Effects of Lactose, Ethanol and Cycloheximide on the Translocation Pattern of Radioactive Compounds and on Sclerotium Formation in *Sclerotium rolfsii*

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SUMMARY

Protein synthesis and translocation of L-[^{14}C]leucine, D-[^{14}C]glucose and inorganic ^{32}P were enhanced in cultures of *Sclerotium rolfsii* Sacc. grown on medium supplemented with 0.5% lactose. Ethanol (2%, v/v) inhibited translocation but not protein synthesis. Neither lactose nor ethanol affected the uptake of radioactive substances by the fungal mycelium. Cycloheximide applied to the colony margins prevented sclerotium formation and protein synthesis without inhibiting translocation. It was concluded that translocation and protein synthesis in *S. rolfsii* are independent processes and are both essential for sclerotium formation.

INTRODUCTION

The growth and development of fungi are intimately linked with the translocation of materials within hyphae (Zalokar, 1959; Watkinson, 1971*a*). Studies with labelled materials indicate that, with certain sclerotium-forming fungi, translocation often results in the accumulation of substances in the developing sclerotia. For example, Littlefield, Wilcoxson & Sudia (1965) showed that translocated ^{32}P accumulated in sclerotia of *Rhizoctonia solani*. Translocation was resumed when sclerotial primordia formed, but not in cultures that formed no sclerotia nor in those from which sclerotia were removed before ^{32}P application. The age, size and location of sclerotia had no consistent effect on the degree of accumulation (Littlefield, 1967). Wilcoxson & Subbarayudu (1968) found that ^{32}P was translocated and accumulated in sclerotia of *Sclerotium rolfsii* and to a significantly greater extent when the direction of translocation was from the old to the young portion of the cultures than when in the reverse direction. Accumulation of ^{14}C -labelled iodoacetic acid in sclerotia of *S. rolfsii* was reported by Chet, Henis & Mitchell (1966).

The purpose of this work was to study the relationship between translocation and sclerotium formation in *Sclerotium rolfsii* under conditions which led to synchronous formation of sclerotia (Okon, Chet & Henis, 1972).

METHODS

Organism and growth conditions. *Sclerotium rolfsii* Sacc. (isolated from sugar beet) was grown on a synthetic agar medium (SM), containing (g/l): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; K_2HPO_4 , 0.9; KCl, 0.2; NH_4NO_3 , 1.0; glucose, 15; Fe^{2+} , 0.002; Mn^{2+} , 0.002; Zn^{2+} , 0.002; thiamine hydrochloride, 0.0001; agar (Difco), 20; in d distilledwater; pH 6.8.

Agar discs (1 cm diam.) covered with mycelium were cut from 5-day-old cultures and

Fig. 1. Diagram of Petri dish culture used for studying translocation in mycelium of *Sclerotium rolfii*.

used as inocula. Plates were incubated at 30 °C. Each treatment within an experiment was replicated six times and each experiment was repeated four times.

Labelled compounds. L-[¹⁴C]Leucine (specific activity, 10 mCi/mM) and D-[¹⁴C]glucose (specific activity, 2.9 mCi/mM) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Inorganic ³²P was obtained from the Nuclear Research Centre, Dimona, Israel.

Translocation and distribution of labelled compounds. A sterile plastic cup (1.2 cm diam. with walls 4 mm high) was placed in the centre of each Petri dish containing 10 ml SM. It was filled with 0.5 ml of the same medium and inoculated with a 1 cm disc. The fungus was grown for 4 days. Labelling was done by adding a distilled water-agar (2 %) disc (1 cm diam.) containing 0.2 µCi of radioactive substance (Fig. 1).

Agar discs (0.8 cm diam.) covered with mycelium (but not sclerotial initials) were taken from the margins of the growing cultures, at intervals, and tested for radioactivity. 'Reversed' translocation was measured by labelling the colony margins with a disc of radioactive agar, and testing agar discs covered with mycelium sampled from the colony centre.

Radioactivity of the discs was measured by a Packard Liquid Scintillation Spectrometer (model 3003). Radial strips (0.5 cm wide) of agar covered with mycelium were cut from the colony 24 and 48 h after labelling and dried by infrared light on Whatman no. 1 filter paper. The distribution of radioactivity along the strip was measured by a Packard Radiochromatogram Scanner (model 7200).

To evaluate the extent of passive diffusion of radioactive compounds into the agar, the fungus was grown on a cellophane membrane placed on the agar and labelled as described above. Agar discs were taken from beneath the cellophane and their radioactivity measured. The possibility of passive transport of radioactive compounds was examined by following translocation in cultures which had been killed by chloropicrin.

