Morphology, Cytology, and Physiology of *Sclerotinia* Species in Culture

Duane Le Tourneau

Department of Bacteriology and Biochemistry, University of Idaho, Moscow, 83843.
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Growth and morphogenesis of fungi may be studied conveniently by using culture media in which various nutritional and non-nutritional factors can be controlled. The life cycle of *Sclerotinia* spp. can be completed on a variety of artificial and synthetic media. As a result, numerous articles reporting various aspects of growth and morphogenesis of *Sclerotinia* spp. have been published.

This presentation will be limited to studies on the formation and germination of sclerotia of *S. sclerotiorum* and closely related fungi. These topics are of interest to plant pathologists because disease control might be possible if the life cycle of the fungus could be broken by preventing the formation or germination of sclerotia. Where possible, I will cite recent papers from which interested readers may obtain references to the earlier literature. Additional references are available in the bibliography, "World Literature of *Sclerotinia sclerotiorum*, Related Species and the Diseases They Cause", which is available from the Plant Protection Institute, U.S. Department of Agriculture, Agricultural Research Center, Beltsville, MD 20705.

When reviewing the results of studies of *S. sclerotiorum* in culture, it is necessary to recognize the high degree of variability among isolates or strains of the fungus. Morrall et al (22), for example, grew 114 isolates of *Sclerotinia* spp. collected from 23 hosts in Saskatchewan on a glucose-salts agar medium. Large variations were noted in numbers, shape, size, and texture of sclerotia and six other characteristics. Such wide local differences undoubtedly explain the variability of morphological and physiological characteristics reported from diverse parts of the world.

DEFINITION OF TERMS

The formation and germination of dark-colored hardened compact masses of fungal tissue known as sclerotia have been studied in numerous fungi. From such studies, certain similarities and differences have been noted. Depending on the manner in which hyphae aggregate, the main types of sclerotium development are classified as terminal, strand, or loose. Further, sclerotium formation occurs in three stages or phases: initiation—formation of sclerotial initials; development—formation of full-sized sclerotia; and maturation—formation of a compact mass with dark-colored external pigmented cells. Once sclerotia are formed they may remain viable for up to several years. Under appropriate conditions, sclerotia germinate in one or more of three ways: myceliogenic—the formation of hyphae; carpogenic—the formation of sexual fruiting bodies; and sporogenic—the production of asexual spores. Both carpogenic and myceliogenic germination occur in *S. sclerotiorum*. Further information on comparative aspects of sclerotium formation, structure, and germination can be found in recent reviews (2,34).

SCLEROTIUM FORMATION AND COMPOSITION

Cytological and morphological aspects. Although there are variations due to isolates, substrates, and conditions, sclerotium formation in *S. sclerotiorum* follows a general sequence. When ascospores, mycelia or sclerotia are placed on a solid nutrient medium, hyphae grow out to form a thin film of growth over the surface. When the colony reaches the edge of the container or when growth is otherwise restricted, the mycelial mat thickens and produces white mounds of mycelium covered with small liquid droplets. As a sclerotium increases in size, the surface begins to darken and larger exudate droplets are apparent. As the surfaces of the sclerotium continue to darken until it is black, the exudate droplets disappear. Within ~ 1 wk or so the process is complete and a mature sclerotium can be removed from the substrate. Sclerotia often form at the edge of the plate but sometimes the sclerotia cover the substrate in a series of concentric circles or some other regular pattern. These patterns appear to be due to endogenous rhythms in some isolates of the fungus (11).

Details of sclerotium formation have been studied by light-, transmission-, and scanning electron microscopy (5,27,33). The vegetative hyphae contain various organelles and a simple, single perforated septum characteristic of other Ascomycetes. Saito (27) also noted a lomasomelike structure between the plasma membrane and the one-layered cell wall and an unidentified tubular vesicular complex in the cytoplasm.

