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REGULATION OF SCLEROTIUM PRODUCTION BY PRIMARY METABOLITES IN *COPRINUS CINEREUS* (= *C. LAGOPUS SENSU* LEWIS)

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Aerial sclerotia of *Coprinus cinereus* (Schaeff. ex Fr.) S. F. Gray were produced most abundantly in conditions which strongly favoured mycelial growth. With the nitrogen concentration maintained at 25 mM ammonium tartrate the number of sclerotia formed increased as the initial maltose concentration of the medium was increased, reaching a maximum at 25 mM. The length of time required for sclerotium maturation was also minimal on media which initially contained 25 mM maltose. Changing the concentration of ammonium tartrate (in media containing 25 mM maltose) did not markedly affect the total number of sclerotium initials which were formed but had a profound influence on sclerotial maturation. With 5 mM ammonium tartrate maturation occurred most rapidly and most completely, with 63 % of the final total sclerotial yield being matured within 7 days, and 91 % within 14 days. Higher ammonium concentrations first delayed and then prevented maturation so that at 100 mM less than 0·3 % of the initials had matured within 21 days. It is suggested that the ability of ammonium ions to regulate sclerotium maturation can be correlated with the ability of the sporophore to excrete ammonia into the medium so as to provide a mechanism for sequencing the two pathways. While sporophores are developing the ammonium concentration will be maintained at a level which prevents sclerotial maturation, thus avoiding competition for metabolities between the two morphogenetic sequences.

A sclerotium is a highly organized structure resulting from a co-ordinated process of growth and branching by hyphae of the vegetative mycelium. Sclerotia serve as perennating organs, germinating when adverse conditions ameliorate. The agaric *Coprinus cinereus* produces sclerotia on both the aerial and submerged parts of the colony, but those formed by the two regions are quite different in origin and structure (Waters, Butler & Moore, 1975). Submerged sclerotia were found to be regions of the submerged mycelium which had simply been separated off by a unicellular rind layer of thick-walled cells. In contrast, aerial sclerotia were the end product of a complex sequence during which, probably from a single originating cell, a compact semi-spherical body (about 0·25 mm diameter) consisting of two quite distinct and specialized multicellular tissues was developed (Waters, Moore & Butler, 1975). Early stages in the morphogenesis of aerial sclerotia are very similar, if not identical, to the very earliest events in sporophore development described by Matthews & Niederpruem (1972). Study of the physiological factors influencing sclerotium formation in *Coprinus* would consequently provide valuable information about a complex developmental system, and, hopefully, eventually provide a link with our parallel studies of sporophore morphogenesis (Stewart & Moore, 1974). This paper reports the results of experiments designed to determine the basic nutritional requirements for the formation of aerial sclerotia in *C. cinereus*.

METHODS

Strains

The organism used was *Coprinus cinereus* (Schaeff. ex Fr.) S. F. Gray (=*C. lagopus sensu* Lewis). Three strains were used: ZBw601/40,40, a monokaryotic wild type which originated in Czechoslovakia, and the dikaryon H1 × BC9/6,6 have been used and described previously (Stewart & Moore, 1974; Waters, Butler & Moore, 1975). They were used here to provide continuity between the different investigations although this study made most use of a dikaryon constructed from BC9/6,6 and ZBw601/40,40 and given the stock number RA1.

Media

For stock cultures and other routine purposes maltose-complete medium (Stewart & Moore, 1974) was used. A fully defined basal medium (SNC) was employed for nutritional experiments. It contained 10 mM-Na_2HPO_4, 10 mM-KH_2PO_4,

2 mM-Na_2SO_4 and 3 μM thiamin hydrochloride. The medium was made up with tap water and solidified with 1·5% (w/v) Difco Bacto-Agar. After addition of appropriate nitrogen sources media were autoclaved for 15 min at 121°. Solutions of carbon sources were autoclaved separately.

