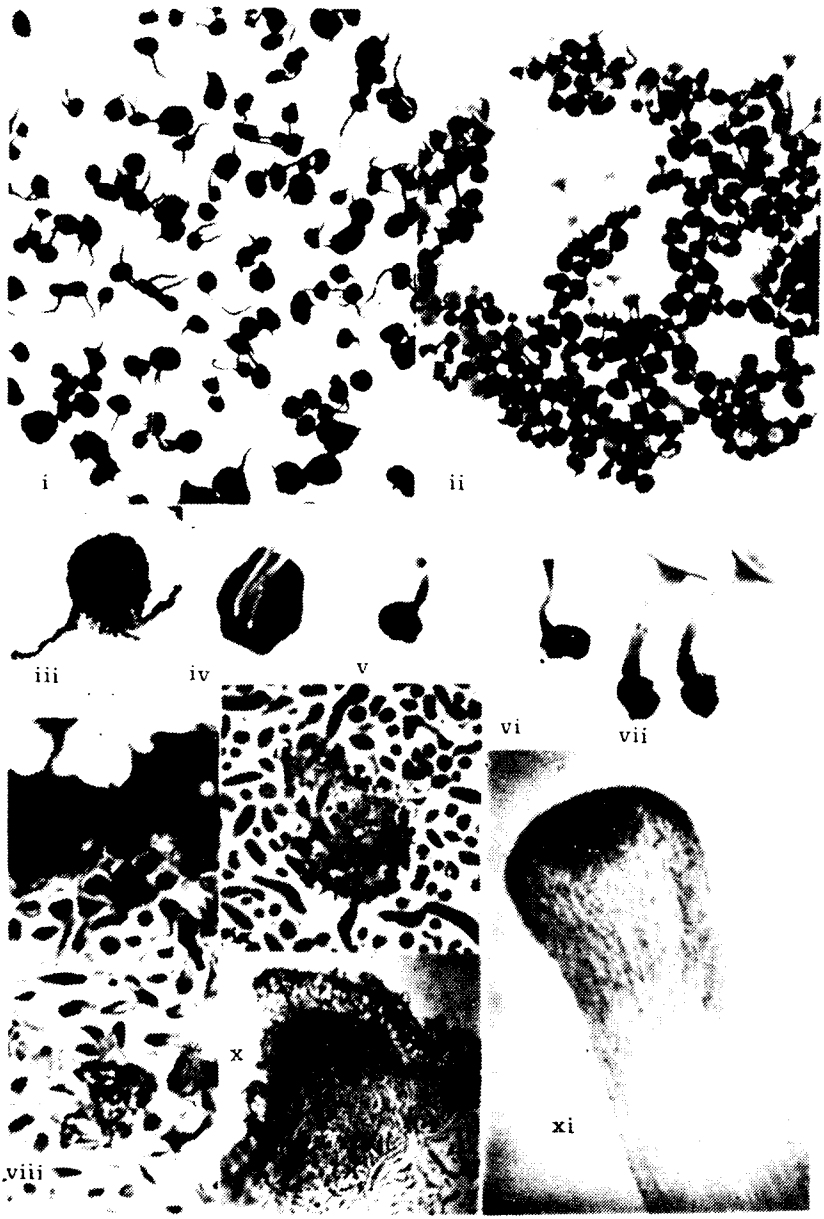

Figure 1

Fig. 1. i. Germinated sclerotia of *S. sclerotiorum* in a dish.

ii. Apothecia of *S. sclerotiorum* on sterilized distilled water.

iii. Section of a young apothecial fundament which resembles a minute bulb.

iv. Elongated, young, apothecial fundaments.

v. The depression of a young apothecium prior to expansion.

vi. A young apothecium about to expand.

vii. Mature, cup-shaped apothecia on sclerotia.

viii. The nest cells in a sclerotium of *S. sclerotiorum*.

ix. Section of nest cells after the initial has started to grow.

x. Section of an apothecial primordium of *S. sclerotiorum* in the process of breaking through the sclerotiorum.

xi. Longitudinal section of a young apothecium showing the sheath cells at the top of the depression; note their smaller size compared with the other hyphae.

small protuberances differentiated into apothecia when conditions were suitable.