Uptake and incorporation of labelled compounds. The fungus was grown for 4 days on a cellophane membrane placed on the surface of a synthetic agar medium containing lactose (0.5 %), ethanol (2 %, v/v) or both. The cellophane with the fungus was transferred to plates containing 20 ml liquid medium, supplemented with leucine or glucose (each at 0.1 µCi/ml), and glass beads as a mechanical support. After 2 h the mycelium was collected from the cellophane, washed with 0.1 M-phosphate buffer (pH 7.0) until no radioactivity could be detected in the buffer, dried at 80 °C for 24 h, weighed and tested for radioactivity.

Incorporation of L-[¹⁴C]leucine into trichloroacetic acid-insoluble material was determined after homogenization of the outer 5 mm of the colony margins by Omnimixer (Sorvall) for

2 min at 8000 rev./min. The homogenate was centrifuged at 10000 g for 10 min. Trichloroacetic acid (final concentration 5%, v/v) was added to the supernatant and the resulting precipitate washed three times with trichloroacetic acid (5%) by centrifugation. Protein content of the pellet was determined with the Folin phenol reagent prepared as described by Lowry, Rosebrough, Farr & Randall (1951).

Extraction and analysis of free amino acids. A culture of *Sclerotium rolfii* was grown on a cellophane membrane placed on SM supplemented with lactose, ethanol or both. After 3 days of incubation, hyphal tips were collected at a distance of 5 mm from the colony margin, homogenized for 2 min with 3 ml of 5×10^{-3} N-HCl (pH 2.5) and centrifuged at 15000 g for 10 min. The supernatant was mixed with trichloroacetic acid (final concentration 5%, v/v) and centrifuged again at 5000 g for 5 min. It was then washed five times with diethyl ether and lyophilized. The residue was dissolved in 2 ml 0.2 N-citrate buffer (pH 2.2) and analysed with a Bio Cal-200 amino acid analyser.

The effect of cycloheximide on sclerotium formation. A circle of Whatman no. 1 filter paper, 0.5 cm wide and 6.0 cm in internal diam., was soaked with 0.2 ml cycloheximide solution (5 mg/ml), air-dried and placed around a 3-day-old colony of *Sclerotium rolfii*. The central region of the agar was isolated by a plastic cup (3 cm diam.).

RESULTS

Effect of lactose and ethanol on translocation. *Sclerotium rolfii* was grown on SM supplemented with lactose, ethanol or both. Labelled compounds were added to the central cup when the colony diameter reached 50 mm on the medium containing lactose or 80 mm on a medium without lactose.

Translocation patterns of radioactivity originating from leucine, glucose and phosphate are shown in Fig. 2.

Passive absorption by the mycelium and diffusion of radioactivity in controls of dead mycelium and agar discs, taken from beneath the cellophane membrane, did not exceed 5%, as compared with living mycelium.

No 'reversed' translocation could be detected. In general, translocation of radioactivity within the fungus was greater when grown on SM+lactose than on SM+lactose+ethanol (Fig. 2). No significant differences in translocation were observed when ethanol alone was added to SM (Fig. 2). When the fungus was grown on SM without ethanol, most of the radioactivity was translocated into sclerotial initials which were not formed in ethanol-containing medium (Table 1).

Application of cycloheximide to the margins of a colony grown on lactose resulted in inhibition of sclerotium formation. However, no effect on translocation was observed (Fig. 2).

Distribution in the fungal mycelium of radioactivity originating from various labelled compounds 48 h after labelling is shown in Fig. 3. Similar patterns were observed after 24 h.

No significant variation was observed among replicates.

Uptake of leucine and glucose. Cellophane-grown cultures were incubated on a liquid medium containing L-[14 C]leucine or D-[14 C]glucose. No significant differences in uptake of radioactivity ($1520 \pm 8\%$ and $800 \pm 6\%$ c.p.m./mg dry wt, respectively) could be detected between cultures grown on SM, SM+lactose, SM+ethanol or SM+lactose+ethanol.

Incorporation of L-[14 C]leucine into the trichloroacetic acid-insoluble mycelial fraction increased in fungus grown on SM+lactose compared to the synthetic medium, and was significantly inhibited by cycloheximide (Table 2).

Fig. 2. The effect of lactose, ethanol and cycloheximide on the translocation of L-[^{14}C]leucine (a); [^{14}C]glucose (b); and inorganic ^{32}P (c) in *Sclerotium rolfsii*. Synthetic medium (SM), ●—●; SM+ethanol (2 %, v/v), ○—○; SM+lactose (0.5 %, w/v), ■—■; SM+lactose+cycloheximide, □—□; SM+lactose+ethanol, △—△.