Estimation of growth rate and sclerotium production

Extension growth rate was determined by measuring the colonies across two diameters at right angles at 24 h intervals. Four replicates were usually made of each treatment. Although this technique does not take into account the density of mycelium it is a useful measure of growth, particularly where some inhibition is occurring (Trinci, 1969). Sclerotium production was quantified by counting with the aid of a dissecting microscope. Immature sclerotia (white tufts) and mature sclerotia (brown–black compact spheroids) were separately enumerated in 25–30 fields of view scattered across two diameters at right angles. About 35% of the total surface area of the Petri dish was examined in this way. Three replicates for each treatment were scored and all counts contributed to the final measure of 'sclerotia per plate'.

RESULTS

Conditions of culture

Taking the radial growth rate and numbers of sclerotia produced as measures to determine optimal culture conditions, a number of physical and chemical factors that might affect growth of the colony on maltose-complete medium were studied. Sclerotia initiated and matured late at acid pH values, a feature which correlated with decreased growth rates below pH 6. Sclerotium production reached a maximum in the pH range 6·8–7·4 and then declined, although growth rate continued to increase up to pH 8. Continuous illumination during incubation inhibited sclerotial production, none being produced over a 28-day period, and encouraged dikaryons to form sporophores. Continuous cycles of 12 h light/12 h dark allowed the formation of both sclerotia and sporophores by the dikaryons (sclerotia only by the monokaryon), but both types of colony produced by far the largest numbers of sclerotia when incubated in continuous darkness. Illumination had no significant effect on growth rate. Over the temperature range 26–37° the growth rate changed very little although no sclerotia were observed in culture grown at 26°. The established optimum temperature for vegetative growth is 37°, and this was clearly also the best for sclerotium formation. The depth of the medium also had more effect on sclerotium formation than on growth rate. No sclerotia at all were found on colonies grown on media less than about 3 mm deep, even when grown in saturated atmospheres in moist chambers. Agar depths of about 6 mm were required for the greatest yields of sclerotia. A general conclusion from these initial studies was that good sclerotium formation accompanied good mycelial growth, although optimum sclerotium production occurred over a narrower range of conditions than optimum growth rate, a feature which probably reflects the difference between extension growth rate and specific growth rate. All subsequent experiments were done using 9 cm plastic Petri dishes containing 35 ml of agar medium at pH 6·8, inoculated plates being incubated at 37° in the dark.

Table 1. *Effect of different carbon sources on the growth rate and sclerotium production of the dikaryon RA1*

Carbon source	Growth rate (μm/h)	Numbers of mature sclerotia/plate	
		Day 14	Day 21
Maltose, 25 mM	414	4820	18700
Trehalose, 25 mM	428	8780	15800
Glucose, 50 mM	305	6300	16100
Fructose, 50 mM	268	6000	18300
Sodium acetate, 150 mM	56	0	0
Starch, 6·7 g/l	379	5900	7070

Nature and concentration of the carbon source

This organism makes use of a very small number of potential carbon sources (Moore, 1969) so there is relatively little scope for investigating the effect of changing the identity of the carbon source. The response of RA1 to sodium acetate and to five carbohydrates is summarized in Table 1. Only sodium acetate stands out as having a profound influence. The low sclerotium yield on starch may be a concentration effect (note the comparability with the other sugars at day 14) and apart from this there was no significant difference between the carbohydrates in their ability to support sclerotium formation. In the absence of detailed knowledge of the relative permeability of the organism and its ability to interconvert different compounds little can be gained from comparative studies of different sugars. Attention was therefore turned to sugar concentration, maltose being chosen, fairly arbitrarily, as the carbon source for further tests (Table 2). Generally there was in all cases a clear optimum of sclerotial

Table 2. *Comparison of growth rates and sclerotium production of three strains of* Coprinus cinereus *grown on agar media containing different initial concentrations of maltose*