Willetts and Wong (33) studied sclerotium development in several *Sclerotinia* spp. In large-sclerotia types (designated as *S. sclerotiorum* and *S. trifoliorum*), sclerotium development was of the terminal type. Initials arose from anastomoses of long primary hyphae and several initials fused to form a large sclerotium. Small-sclerotia types (designated as *S. libertiana* and *S. minor*) formed initials by a special type of strand development. Initials arose in a position laterally to the main hyphae from short aerial hyphae which became interwoven.

With continued growth, the internal (medullary) cells became compact and rind cells began to form beneath the surface mycelial network. During this period, intercellular spaces filled with liquid which was continuous with the surface droplets (5). Cells of the developing sclerotia contain numerous ribosomes and multivesicular and multitubular lomasomes. Invaginations of the plasma membrane were noted frequently. As the sclerotium matured, the original chitinous wall of the internal hyphae became covered with a thick fibrillar layer. Histochemical tests in combination with enzyme treatments indicated that this layer is composed of $\beta 1 \rightarrow 3$ glucan and protein (27). These same hyphae contained a polysaccharide, probably glycogen, within the cells. The cristae of the mitochondria became indistinct and electron-dense materials, possibly polyphosphates, accumulated in the vacuoles as the sclerotium matured. Mature rind cells, reported to contain melanin (13), have numerous pores or openings.

When mature, a sclerotium consists of a black rind approximately three cells wide and a medulla of prosenchymatous tissues embedded in a fibrillar matrix (5,27). Others (15) have described a cortical layer two to four cells thick between the rind and the medulla.

Composition of sclerotia. Results of chemical determinations supplement the histochemical data and demonstrate differences in composition between sclerotia grown in culture and those obtained from field collections. Proximate chemical analyses (Le Tourneau, *unpublished*) of sclerotia collected from commercial bean and pea-cleaning operations showed less than 2% crude fat (diethyl ether extract), 3.5–5.0% ash, and 20–25% protein (total Kjeldahl N × 6.25). A considerable proportion (20–25% of the dried ground sclerotia) was soluble in ethanol. A protein content of 10–15% was obtained if the calculation was based on alcohol-insoluble nitrogen. Sclerotia grown on a synthetic agar medium contained

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less crude fat, alcohol-soluble material, and protein than did sclerotia from field collections. Other analyses of the component hydrocarbons, free fatty acids, and amino acids in various fractions of such sclerotia are available (32).

Carbohydrates make up the greatest portion of the dry matter of mature sclerotia. In support of histochemical results, chemical analyses (Le Tourneau, *unpublished*) of sclerotia indicated about 25% β-glucan. Histochemical evidence (27) shows the presence of the polysaccharides, chitin and glycogen; infrared spectra studies did not indicate the presence of cellulose (20). Trehalose, a nonreducing disaccharide, and mannitol, a sugar alcohol, are the major low-molecular weight carbohydrates found in sclerotia. Monosaccharides, such as glucose or fructose, usually are present in the mycelia and sclerotia. The occurrence of other sugars and sugar alcohols is dependent on the carbon source in the medium (29).

Metabolic changes associated with sclerotium formation. The formation of sclerotia entails extensive cellular changes and the mobilization and deposition of many materials. As yet, these changes and their control mechanisms are but poorly understood.

The factors involved in the initiation phase have received little attention. As pointed out by Willetts (34), vegetative hyphae usually grow away from one another. In the formation of initials, there must be an attraction so that hyphal fusions occur. Because initials usually form after growth has covered the substrate, their formation may be a response to changes in nutrient availability. The fact that several fungi, including *S. sclerotiorum*, form sclerotia when mycelial mats are transferred to a medium low in nutrients supports this hypothesis (3).

