The Potential for the Formation of Sclerotia in Submerged Mycelium of *Sclerotium rolfii*

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Synchronous formation of sclerotia in *Sclerotium rolfii* was induced in both solid and liquid synthetic media. Sclerotial initials were formed when mycelium was forced to pass from the submerged to the aerial growth phase in one of the following ways: (i) by removal of aerial mycelium with a scalpel; (ii) by growing the mycelium under a cover glass which was later removed; (iii) by inverting the agar in a Petri dish containing the fungal culture; (iv) by pouring a layer of agar over a colony. In all cases hyphae which emerged from the submerged phase produced many sclerotia synchronously. When grown in shaken liquid culture *S. rolfii* did not produce any sclerotia while submerged, but when samples of the shaken culture were poured into a Petri dish 50 to 90 h after inoculation and incubated further, sclerotial initials were formed synchronously within 16 h on the liquid surface.

INTRODUCTION

When grown on an agar medium *Sclerotium rolfii* Sacc. forms a submerged vegetative mycelium consisting of single hyphae and an aerial mycelium composed of both single hyphae and hyphal strands. Sclerotial initials are formed on these hyphal strands (Townsend & Willetts, 1954). Sclerotium formation on agar plates usually occurs when the mycelium approaches the edge of the Petri dish (Wheeler & Waller, 1965); it can also be induced by cutting the mycelium with a cork borer (Henis *et al.*, 1965). Synchronization of sclerotium formation can be induced by the addition to the growth medium of iodoacetic acid (Chet *et al.*, 1966), lactose (Okon *et al.*, 1972) or threonine (Kritzman *et al.*, 1976).

When grown in liquid medium *S. rolfii* produces an extracellular viscous polysaccharide (Kritzman *et al.*, 1979) but does not produce any sclerotia (Okon *et al.*, 1974). In this paper we report on the development of a sclerotium-producing potential in the submerged mycelium of *S. rolfii* which can be revealed when mycelial growth is transferred from a submerged to an aerial environment.

METHODS

Strain and growth conditions. *Sclerotium rolfii* type A ATCC 26325 (Chet & Henis, 1972) was grown at 30 °C either on an agar medium in Petri dishes or in Erlenmeyer flasks containing liquid synthetic medium (Okon *et al.*, 1973). Plates containing 15 ml solidified medium were centrally inoculated with agar discs (0.5 cm diam.) covered with fungal mycelium which had been cut from a 5 d colony. Flasks containing 50 ml medium were inoculated with 1 ml suspension containing 10 mg (dry wt) mycelium and incubated at 30 °C in a rotary shaker (New Brunswick Scientific) at 150 rev. min⁻¹ for 3 d. Inoculum was prepared by homogenizing mycelium in the culture flask with an Ultra-Turrax homogenizer (Janke & Kunkel KG., West Germany) operated at one-third of maximum speed (Zweck *et al.*, 1978).

Analyses. Mycelium was separated from extracellular polysaccharide by centrifugation at 12000 g for 20 min at 4 °C. Polysaccharide was precipitated by mixing the supernatant with 2 vol. ethanol. The precipitate was collected on a glass rod, washed three times with cold ethanol, lyophilized and weighed. To determine dry weight, samples

were dried at 70 °C in an oven to constant weight. Glucose was determined using the glucose oxidase reagent (Sigma) according to the directions of the manufacturer.

RESULTS

Induction of synchronous sclerotium production by transition from submerged to aerial growth

When the aerial mycelium of *Sclerotium rolfsii* grown on agar was removed with a scalpel, the hyphae rising from the submerged mycelium simultaneously produced sclerotial initials within 16 h (Fig. 1). The response of *S. rolfsii* to removal of its aerial mycelium depended both on the age of the colony and on the agar concentration. Highest numbers of sclerotia were produced in a medium containing 4% (w/v) agar (Fig. 2). For all the agar concentrations tested the maximum production of sclerotial initials occurred when the aerial mycelium was removed 3 to 4 h prior to sclerotial initiation in the control. Earliest initiation appeared in the area of a 64 h colony from which aerial mycelium had been removed at 48 h. Numbers of initials formed in the treated colony area increased with colony age. However, removing aerial mycelium which had already formed sclerotial initials resulted in a reduced number of new initials. Moreover, a second removal of newly formed aerial mycelium and initials from a previously treated area did not result in sclerotium formation, indicating that the same area of a *S. rolfsii* colony could not produce sclerotia twice.

According to Wheeler & Waller (1965), untreated colonies of *S. rolfsii* do not produce sclerotial initials until the mycelium approaches the edge of the Petri dish regardless of the size of dish used. In our system, however, removing the aerial mycelium from a colony grown in a 15 cm diam. Petri dish 64 h after inoculation resulted in the production of large numbers of initials, although the untreated parallel area of the same thallus had not yet produced any initials (Fig. 1*b*). Thus, the removal of aerial mycelium of *S. rolfsii* revealed the potential for production of sclerotia long before fungal growth had reached the edge of the Petri dish.