Strain		Maltose concentration (mM) 5	10	25	50	100	250	500
H1 × BC9/6,6 (dikaryon)	Mature sclerotia counted on day 7	0	0	0	0	0	0	0
	Mature sclerotia counted on day 14	20	50	316	40	0	0	0
	Growth rate (μm/h)	396	371	472	396	330	220	198
RA1 (dikaryon)	Mature sclerotia counted on day 7	2750	1810	4380	3670	4610	3150	0
	Mature sclerotia counted on day 14	5900	3210	39900	14300	13200	8990	0
	Growth rate (μm/h)	668	652	671	662	666	511	220
ZBw601/40,40 (monokaryon)	Mature sclerotia counted on day 7	0	40	2320	10	10	0	0
	Mature sclerotia counted on day 14	568	1050	2730	220	125	0	0
	Growth rate (μm/h)	345	347	302	342	310	329	241

Table 3. *Sclerotium production by the dikaryon RA1 grown on different initial maltose concentrations*

Incubation (days)	Sclerotium type	Maltose concentration (mM) 5	10	25	50	100	250
7	Mature	2750	1810	4380	3670	4610	3150
	Immature	2040	2920	12400	12100	9500	6110
14	Mature	5900	3220	39900	14300	13200	8990
	Immature	1940	1890	9160	12400	11000	11500
21	Mature	7050	17000	56900	22200	13000	9490
	Immature	2880	4960	18000	9490	8210	5280

Entries in the Table are average numbers of sclerotia per Petri dish. No sclerotia were formed on media containing 500 mM maltose.

production at 25 mM maltose. The numbers of mature sclerotia gradually increased as the initial maltose concentration was increased to this level, but at higher concentrations the yield was reduced. A similar inhibition was seen in the rate of growth. The progressive production of sclerotia is shown by the increase in sclerotial numbers counted on the two sampling dates, and it is in this respect (other than in the absolute numbers of sclerotia formed) that differences between the strains are also obvious. RA1 shows a considerable increase in sclerotial numbers during the second week of incubation on maltose concentrations above 25 mM, whereas in the other strains the greatest increases in sclerotium numbers during this time occurred on media which initially contained less than 25 mM maltose. Detailed investigation was made of strain RA1 over a 21-day period using the same maltose concentrations. Sclerotium initials were first seen after 96 h incubation, and by the seventh day it was easy to distinguish and count mature and immature sclerotia (Table 3). An outstanding feature of these data is that at concentrations up to about 25 mM (which is again identified as optimum right up to the twenty-first

Table 4. *Effect of different sources of nitrogen on growth rate and sclerotium production by the dikaryon RA1*

Nitrogen source	Growth rate (μm/h)	Mature sclerotia on Day 14	Day 21
25 mM-ammonium tartrate	414	4820	18700
50 mM-ammonium chloride	377	Not done	6500
25 mM urea	618	2110	6040
25 mM asparagine	632	14300	60000

Table 5. *Comparison of growth rates and sclerotium production of two strains of* Coprinus cinereus *grown on agar media containing different initial concentrations of ammonium tartrate*

Strain		Ammonium tartrate concentration (mM)						
		5	10	25	50	100	250	500
RA1 (dikaryon)	Mature sclerotia counted on day 7	39000	6570	4380	442	0	0	0
	Mature sclerotia counted on day 14	56400	38300	39900	5050	0	0	0
	Growth rate (μm/h)	673	660	637	560	342	100	44
ZBw601/40,40 (monokaryon)	Mature sclerotia counted on day 7	8400	25300	1890	0	0	0	0
	Mature sclerotia counted on day 14	96500	67200	103000	4650	30	0	0
	Growth rate (μm/h)	328	302	289	210	90	89	0

Table 6. *Sclerotium production by the dikaryon RA1 grown on different initial concentrations of ammonium tartrate*