Initials may form only after certain metabolic products are produced in the mycelium or in the medium. Organic acids, long known to be produced by *Sclerotinia* spp., may play a role in sclerotium formation (12,29). While the amount and kinds of organic acids vary with the isolate and the medium, oxalic, fumaric, malic, and succinic acids usually are produced by *S. sclerotiorum* (6,12). Other acids (eg, citric, glycolic, glyoxylic, and α-ketoglutaric) also have been detected (4,12). Enzymes for the production of Krebs cycle acids and oxalic acid are present in *S. sclerotiorum* (6,19).

When *S. sclerotiorum* was grown on a liquid medium, the pH of the culture filtrate dropped to pH 3–4 and then tended to increase slightly (29). The drop in pH occurred at the time of maximum acid production and when the specific activity of Krebs cycle enzymes was high. Numerous sclerotia formed when the fungus grew on a medium which supported good growth and the initiation of sclerotia tended to coincide with the pH drop. When the fungus was grown on a medium which supported very little growth or sclerotium formation, the pH of the culture filtrate usually increased (29). The addition of oxalic and Krebs cycle acids to the medium did not enhance sclerotium formation (12,29).

Once initials have formed, materials must be supplied for the synthesis of reserves and other compounds that are present in the mature sclerotium. Studies of translocation of solutes have been summarized (2,34) and will not be discussed here.

As indicated earlier, developing sclerotia are covered with droplets that are continuous with the interior of the developing sclerotium (5). These droplets are covered by a membrane and contain a variety of inorganic and organic materials, including several enzymes, which change in concentration as the sclerotium matures (4). The precise role of the exudate is still open to interpretation. It may play an osmotic role in translocation or it may be involved in the loss of water from the sclerotium during maturation (34).

Although many enzymes have been detected in sclerotia, it is not yet clear how the activity of these enzymes is controlled or coordinated or which enzymes may play key roles in morphogenesis. From results of polyacrylamide-gel electrophoresis studies, Wong and Willetts (35) suggested that suppression of glycolysis and the Krebs cycle and stimulation of the pentose phosphate pathway were involved during the compaction and maturation of sclerotia. Various enzymes of phenol oxidation have been detected (4,13,35). Tyrosinase may be involved in the initiation of sclerotia (35) and other phenol oxidases may be involved in the formation of the darkened rind.

FACTORS AFFECTING SCLEROTIUM FORMATION

Nutritional factors. Studies with synthetic media have shown that *Sclerotinia* spp. utilize many organic compounds as carbon sources for growth and sclerotium production (29). Trehalose, sugar alcohols, and organic acids commonly produced by and present in the fungus and the exudate droplets generally are poor carbon sources. The same amount of carbon supplied as a mixture of two poor carbon sources sometimes supported better growth than either source alone. The inability to use endogenous carbon sources normally present in the sclerotium for mycelial growth may be part of a control mechanism related to carpogenic and myceliogenic germination (29).

Other studies have shown that a number of inorganic and organic compounds are utilized as nitrogen sources. Amino acids closely related to organic acids of the Krebs cycle were particularly good nitrogen sources for sclerotium formation (30). The C/N ratio and the form of nitrogen in relation to the C/N ratio may also affect sclerotium production (17).

Growth and sclerotium formation of *S. sclerotiorum* occurred only when the inorganic macronutrients P, K, Mg, and S were present in the medium and was enhanced by the addition of an inorganic micronutrient mixture (24). When the fungus grew in a liquid medium containing purified chemicals in plastic flasks, less dry weight was produced and three of four isolates did not produce sclerotia in the absence of Zn (31).

Non-nutritional factors. The effects of light, temperature, pH of the medium, composition of the atmosphere, and osmotic potential on the production of sclerotia by several fungi are summarized in recent reviews (2,34).

