Conservation of Mycelial Constituents in Four Sclerotium-Forming Fungi in Nutrient-Deprived Conditions

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ABSTRACT

Sclerotia were formed when washed mycelial mats of *Sclerotinia sclerotiorum, Sclerotium rolfsii, Sclerotium cepivorum*, and *Rhizoctonia solani* were incubated on soil, on a bed of glass beads leached with slowly running water, or on a bed of water-saturated glass beads without leaching. Sclerotium formation was accompanied by extensive mycelial lysis. For mycelia incubated on leached glass beads, 39-52% of dry matter, 27-58% of carbohydrates, and 42-79% of nitrogen originally present were conserved in sclerotia of the four fungi. For mycelia incubated on soil, amounts conserved were usually slightly higher. When mycelia of *S. rolfsii* were incubated on leached glass beads, 42% of the total mycelial carbohydrate was incorporated into sclerotia, 25% was lost in exudates, 26% was lost as CO_2, and 7% was recovered in the residual mycelium; 44% of the total nitrogen was incorporated into sclerotia, 43% was lost in exudates, and 13% was recovered in the residual mycelium. Nutrient deprivation may induce the formation of sclerotia from mycelial reserves and products of mycelial lysis.

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Additional key words: lysis, *Sclerotinia sclerotiorum, Sclerotium rolfsii, Sclerotium cepivorum, Rhizoctonia solani.*

Little is known of the environmental stimuli bringing about the change from the vegetative to the resting phase in root-infecting fungi. Formation of sclerotia is known to be influenced by nutrient conditions. The time of maturation of sclerotia of *Rhizoctonia solani* and *Sclerotium rolfsii* was closely related to the time of depletion of carbohydrates in the media (13). Transfer of mycelia of *S. rolfsii* from nutrient-rich to poor media enhanced the rate of production of sclerotial initials (14), whereas

maintenance of an enriched nutrient status through successive increments of carbohydrate (13), or transfer of mycelia to fresh media (14) delayed sclerotial production.

The possible dependence of sclerotial production on materials accumulated in the mycelia apparently has not been investigated. However, this is suggested by results indicating strong polar transfer of materials from mycelium to sclerotia as determined for *S. rolfsii* using ^{14}C-iodoacetate (3), for *R. solani* using ^{32}P-phosphate (9), and for *Sclerotinia sclerotiorum* using ^{14}C-glucose (4). The withdrawal of nutrients from established mycelium during fruit body formation has been shown for *Coprinus lagopus* (11) and *Agaricus campestris* (1).

A crucial role for energy source deprivation as a determining factor in inducing fungistasis and lysis of mycelium in soil has been proposed (7, 8). Accordingly, an attempt was made to determine whether nutrient deprivation could also act as a stimulus for sclerotial formation in several root-infecting fungi. In addition, an estimate was made of the efficiency of the transformation from the vegetative to the sclerotial phase using mycelia as the sole source of materials.

MATERIALS AND METHODS.—*Fungi.*—Cultures of *Sclerotinia sclerotiorum* (Lib.) d By., *Sclerotium rolfsii* Sacc., *Sclerotium cepivorum* Berk., and *Rhizoctonia solani* Kühn were maintained on potato-dextrose agar (PDA) slants. Mycelia were grown from a single sclerotium added to 50 ml potato-dextrose broth (PDB) at 25 C. When mycelial growth had nearly covered the medium (4-7 days), but before the appearance of sclerotial initials, mycelial mats were washed three times in separate 100-ml portions of distilled water.

Substrates for sclerotia production.—Mycelia were incubated on natural soil or were exposed to artificial nutrient stress conditions by incubation on a bed of glass beads either undergoing leaching or not undergoing leaching. The soil, sieved Conover loam at pH 6.7 (8) and 25% moisture was added to a petri dish (25 cc soil/9-cm diam dish) and the soil surface made smooth. The leaching system was a modification of that originally designed by Ko & Lockwood (8). It was composed of a separatory funnel equipped with a sealed-in dripping tip (Kontes Glass Co., Vineland, N.J.), to maintain a constant flow rate. The funnel stem was connected by means of plastic tubing to a needle valve, then to a glass petri dish fitted with an inlet at one side of the lid, and an outlet in the bottom on the opposite side. The petri dish contained a 5-mm bed of Pyrex glass beads of 400-600 μ diam through which the leaching fluid percolated, and on which the mycelia were borne. All glass components were acid-washed. The separatory funnel was filled with glass distilled water or other leaching fluid and the rate of flow adjusted to ca. 30 ml per hr with the needle valve. The use of a constant head funnel combined with the needle valve permitted precise adjustment and constancy of the flow rate. In some experiments the leaching solution was 0.025 M phosphate buffer at pH 6.2 or 7.0, or a mineral salt solution that contained, per liter of water: 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$; or 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4$. Some mycelial mats were incubated on a 5-mm bed of glass beads saturated with the appropriate medium, but without leaching, in ordinary covered petri dishes. Incubation was at 24 C in the diffuse light of the laboratory.