Incubation (days)	Sclerotium	Ammonium tartrate concentration (mM)					
		5	10	25	50	100	250
7	Mature	39000	6570	4380	442	0	0
	Immature	4050	11700	12400	13200	9640	0
14	Mature	56400	38300	39900	5050	0	0
	Immature	5140	9850	9160	6160	18200	0
21	Mature	58600	41200	56900	6390	90	0
	Immature	3500	4400	18000	28400	34100	0

day) maturation required about seven days. The number of mature sclerotia on day 14 closely approximates the sum of matures and immatures counted on day seven. The same is essentially true at concentrations above 25 mM, but the picture is quite different at the 25 mM concentration, for the sum of the day seven counts represents less than 50% of the number of mature sclerotia counted on day 14, implying that the initiation–maturation cycle takes less than seven days at this concentration. A final point is that at initial concentrations of 50 mM and below sclerotium production continues for three weeks, whereas at higher maltose concentrations a plateau is apparently attained after two weeks, as the situation on the twenty-first day differs little from that of day 14. The carbon source evidently controls sclerotium formation in a fairly direct manner. As the concentration is increased so the yield of sclerotia increases up to an optimum concentration of 25 mM. This concentration is optimal for the rate of sclerotium formation as well as for the final numerical yield. Substrate inhibition was apparent at higher concentrations; fewer sclerotia were formed and not all of the initials finally matured. The strains differed in their sensitivity to this inhibition, RA1 being the least sensitive.

Nature and concentration of the nitrogen source

C. cinereus will not grow on nitrate, requiring nitrogen in a reduced or organic form. Table 4 records growth rates and sclerotium yields of RA1 grown with four nitrogen sources. Asparagine was apparently the best nitrogen source, supporting very good mycelial growth and a large number of sclerotia. However, further work was done using ammonium tartrate so as to avoid contribution to the carbon pool; the tartrate ion is not used as a source of carbon, while the carbon skeleton of asparagine is utilized synergistically in this way (Moore, 1969). Assessment of the effect of varying the initial concentration of ammonium tartrate (Tables 5, 6) revealed that sclerotium production was enhanced at very low concentrations. Concentrations of 100 mM and above inhibited sclerotium production; extension growth rate was also affected but sclerotium formation was much more seriously reduced. It is clear that sclerotium development demands very low quantities of nitrogen, but until the initial concentration reaches a level which very seriously inhibits mycelial growth the major effect of ammonium is one which is exercised on the progress of the morphogenetic process. Table 6 shows that over a

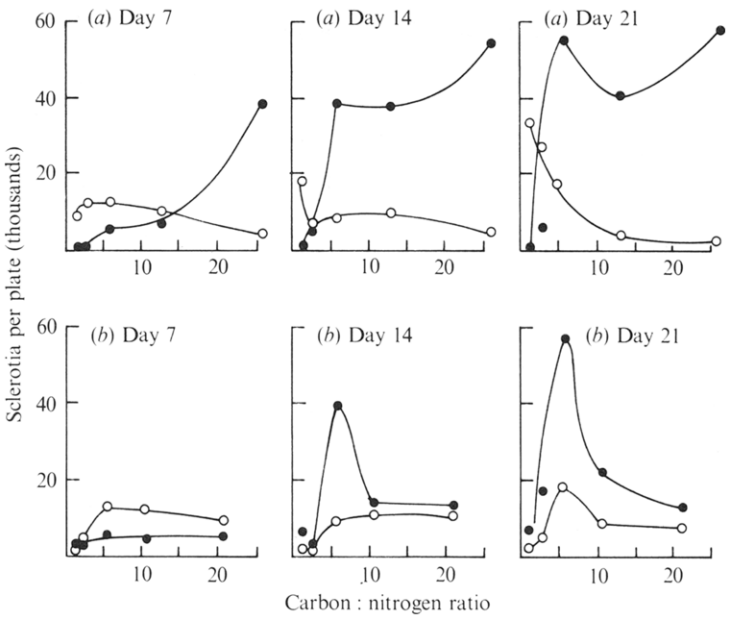

Fig. 1. Sclerotium production of strain RA1 expressed as a function of the carbon:nitrogen ratio. In each case the abscissa shows the C:N ratio which in (*a*) was altered by maintaining the initial carbon concentration (at 25 mM maltose) and changing the ammonium tartrate concentration, while in (b) the initial ammonium tartrate concentration was held at 25 mM and the maltose concentration was varied. The ratios were calculated from maltose and ammonium tartrate concentrations expressed respectively as g carbon/litre and g nitrogen/litre, the tartrate ion being ignored. After 7, 14 and 21 days incubation the numbers of mature (closed symbols) and immature (open symbols) sclerotia formed by colonies growing on the different media were separately counted.