Nests (< 40 µm diam.) of small, thin-walled, closely fitting pseudoparenchymatous cells with dense contents were observed in the cortical and medullary regions of the sclerotia (Fig. 1.viii and ix). The nests were numerous in some sections. The majority were formed near the surface of the sclerotium but occasionally some were observed almost in the centre of the medullary tissue. Most of the small clusters of cells were brown in colour with the intensity of coloration very variable, but all were very prominent when examined under low magnification. Closer microscopic observations of the sections revealed smaller nests in which the cells were hyaline and therefore not obvious. The brown pigmentation is similar to that obtained when oxidized polyphenols accumulate in fungal tissues. Mechanical damage of mycelium of some members of the Sclerotiniaceae causes this type of discoloration; sectioning of the sclerotia and exposure to air could be responsible for pigmentation in regions in which there is high activity of polyphenol oxidases (tyrosinase and laccase). Therefore, some sclerotia were fixed in FAA before sectioning so that the enzymes were inactivated. It was found that most of the nests remained hyaline when cut after fixation although some dark areas were still apparent. Possibly the hyphae in these deeply pigmented nests had previously undergone autolysis and were not viable

Using the light microscope it was difficult to distinguish any particular pattern in which the closely interwoven, thin-walled hyphae of the nests were arranged. Although the nest hyphae were different in appearance from the sclerotial hyphae, no structures resembling archegonia, trichogyne, phialides or microconidia were observed in the initials, nor in the adjacent sclerotial hyphae.

Figs. 2i and ii show the appearance of an apothecial initial when viewed with a transmission electron microscope. The diameters of the hyphae of the initial were smaller (0.5 - 2.5 µm) than those of the sclerotial hyphae (5 µm); the hyphae tended to be arranged parallel to each other and were not as interwoven as the sclerotial hyphae. Most of the latter contained many lipid bodies while the former had only a few. In both sclerotial and initial hyphae there were small electron-dense structures which were not identified. Sometimes they were present within the lipid bodies and they may have been formed by the hydrolysis of the fatty material in the globules.

Active division of the cells forming the nests produced a knot of closely interwonen hyphae, each hypha about 1 - 2 µm in diameter and containing dense cytoplasm. Within 20 days the young initials became visible from the surface of the

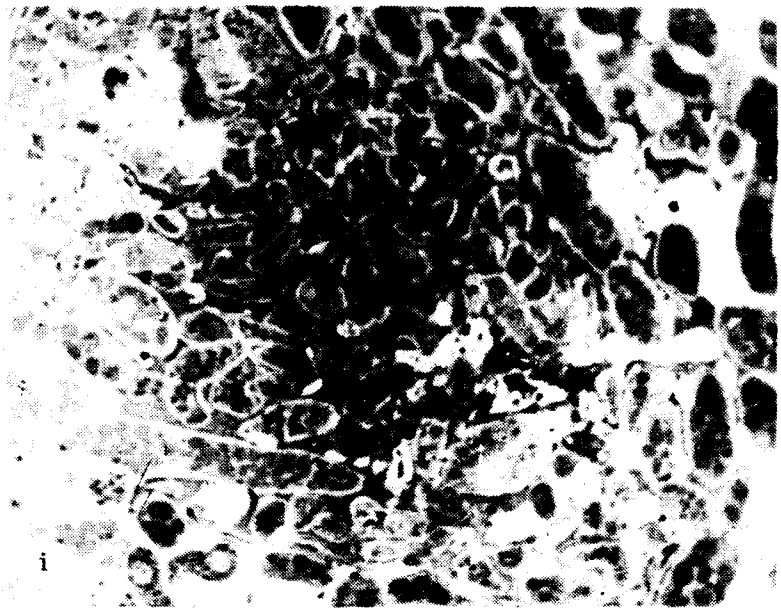

Fig. 2.i. Ultrathin section through the nest cells, showing the differences between the nest cells, and the other sclerotial hyphae. x 9.000.