Results of studies on the effects of light on sclerotium formation of *Sclerotinia* spp. are conflicting. Marukawa et al (17) grew an isolate identified as *S. libertiana* on a Czapek-Dox agar medium in the dark and under fluorescent light of 1,000–2,000 lux. Under these conditions, light caused a reduction in the number of sclerotia produced and in the total dry weight of sclerotia. In later research, 13 or 16 single-ascospore isolates of *S. sclerotiorum* grown on a glucose-asparagine yeast extract agar medium produced more sclerotia in the light than in the dark (10). However, the dry weight per sclerotium was greater when the fungus grew in the dark. Light was most effective in increasing the number of sclerotia when the fungus was illuminated before the formation of initials. When different wavelengths of light were used, ultraviolet and blue light were most effective in increasing the number of sclerotia. Cultures illuminated with green, red, and infrared light produced as many sclerotia as those held in darkness.

Depending on the isolate and other environmental conditions, *S. sclerotiorum* grows and produces sclerotia over a temperature range from near 0 C to 30 C. Fewer, but larger, sclerotia may be produced at lower temperatures (see, for example, 17).

Most workers have found that *S. sclerotiorum* will grow and produce sclerotia on media with an initial pH in the range 2.5–9 (17). The results of such studies undoubtedly depend on the type of medium, especially the buffering capacity. As indicated elsewhere (6,29) the pH of the medium may be changed during growth by the production of organic acids.

When salts or sucrose were added to an agar medium, mycelial growth of *S. sclerotiorum* was stimulated by decreasing the osmotic potential from −1 to −14 bars. Growth decreased below this value, but there was measurable growth at −100 bars. Sclerotia formed at −65 bars but not at −73 bars (8).

Sclerotium-forming compounds. Some isolates of *Sclerotinia* spp. lose the ability to produce sclerotia after repeated subculturing (18). This may be due to the inability of the fungus to synthesize specific compounds required for sclerotium formation, and several investigators have attempted to isolate such hypothetical compounds. In such studies, sclerotium production (number or weight) is measured after old culture filtrate (staling products) is added to a fresh medium. With such a bioassay, it is possible to

fractionate active preparations. In a recent study, Humpherson-Jones and Cooke (12) showed that two unidentified acids enhanced sclerotium production. Research by Satomura's group (18) suggests that sclerin, a known metabolite of *Sclerotinia* spp. in combination with other compounds, such as phenols, is involved in melanogenesis and formation of hyphal aggregates.

Inhibitors of sclerotium formation. The failure of some isolates of *Sclerotinia* spp. to produce sclerotia might be due to the production of inhibitors. While no one seems to have investigated this possibility, several workers have tried to find compounds that would permit mycelial growth but block sclerotium formation. Some compounds and the approximate concentration required to inhibit the formation of sclerotia are: *p*-aminobenzoic acid, 6 mM (17); phenylthiourea, 1 mM (16); Al^{+++}, 0.6 mM (23); and fluorophenylalanine, 0.1 mM (7). It is of interest that *p*-aminobenzoic acid and phenylthiourea are known inhibitors of polyphenoloxidase. Further studies should be made to find more potent inhibitors and to determine the mode of action of these inhibitors.

SCLEROTIUM GERMINATION

Carpogenic germination. If sclerotia are placed on a substrate low in nutrients (moistened sand, cotton, or polyurethane, water agar, or water) they will, under proper conditions, produce one or more stipes each with an apothecium. Recent studies have shown that low osmotic potential will inhibit the formation of apothecia (8,21). While some workers have recorded carpogenic germination in sclerotia taken directly from host tissue or laboratory media (15), others condition sclerotia for several weeks or longer in a moistened state (25,28).

The sequence of events for apothecium production from sclerotia has been studied by light- and electron microscopy in conjunction with various histochemical tests (14,15,25). Apothecial initials arise in the cortex or medulla as brown to hyaline clusters or nests of interwoven hyphe. Active division of hyphae produces a knot of closely interwoven hyphae with dense cytoplasm. Eventually a few of the primordia erupt through the surface of the sclerotium. Numerous clusters of microconidia were observed, but there is no evidence that spermatization is a prerequisite for apothecial initiation (15).