Aseptic conditions were used unless indicated otherwise.

Analysis of sclerotia and mycelia.—Mycelia and sclerotia were analyzed for total carbohydrates using the anthrone reagent (12) with glucose as a standard, and for total nitrogen by the semi-micro Kjeldahl procedure (2). The entire crop of mature sclerotia of *S. rolfsii, S. sclerotiorum*, or *R. solani* was collected from cultures with forceps; the tiny sclerotia of *S. cepivorum* were collected by means of a Pasteur pipette plugged with cotton and attached to a vacuum line. The sclerotia were separated from mycelial fragments by sedimentation in water. Sclerotia were oven-dried for 48 hr at 102 C and weighed before analysis.

All treatments were in duplicate and all experiments were repeated at least once.

RESULTS.—*Sclerotium formation.*—All four fungi, when transferred to soil or to glass beads leached or not leached with water, made sparse radial growth which ceased within 24 hr except for *R. solani* which grew more extensively on soil. Sclerotial initials of all fungi first appeared within 18-24 hr; by the 4th day these had reached the size, shape, and color characteristic of mature sclerotia of the species. Extensive mycelial lysis accompanied formation of sclerotia in all three incubation conditions. Thus, the pattern of sclerotium formation was the same on leached or non-leached glass beads as on soil. Mycelia growing in PDB did not produce sclerotia until the mycelia had reached the walls of the culture flasks.

Additional experiments were done with *S. rolfsii* to evaluate factors other than nutrient deprivation which may have been involved in the induction of sclerotia formation on leached glass beads. To test the possibility that mycelia incubated on leached sand sustained an osmotic shock, washed mycelia were incubated on glass beads leached with either of two salt solutions or water. Sclerotia formed readily in all three treatments, with no significant difference between dry weights of sclerotia formed. Aqueous washing of mycelia did not induce sclerotium formation; mycelia washed three times in 100-ml portions of distilled water, then returned to PDB did not form sclerotia until the fungus had covered the medium.

The possible effect of leaching per se on sclerotium formation was tested using a nutrient medium. Small (5- to 6-cm diam.) mycelial mats were transferred to glass beads and leached with PDB for 3-4 days during which growth continued. Then, PDB was withdrawn and the system was leached with sterile distilled water. Within 24 hr growth ceased and sclerotial initials formed; these matured at the usual

TABLE 1. Conservation of fungal dry matter, total carbohydrates and total nitrogen in sclerotia formed from mycelia first grown on potato-dextrose broth, then washed and incubated on soil or on a bed of leached glass beads to induce sclerotium formation

Fungus	Soil or leaching system (L.S.)[a]	Dry wt (mg)	Carbohydrates[b] (mg)	Nitrogen[c] (mg)
Sclerotinia sclerotiorum				
Original mycelia		120.6	74.8	5.2
Sclerotia	Soil	66.6	53.8	2.5
	L.S.	48.0	43.0	2.2
Sclerotium cepivorum				
Original mycelia		226.0	187.0	4.3
Sclerotia	Soil	154.2	44.0	3.9
	L.S.	118.3	77.0	3.0
Sclerotium rolfsii				
Original mycelia		380.3	240.0	5.5
Sclerotia	Soil	193.4	151.0	6.0
	L.S.	142.4	124.0	4.3
Rhizoctonia solani				
Original mycelia		58.0	34.0	3.6
Sclerotia	Soil	114.0	36.5	5.8
	L.S.	22.5	9.0	1.8

[a] Soil was natural Conover loam. The leaching system consisted of a petri dish containing a bed of glass beads which supported the mycelia and which were continually leached with distilled water at the rate of ca. 30 ml/hr, under aseptic conditions.

[b] Total anthrone-positive materials.

[c] Total nitrogen was determined by the Kjeldahl method.

rapid rate. Mycelia subjected to continual leaching with PDB did not form sclerotial initials until dense mycelia had completely filled the petri dish forming a barrier to the entry of PDB into the substrate. This usually required ca. 8 days after transfer of mycelia. Even then, maturation of sclerotia was delayed compared with those formed from mycelia leached with water. Similar results were obtained with mycelia grown from sclerotia germinated on glass beads leached with PDB, then left in situ.

Mycelia incubated on glass beads leached with phosphate buffer at pH 6.2 or 7.0 formed sclerotia as readily as those leached with water; the pH of the aqueous leachate was 3.6 - 4.2 and that of the buffered leachates was 6.2 - 6.3 and 6.9 - 7.0, respectively, when measured 2-3 times daily for 5 days. Mycelia incubated on beads leached with PDB having an initial pH of 6.7 were not induced to form sclerotia, as described above, yet the leachate had a pH of 3.8 - 4.5. Therefore, pH change did not appear to be required for sclerotium formation.

The most probable factor inducing sclerotium formation in the model system appeared to be energy deprivation.