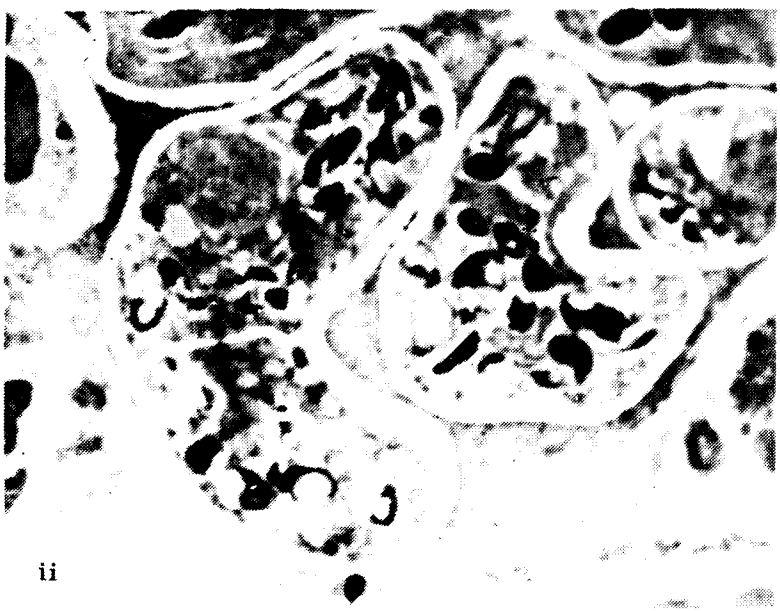

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Fig. 2.ii. Enlargement of the nest cells. x 14.500.

sclerotium as small protuberances (Figs. 1.i and iv); a section of one of these is shown in Figs. 1.iii and x. The continued multiplication and growth of the hyphae within the sclerotium ruptured the rind and a young stipe which was light buff in colour and had a slightly pointed tip, emerged from the surface. The torn rind could be clearly seen at the base of the stipe (Figs. 3.ii and iii).

Later development of the apothecium

When the apothecial initial had emerged from the sclerotium the hyphae of the primordium became elongated, flattened and were of smaller diameter (about 1 - 2 µm) than the sclerotial hyphae (Fig. 1.x). The stipe grew in length, the direction usually being toward the light source. If the cultures were incubated in the dark, the stipe continued to elongate for a time and then growth ceased. Aborted stipes up to 50 mm in length were observed. Under illumination, the stipe grew to a length of about 7 mm and a depression appeared at the tip (Figs. 1.v, vi, xi and 3.iv). Sometimes very short stipes bore mature discs. The hyphae lining the depressed area formed a palisadelike tissue of long narrow hyphae. There were young paraphyses and at first they were not exposed to the atmosphere since the margins of the depression were inverted and a thin membrane covered the surface. As the disc enlarged, the protecting membrane ruptured exposing the well-developed paraphyses. The latter were long (100 µm) compared with their diameter (1 - 2 µm), slightly swollen at their tips, multinucleate, sparsely septate and occasionally branched at their bases (Fig. 3.vii).

The tips of ascogenous hyphae appeared in the subhymenium at the time the membrane broke; these hyphae were approximately 2 µm wide and the tip cells were binucleate. From their tips minute croziers developed and there was fusion of the two nuclei in the penultimate cell. The young asci elongated and pushed upwards between the paraphyses. The fusion nucleus increased in size and was usually found near the tip of the ascus. In the young ascus the cytoplasm was very dense and the tip was narrow and pointed. As the ascus elongated, the cytoplasm became vacuolated with most of the cytoplasm at the tip so that the basal part was almost devoid of stainable contents. As the young ascus elongates it has been shown in other Discomycetes that the fused nucleus passed through one meiotic and two mitotic divisions. Eight nuclei were distinguished at the end of the divisions. Daughter nuclei produced by mitotic division and a clear area which mark the position of the spindle could be seen clearly. Each nucleus with some cytoplasm and the development of a wall became an ascospore.