Saito (25) divided apothecial genesis into four stages. He concluded that many primordia form but only a few erupt through the surface. He believed that the reserve materials of sclerotia would be depleted if all the primordia developed. This suggests that some control mechanism(s) within sclerotia regulates the development of initials.

Once the pointed, buff-colored initials erupt through the surface they continue to grow and develop. Stipes are positively phototropic and will not differentiate into disks unless they receive light. Recently it was shown that only light below 390 nm is effective in inducing apothecium formation (9). A depression develops at the tip of the apothecial initials and paraphyses develop. Ascogenous hyphae with croziers form in the subhymenium. After differentiation, the ascus contains eight ascospores (15).

The fully differentiated stipe consists of an outer layer of compressed hyphae and a medulla of elongated, septate, sparingly-branched hyphae. Following differentiation, the disk of the mature apothecium consists of (i) an ectal excipulum, (ii) a medullary excipulum, (iii) the hymenium, and (iv) the subhymenium (15). Additional details of cellular structure, histochemical tests, and ascospore production are found in the papers cited above.

Relatively little is known about the biochemical events during carpogenic germination. By analogy with other germinating structures, it would be expected that reserve materials are metabolized to provide "building blocks" which in turn are used for synthesis of the components of the stipe and apothecium. Histochemical studies and chemical analyses indicated intense activity of hydrolytic and synthetic enzymes in the area of the apothecial initials (15) and that β-glucanase was the only enzyme of carbohydrate metabolism that increased in activity during germination (26).

Myceliogenic germination. As indicated earlier, comparative studies with many fungi have shown that sclerotia may germinate by producing mycelia (2,34). *Sclerotinia* spp. have been shown to produce mycelia by hyphal or mycelial germination (1).

In hyphal germination, sclerotia placed on a substrate low in nutrients usually will produce sparse mycelial growth from hyphae which emerge through the rind. In some cases this mycelial growth may produce smaller secondary sclerotia (28). If hyphal germination occurs on a medium containing nutrients, the mycelia will continue to grow and produce new sclerotia.

Mycelial germination was observed with isolates that produce small sclerotia, eg, *S. sclerotiorum* 'minor' (1). After a dormant period, sclerotia placed on a moistened medium developed one or more bulges. These bulges grew larger, finally ruptured, and released a plug or mass of dense mycelium that was visible to the unaided eye. In some instances, two or more of these masses from the same sclerotia coalesced. Such mycelial masses grew from the sclerotia for distances of 2–3 mm.

Inhibition of germination. Very few workers have studied the possibility of inhibiting various types and stages of sclerotium germination. In one recent report, Steadman and Nickerson (28) found differential inhibition; ie, different concentrations of an inhibitor often were needed to prevent stipe formation, apothecium formation, and myceliogenic (hyphal) germination. A number of commercial fungicides and transition metal ions, especially cadmium, were inhibitory in the range of 1 μM to 0.1 mM. Higher concentrations (1–100 mM) of several common buffers (acetate, phosphate, and Tris), cations (sodium and lithium), anions (nitrate and sulfate), and low-molecular-weight carbohydrates (glucose, mannitol, sucrose, and trehalose) also inhibited germination. Potassium and chloride ions were not inhibitory. These authors also concluded that germinating sclerotia may affect the pH of the germination medium through metabolite release. Further studies on the inhibition of germination, including the possible occurrence of endogenous inhibitors of carpogenic and myceliogenic germination should be fruitful toward gaining an understanding of this phase of sclerotium morphogenesis.

SUMMARY

Obviously the formation and germination of sclerotia of *Sclerotinia* spp. are programmed and controlled processes. The results of studies in culture summarized in this paper highlight the deficiencies in our knowledge. While the general sequence of events and some of the factors affecting these processes have been described, little is known about the control mechanisms involved and the concomitant biochemical changes in the various stages of morphogenesis. Much additional basic research is needed to provide the information necessary to develop effective disease control measures.

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