Fig. 1. Synchronous sclerotium formation as a result of removal of aerial mycelium of *Sclerotium rolfsii* 72 h after inoculation in 9 cm (a) and 15 cm (b) diam. Petri dishes, photographed 24 h later (left, control; right, mycelium removed from half of the colony).

Fig. 2. Effect of removal of aerial mycelium on sclerotium formation by *Sclerotium rolfsii* as a function of colony age at different agar concentrations: ▲, 0.7%; △, 2%; □, 4%.

Fig. 3. Sclerotium formation (▲) by *Sclerotium rolfsii* 16 h after transfer from shaken to stationary incubation. Glucose utilization (○), polysaccharide concentration (△) and dry weight (●) at time of transfer are also shown.

To examine the possibility that induction of sclerotia might have resulted from mechanical damage (Henis *et al.*, 1965), growth of aerial mycelium was prevented by covering the growing thallus with a cover glass placed at the edge of a 24 h colony. Under these conditions, the culture underneath the cover glass consisted of submerged mycelium only. Removal of the cover glass after 48 h was followed by an extensive outgrowth of new aerial mycelium, with the concomitant appearance of sclerotial initials. Removal of the cover glass either 24 h before or after the optimal time (i.e. 72 h) did not result in the formation of any sclerotial initials.

Induction of sclerotia without damage to the mycelium was also achieved either by inverting the agar medium in Petri dishes containing 72 h colonies so that aerial mycelium came in contact with the bottom of the dish, or by pouring 10 ml water agar over the culture surface and incubating further. Under these conditions new hyphae grew through the upper layer and produced initials on the surface within 24 h.

Capacity for sclerotium production of submerged liquid cultures

When *S. rolfsii* was grown in a shaken liquid medium, no sclerotia were produced in the submerged culture (Okon *et al.*, 1974). Moreover, when these flasks were transferred to

stationary incubation within the first 40 h of shaking, the liquid surface became covered with vegetative mycelium only. Cultures transferred to stationary incubation after 50 to 90 h of shaking produced many sclerotial initials synchronously. Similar results were obtained when 15 ml samples of the liquid cultures were poured into Petri dishes after the same period of shaking, and incubated for a further 12 to 16 h. Production of fungal biomass (dry weight) and extracellular polysaccharide reached a plateau 52 h after inoculation, by which time most of the glucose had been utilized. The capacity for sclerotium production decreased 95 h after inoculation and at the same time there was a significant decrease in the polysaccharide content of the medium (Fig. 3).

DISCUSSION

Our results suggest that the submerged mycelium of *Sclerotium rolfsii* plays an active role in the regulation of sclerotium production. Synchronous formation of sclerotia could be triggered by changing the relative proportions of aerial and submerged mycelium in favour of the latter. Sclerotia are always produced on strands of aerial hyphae (Townsend & Willetts, 1954) and never in submerged mycelium, whether growth is on solid or in liquid medium (Okon *et al.*, 1976), in accord with the observation that under field conditions sclerotia of *S. rolfsii* are formed on the surface of, rather than inside the infected plant tissue (Aycock, 1966). Sclerotium production by the aerial mycelium requires oxygen concentrations higher than 15% which are never reached inside agar or liquid media, yet hyphal growth is not inhibited at lower oxygen concentrations (Griffin & Nair, 1968). Our results indicate that both submerged and aerial mycelia participate in sclerotium production: the site of sclerotium formation is restricted to the aerial mycelium and the initiation of the process is regulated by the submerged mycelium. The capacity to induce sclerotia develops in the submerged mycelium within 72 h and declines with further incubation.

These results are consistent with working hypotheses suggested by other authors to explain the mechanism involved in induction of sclerotium formation by *S. rolfsii*. Thus, Wheeler & Waller (1965) claimed that sclerotial initiation and growth of vegetative mycelium competed with each other for metabolites essential for sclerotium production. Goujon (1970) proposed that a 'morphogenetic factor', probably a protein, must reach a critical concentration inside the hyphae. Kritzman *et al.* (1976) suggested that formation of sclerotia required an increased supply of carbohydrates and energy which were mainly provided by the glyoxylate pathway. In none of these studies, however, were the physiological differences between the submerged and the aerial mycelia and their possible role in sclerotial initiation considered.

The relationship between the age of the submerged mycelium and its potential to produce sclerotial initials suggests that metabolites or enzymes formed in the submerged growth phase play a role in the aerobic growth phase. These factors are probably transported to the aerial mycelium where they induce sclerotium formation.

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