considerable range of concentrations the final yield of both mature and immature sclerotia is relatively little changed; the final total count of matures plus immatures reduces from about 60000/plate to about 30000/plate as the initial ammonium level is increased from 5 to 100 mM, and the decline in growth rate (673 μm/h to 342 μm/h) may account for this. However, at low ammonium concentrations the initiation–maturation cycle is considerably accelerated, the yield from two weeks incubation on 5 mM ammonium tartrate is not attained until the third week on 25 mM ammonium tartrate, and at higher concentrations although the numbers of initials produced are not seriously reduced their maturation is completely prevented; the count of mature sclerotia declining from 60000 to less than 100 between 5 mM and 100 mM ammonium tartrate.

Because of the complex interrelationships between carbon and nitrogen metabolism it may be that the carbon to nitrogen ratio is more important than the concentrations of individual compounds. The data of Tables 3 and 6 are summarized in this form in Fig. 1. These displays emphasize the differences between the two primary metabolites and, incidentally, show that in this system at least the metabolite concentration is of major importance and that it can be misleading to ascribe any importance to a particular C:N ratio without specifying the concentrations.

DISCUSSION

The experiments described here show that the nutritional requirements for sclerotium production by *C. cinereus* are quite similar to those of other fungi. Mono- and disaccharides have generally been identified as being particularly favourable for both mycelial growth and sclerotium production (Page, 1956; Townsend, 1957; Henis, Chet & Avizohar-her-Shenzon, 1965). It is also generally true that increasing the concentration of the carbon source enhances the yield of sclerotia though high concentrations tend to inhibit, retarding maturation rather more than initiation. Less attention has been paid to the effects of the nitrogen source, so generalization is less easy. Dependence of sclerotium formation on nitrogen concentration has been demonstrated

(Wheeler & Sharan, 1965) and inhibition by high nitrogen concentrations is not unusual (Townsend, 1957). However, the exceedingly clear distinction between the ways in which carbon and nitrogen sources affect sclerotium morphogenesis which is evident in these experiments with *C. cinereus* is rather unusual. Ammonium is demonstrated to have a fundamental regulatory influence on the progress of sclerotium morphogenesis. Something similar has been demonstrated in *Botrytis* by Peiris (1947), who found that sclerotium production increased when glucose concentration was increased, while the production of conidia was improved by increases in the peptone concentration. In *C. cinereus* the sclerotial behaviour seems to correlate with events which accompany sporophore development. Sclerotia and sporophores are both produced by the dikaryon, and in artificial culture they are each formed quite readily. They are also, apparently, alternate outcomes of a single initiating process. The two morphogenetic pathways must be in danger of competing for metabolite supplies, yet in the wild one would expect there to be a need for avoiding such internecine competition. Indeed, consideration of the biological functions of the two structures would lead to the prediction that the large-scale dispersal of genetically recombinant spores offered by the sporophore would take precedence over simple preservation of the parental colony by the formation of relatively few sclerotia. A previously puzzling aspect of sporophore physiology provides a mechanism which could achieve this sort of balance between the two pathways. We have shown that sporophore production coincides with exhaustion of the reducing sugar and α-amino nitrogen supplies of the medium (Stewart & Moore, 1974). However, the same analyses revealed that the concentration of ammonia in the medium *increased* by a factor of three over the four to five days between first appearance of sporophore initials and first discharge of mature spores. The reasons for this phenomenon and the metabolic origin of the ammonia are unknown, but one consequence of this behaviour will be that the development of sporophores, because of their excretion of ammonia, will delay sclerotium maturation. Since sclerotia and sporophores initiate through the same pathway, control of initiation would be ineffective in sequencing the developmental alternatives. However, the dependence of sclerotial maturation on ammonium concentration coupled with the peculiar excretion of ammonia by sporophores will facilitate such sequencing. As a result preference will be given to sporophore development, and only when conditions are no longer favourable to this more demanding pathway will sclerotium maturation be released from its inhibition.

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