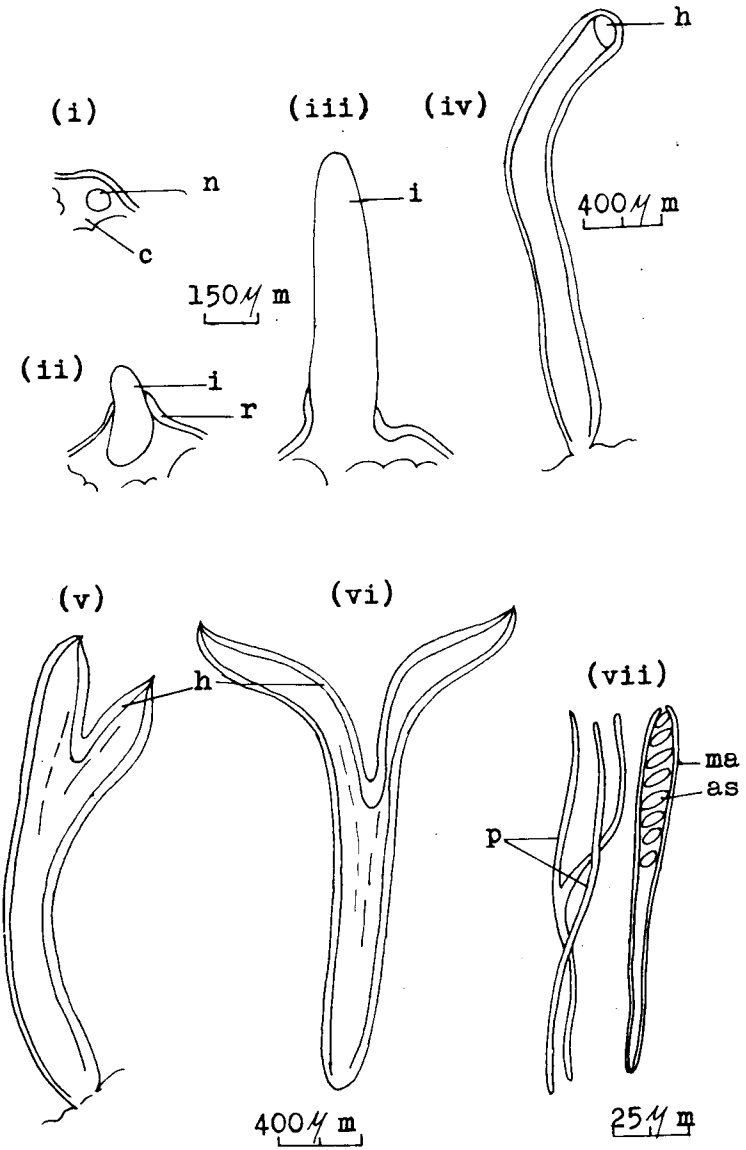

Figure 3

Fig. 3. Developmental stages of apothecia of *S. sclerotiorum*.

i. Very young apothecial initial consisting of a nest of hyphae (n) in the cortex (c) of a sclerotium.

ii. The initial (i) after rupturing the sclerotial rind (r).

iii. An elongating initial with the tip slightly pointed and no evidence of disc differentiation.

iv. Very early stage of disc differentiation with the hymenium in a depression at the tip of the young apothecium.

v. Longitudinal section of a young apothecium showing the hymenial region (h).

vi. Longitudinal section of a mature apothecium with the hymenium exposed by expansion of the disc.

vii. Paraphyses (p) and a mature ascus (ma) containing ascospores (as).

When fully differentiated the asci were 112.0 - 120.0 µm long, i.e., the same length as the paraphyses, clavate-cylindrical, tapering gradually to their bases. Each ascus contained eight ascospores arranged linearly and approximately 9.5 - 15.0 x 4.5 - 6.0 µm in size.

Structure of mature apothecium (Fig. 4)

(a) The stipe or stalk.

The ectal layer of the stipe consists of closely compressed textura porrecta to textura intricata, with occasional tomentum hyphae. Inside this layer, the hyphae are much more elongated and more or less parallel in arrangement, septate and mostly unbranched (Figure 4.v). Presumably these hyphae are translocatory and function in the transport of the nutrient from the sclerotium to the head of the apothecium.

(b) The disc or head (Figure 4.ii).

Axial sections of the mature apothecium reveal that the hymenium is 160.0 - 190 µm thick, subhyaline to light brown. The asci are inoperculate, cylindrical, and narrowed below to a short stalk, rounded at the apex. The latter is thickened and possess an iodine-positive pore.

Ascospores are hyaline, ellipsoid and smooth, usually biguttulate to triguttulate, uniseriate and oblique.

Paraphyses are filiform, septate, simple or branched, 1 - 2 µm in diameter and slightly enlarged toward the tip.