Table 1. Effect of ethanol on accumulation of radioactive compounds in mycelium and sclerotial initials of *Sclerotium rolfsii*

Growth medium	Structure	Radioactive compounds (c.p.m./mg dry wt)		
		[^{14}C]Leucine	[^{14}C]Glucose	[^{32}P]Phosphate
SM	Initials	690	190	110
	Mycelium	114	90	33
SM+ethanol 2 % (v/v)	Mycelium	48	52	33

Fig. 3. The distribution of L-[¹⁴C]leucine (a), [¹⁴C]glucose (b), and inorganic ³²P (c) along dried radial strips of *Sclerotium rolfii* mycelium as detected by a Radiochromatogram Scanner. Synthetic medium (SM), 1; SM + ethanol (2 %, v/v), 2; SM + lactose (0.5 %, w/v), 3; SM + lactose + ethanol, 4. Radioactivity in radial strips was measured from colony centre (C) to colony margins (M). Petri dish walls, W.

Table 2. Incorporation of L-[¹⁴C]leucine into the trichloroacetic acid-insoluble fraction in *Sclerotium rolfii*

Growth medium	Radioactivity (c.p.m./mg protein)
SM	5285 b*
SM + lactose 0.5 % (w/v)	17540 a
SM + lactose + cycloheximide	1300 c
SM + ethanol 2 % (v/v)	4990 b
SM + lactose + ethanol	14000 a

* Numbers accompanied by the same letter did not differ significantly at the 5 % probability level, using Duncan's multiple-range test.

Table 3. *Amino acid pool composition ($\mu\text{g}/\text{mg}$ dry wt) in colony margins of *Sclerotium rolfsii* grown on different media*

	Growth medium					
	SM	SM + cycloheximide	SM + lactose	SM + lactose + cycloheximide	SM + ethanol	SM + lactose + ethanol
Asp	6.6	9.3	5.1	4.9	5.6	2.7
Thr	1.5	2.1	1.1	1.7	2.1	1.9
Ser	4.0	5.7	1.6	2.2	2.3	0.8
Glu	20.6	32.3	14.7	35.3	23.0	14.0
Gly	1.0	1.4	0.8	1.4	1.2	1.0
Ala	3.5	8.9	1.3	2.2	3.7	1.1
Cys	0.9	0.7	0.4	0.9	1.4	0.6
Val	0.6	1.4	1.0	1.6	1.7	0.9
Met	0.5	0.6	0.1	0.4	0.2	0.1
Ile	0.6	0.9	0.4	1.0	0.4	0.2
Leu	1.2	3.0	0.9	1.9	1.3	0.5
Tyr	2.0	4.9	0.9	3.6	2.1	1.0
Phe	2.1	8.5	1.5	3.3	2.2	1.3
Lys	1.2	1.8	0.6	1.1	1.6	1.1
His	1.6	1.9	0.5	1.4	2.7	0.4
Arg	2.6	2.3	1.6	2.7	4.5	2.7
Total	50.5	85.7	32.5	65.6	56.0	30.3

Free amino acid content of hyphal tips. The free amino acid pool in the colony margins of the fungus grown on lactose medium was significantly lower than that of a colony grown on a non-supplemented medium. On the other hand, cycloheximide appreciably increased the free amino acid pool in both treatments (Table 3).

DISCUSSION

Wilcoxson & Subbarayudu (1968), working on fungal translocation, noted that their data did not completely separate absorption by mycelium from translocation. Watkinson (1971*b*), who used the split-plate method of Lucas (1960), killed the mycelium and showed that there was no diffusion between the two halves of the plate.

The techniques described in this paper provide an efficient method for studying both distribution (by means of the Radiochromatogram Scanner) and translocation of radioactive compounds in fungi and enable a distinction to be made between absorption by mycelium, diffusion in agar and translocation.

Translocation of various radioactive compounds was enhanced in cultures grown on lactose-supplemented (as compared with a non-supplemented) medium, could be detected before the onset of any morphological changes, and accompanied sclerotium formation. This enhanced translocation, with the concomitant partial inhibition of mycelial linear growth, could be the cause of the formation of a circle of sclerotia at the colony margins.

In contrast to lactose, ethanol drastically decreased translocation of the compounds tested with only a slight inhibition of linear growth (Okon *et al.* 1972), but with a total inhibition of sclerotium production. Neither lactose nor ethanol affected uptake of radioactive compounds by the fungal mycelium. These results support the suggestion that internal translocation is related to morphogenesis (Zalokar, 1959; Watkinson, 1971*a*).

In addition to its effect on translocation, lactose induced a preferential incorporation of [¹⁴C]leucine into trichloroacetic acid-insoluble material at the colony margins, with a concomitant decrease in the free amino acid pool, indicating an increase in protein synthesis before the appearance of sclerotial initials at this site.

Though it affected translocation, ethanol did not affect the composition and level of the amino acid pool nor leucine incorporation into the trichloroacetic acid-insoluble fraction.

Application of cycloheximide to colony margins prevented sclerotium formation but not the translocation of radioactive compounds, either in the presence or absence of lactose. It can therefore be concluded that lactose stimulates amino acid translocation and incorporation independently, both processes being essential for sclerotium formation in *Sclerotium rolfsii*.

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