Analysis of sclerotia formed on soil and in the leaching system.—Amounts of dry matter, carbohydrate, and nitrogen in mycelia and in sclerotia formed from mycelia on soil and in the leaching system were determined for the four fungi (Table 1). Sclerotia formed on soil contained 39-52% as much dry matter, 27-59% as much carbohydrate, and 42-79% as much nitrogen as was present in the original mycelium. Data for soil and the leaching system were in reasonable agreement for *S. rolfsii*, *S. sclerotiorum*, and *S. cepivorum* though values for the soil treatment tended to be higher than those for the leaching system. For example, dry matter in sclerotia of these three fungi formed on soil was 51, 55, and 68%, respectively, of that in the original mycelia; for the leaching system values were 38, 40, and 52%, respectively. *R. solani* was exceptional in that more dry matter, carbohydrate, and nitrogen were present in the sclerotia formed on soil than were present originally in the mycelium, apparently due to the ability of this fungus to grow in natural soil without added energy sources (10). When *R. solani* was incubated on glass beads, amounts of dry matter, carbohydrate, and nitrogen were less than those in the original mycelium.

Fate of mycelial carbohydrate and nitrogen during sclerotial formation in S. rolfsii.—Total carbohydrate and nitrogen recovered in sclerotia formed on soil and in the leaching system comprised only a part of the total nitrogen and carbohydrate content of the mycelium which gave rise to the sclerotia. Therefore, it was of interest to determine the fate of that portion of the components which did not become incorporated into the sclerotia. The leaching system provided a means to do this. Duplicate mycelial mats of *S. rolfsii*, grown in PDB in synchronous cultures, were incubated for 7 days on glass beads in leaching systems to induce sclerotium formation. The sclerotia, the remaining mycelium, and the leachate were collected and analyzed for total nitrogen and carbohydrate. Of the total carbohydrate content of the original mycelium, 42% was recovered in the

sclerotia, 25% in the leachate, and 7% in the residual mycelium (Fig. 1). The 26% unaccounted for was thought to represent loss as CO_2. Of the total nitrogen content of the mycelium, 44% was recovered in sclerotia, 43% in the leachate, and 13% in the residual mycelium.

DISCUSSION.–The similarity in the results obtained for mycelia incubated on soil with those for mycelia incubated on glass beads suggest that nutrient deprivation may be a stimulus for sclerotium production in natural situations. Soils are known to be deficient in energy (5), and the glass bead systems provided a means for experimentally isolating energy deprivation as an environmental factor. The results confirm an earlier report of rapid sclerotium induction on transfer of mycelia from nutrient-rich to nutrient-poor media (14). In contrast, we found that vegetative growth was prolonged and sclerotium formation delayed in a medium continually supplemented to maintain a high energy status, a finding that has also been previously reported (13). These findings do not exclude the possibility that other stimuli may be involved in sclerotium formation. For example, Henis & Inbar (6) showed that *Bacillus subtilis, B. licheniformis*, and a factor produced by *B. subtilis* induced sclerotium production in *Rhizoctonia solani*.

The leaching system provided a means of estimating the efficiency of sclerotium formation in conserving fungal material by providing a chemically clean environment in which sclerotia developed exclusively from materials available in the mycelium. The relatively high amounts of mycelial dry matter, carbohydrate, and nitrogen converted to sclerotia indicates that these fungi have efficient systems for conserving mycelial reserves in resting structures when confronted by starvation conditions.

The results suggest that under conditions of nutrient deprivation, constituents of established mycelia become translocated to sites of sclerotial synthesis. Utilization of mycelial reserves in fruit body production in some Basidiomycetes is documented (1, 11), and translocation of materials from mycelia to developing sclerotia also has been shown (3, 4, 9). The well-known development of sclerotia in mycelia at sites of wounding may be due to severance of translocating hyphae and consequent accumulation of materials at these sites. In view of the high efficiency of conversion demonstrated in these results, it seems likely that some of the materials utilized in sclerotial formation must have come from cell wall materials made available through autolysis. This suggestion is supported by the virtually complete disappearance of mycelia that occurred during sclerotial formation. Nutrient deprivation has previously been implicated in the autolysis of fungal hyphae in soil (8).

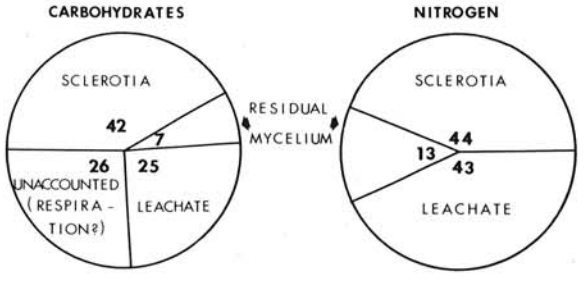

Fig. 1. Distribution of carbohydrates (anthrone method) and nitrogen (Kjeldahl method) of *Sclerotium rolfsii* in sclerotia, residual mycelium, and leachate following transfer of washed mycelium, originally grown in potato-dextrose broth, to a bed of glass beads leached aseptically with distilled water. Total carbohydrates and nitrogen were 197 and 3.8 mg, respectively, per mycelial mat; values given are percentages of those in the original mycelium.

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