The ectal excipulum is about 45.0 - 65.0 µm thick, subhyaline to light brown, of textura angularis to textura prismatica, projecting to form hyaline smooth hair-like processes, septate, approximately 5 µm wide and rounded at the tip (Figs. 4.ii and iv).

The medullary excipulum is 140.0 - 175.0 µm thick, hyaline, of textura intricata to epidermoidea, hyphae smooth to granulate roughened, more or less parallel to the outer surface of the disc, branched, 3.5 - 5.2 µm wide, septate, developing at the margin of the disc a palisade up to 60 µm thick of paraphysis-like compact, parallel hyphae, up to 4.5. µm wide, light brown.

DISCUSSION

Apothecia of this isolate of *S. sclerotiorum*, have been obtained in our laboratory from single ascospore, cultures

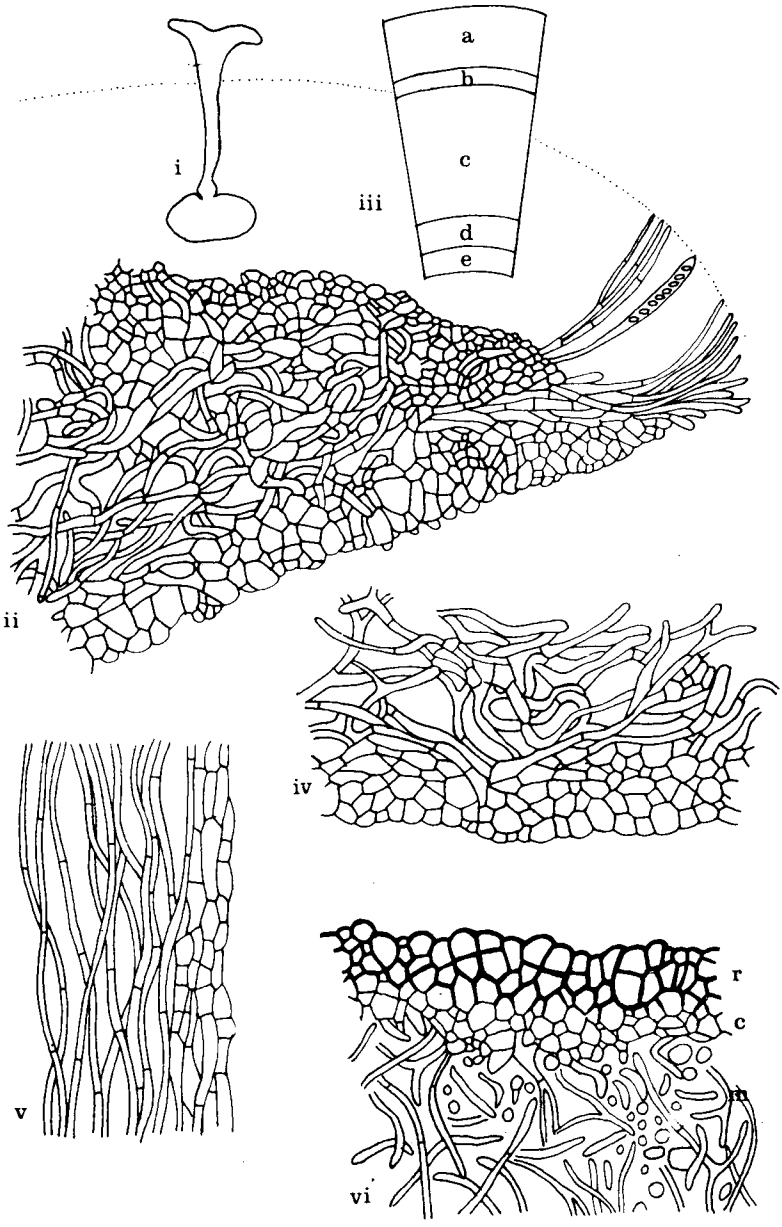

Figure 4

Fig. 4. Camera lucida drawings of sections of the sclerotium and of the apothecium.

i. Whole apothecium arising from a sclerotium. ca. x 4.

ii. Median longitudinal section of apothecium at margin, showing hymenium, medullary excipulum, and extal excipulum. x 125.

iii. Diagrammatic representation of relative distribution of tissues at midpoint between margin and stipe, from top to bottom; hymenium (a), subhymenium (b), medullary excipulum (c), inner ectal excipulum (d), outer ectal excipulum (e). x. 60.

iv. Portion of cup showing outer excipulum and medullary excipulum. x 125.

v. Median longitudinal section through portion of stipe showing inner cells and outer cells. x 125.

vi. Cross section of portion of sclerotium showing rind (r), cortex (c) and medulla (m). x 40.

(Wong, unpublished) indicating that the fungus is self compatible. There were no indications during this investigation of the factors that are responsible for the differentiation of hyphae into apothecial initials in certain areas of the sclerotium. In many Ascomycetes it has been found that ascocarp development starts after the fusion of spermatized cells and patterns of spermatization in this group of fungi have been recently reviewed by Kamat and Pande-Chiplonkar (1971).

Microconidia (spermatia) of *S. sclerotiorum* are formed in clusters on the surfaces of sclerotia and on adjoining mycelium but none were observed within sclerotial tissue. Also there were no indications of hyphae joining the very young apothecial initials with the surface of the sclerotium and along which nuclear movement could take place. Possibly, spermatization could take place by the union of microconidia with physiologically specialized hyphae on the surface of initials after the elongating "stipe" has ruptured the rind but there is no evidence to support this.

Björling (1951) described the cytology of the apothecium of *S. trifoliorum* and he suggested that microconidia of this fungus do not function in spermatization, as it is self fertile and its dikaryotic phase arises directly from vegetative homokaryotic hyphae. Heuberger (1934) did similar work for *Monilinia fructicola*. No workers have been able to show that the microconidia of these fungi are functional.

Even when material was embedded in paraffin it was difficult to obtain good sections for light microscope observations of apothecial initials. This was mainly due to tearing of the small, fragile nest cells when cut and their irregular arrangement and interweaving. Thus it was difficult to study the cytology and nuclear behaviour in the initial and the hyphae adjacent to it. Better preparations were obtained from material embedded in araldite (Figures 2.i and ii). The sclerotial hyphae adjacent to the initials are very rich in lipid reserves which, presumably, are hydrolysed to provide nutrient and energy for apothecial development.

There appears to be some close similarities between the early stages of perithecial development of *Hypoxylon* sp. as described by Parguey-Leduc (1972) and the observations made in this investigation on the morphogenesis of apothecium of *S. sclerotiorum*. In the medulla of the stroma of *Hypoxylon* perithecial initials form, each one consisting of a big uninucleate, pigmented globose cell and several other cells which differentiate to form the wall of the perithecium. The large cell or Woronin hyphae becomes elongated to form a trichogyne but fusion with another cell was not observed. The cell becomes binucleate but Parguey-Leduc regarded this as being by means of mitosis. The nuclei of the Woronin hypha divide and from the latter, asci develop. In the apothecial initials of *S. sclerotiorum* the development of asci does not

take place at the site of initiation but in the apothecial disc which develops exogenously. Possibly *Sclerotinia sclerotiorum* has dispensed with the need for spermatization or plasmogamy for the complete differentiation of the ascocarp; subsequently trichogynes are not produced and the microconidia have become non-functional.

The later stages of development of apothecia have been well documented for a number of the Ascomycetes (Wormald, 1921; Ezekiel, 1924b; Harrison, 1928; Whetzel. 1929 and 1945; Drayton, 1934 and 1937; Gregory, 1941; Elliott, 1962 and 1966). No obvious departures from the data given by these workers were observed.

Almost all the data available on apothecial structure are on the general shape and size of the apothecia, asci, ascospores and paraphyses. This has been emphasised by Korf and Dumont (1968) and Dumont (1973) who regard the sterile tissues as being of diagnostic value in the taxonomy of the Sclerotiniaceae. At present they are in the process of reappraising the taxonomy of *S. sclerotiorum* and related species. The isolate of *S. sclerotiorum* used in this investigation has apothecia in which the ectal excipulum is in the form of well developed textura angularis tissue and at the base this becomes textura prismatica. The medullary excipulum consists mainly of textura intricata which is textura angularis at the base of the hymenium. The outermost region of the disc is of textura prismatica tissue and the inner regions are of textura porrecta.

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