Effect of Carbon Dioxide on Growth and Carbohydrate Metabolism in *Sclerotium rolsii*

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SUMMARY

Carbon dioxide at a concentration of 1 to 2 % (v/v) in air enhanced the growth rate and inhibited sclerotium formation in the fungus *Sclerotium rolsii* Sacc. A CO₂ concentration of 10 % inhibited growth. Similar growth patterns were observed when the fungus was grown on a medium supplemented with the fungicide carboxin, which inhibits succinate dehydrogenase. A high CO₂ concentration (1 to 10 %) or growth on carboxin-supplemented medium caused a decrease in succinate dehydrogenase activity and significant increases in isocitrate lyase, isocitrate dehydrogenase, malate synthase and malate dehydrogenase activities. Mycelium of *S. rolsii* grown at a high CO₂ concentration contained less glyoxylate, lipids and glycogen than mycelium grown in air. It is suggested that sclerotium formation in *S. rolsii* requires a balanced supply of carbohydrate intermediates and energy.

INTRODUCTION

It has long been realized that carbon dioxide is required for growth of bacteria, yeasts and filamentous fungi (Rockwell & Higherberger, 1927) and its role in the biochemical processes controlling growth, fructification, sporulation and spore germination of fungi has been reviewed (Cantino & Lovett, 1964; Niederpruem, 1965; Tabak & Cooke, 1968). According to Cantino (1966), bicarbonate or CO₂ induce multiple enzymic lesions in the tricarboxylic acid cycle, increase the activity of isocitrate dehydrogenase and cause replacement of oxidative decarboxylation of isocitrate to α -ketoglutarate by reductive carboxylation of α -ketoglutarate in *Blastocladiella emersonii*. These changes, believed to be the 'trigger' reactions in the morphogenetic response, also indicate an effect of CO₂ on the glyoxylate shunt. Isocitrate is converted to succinate and glyoxylate in *B. emersonii* (Cantino, 1966), *Fusarium* (Willetts, 1972*a*) and *Penicillium* (Willetts, 1972*b*). In *Blastocladiella*, glyoxylate is further transaminated to glycine (Cantino, 1966). Another pathway of glyoxylate metabolism involves its conversion to malate by malate synthase (Willetts, 1972*a, b*; Kritzman *et al.*, 1976). The glyoxylate shunt, however, only operates when there is a shortage of succinate (Krebs & Lowenstein, 1960). The fungicide carboxin (5,6-dehydro-2-methyl-1,4-oxathiin-3-carboxanilide), was studied by White (1971), Georgopoulos, Alexandri & Chrysai (1972), Ulrich & Mathre (1972) and Ben-Yephet, Dinor & Henis (1975), who found that its site of action was the succinic dehydrogenase system. In strains of *Ustilago hordei* tolerant to carboxin, succinate : 2,6-dichlorophenolindophenol reductase activity was inhibited while that of the alternative glyoxylate cycle increased (Ben-Yephet *et al.*, 1975).

Recently, Kritzman *et al.* (1976) demonstrated a relationship between the metabolism of L-threonine via the tricarboxylic acid and glyoxylate cycles, and the morphogenesis of *S. rolsii*. The purpose of this work was to study the effect of CO₂ concentration on carbohydrate metabolism, growth and sclerotium formation in *S. rolsii*.

METHODS

Strain and growth conditions. *Sclerotium rolfsii* Sacc. type R ATCC26326 (Chet & Henis, 1972) was grown at 30 °C on a cellophane membrane in Petri plates (8.5 cm diam.) containing 15 ml of synthetic medium (SM) (Okon, Chet & Henis, 1973). The plates were inoculated in the centre with agar discs (0.5 cm diam.) from a 5-day-old colony. No degradation of the cellophane was observed when *S. rolfsii* was incubated in the presence of glucose for 10 days. The following supplements (final concentrations) were separately added to the growth medium: L-threonine (10 mM); carboxin (0.05 µg ml⁻¹), formulated as dust containing 75 % active ingredients (Uniroyal Chemical Division, Naugatuk, Connecticut, U.S.A.).

Sclerotium rolfsii was grown under controlled atmospheres containing different concentrations of CO₂ in BBL anaerobic jars. Radial linear growth was measured at intervals. Mycelial dry weight was determined after separating the mycelium from the cellophane membrane with tweezers and drying at 80 °C for 24 h.

Enzyme assays. Mycelium (about 3 g wet wt) was homogenized in 3 ml buffer for 1 min at 4 °C using an Ultra-Turrax homogenizer (Janke & Kunkel K.G., Staufen, West Germany). The homogenate was centrifuged at 18 000 g for 20 min at 4 °C. Unless otherwise stated, low molecular weight compounds were removed from the supernatant fluid by overnight dialysis against distilled water at 4 °C. The protein content of the crude extract was determined with the Folin phenol reagent (Lowry *et al.*, 1951). Enzyme activities in the crude extract were determined by published methods: isocitrate dehydrogenase, EC. 1.1.1.42 (Wang, 1955); isocitrate lyase, EC. 4.1.3.1, and glyoxylate dehydrogenase, EC. 1.2.1.17 (Maxwell & Bateman, 1968*b*); succinate dehydrogenase, EC. 1.3.99.1 (Ulrich & Mathre, 1972); malate synthase, EC. 4.1.3.2 (Maxwell & Bateman, 1968*a*); malate dehydrogenase, EC. 1.1.1.37 (Akira, 1969).

Chemical analysis. Oxalate and glyoxylate production were determined according to Maxwell & Bateman (1968*b*). Other analytical methods were: lipid (Chiang, Gessler & Lowry, 1957); glycogen (Van Handel, 1965); glucose (*Sigma Technical Bulletin* no. 510, 1973); and glucosamine (Elson & Morgan, 1933). Hyphal wall preparation and its chemical analysis was carried out as described by Chet, Henis & Mitchell (1967).

L-Threonine uptake and transport. For studies of the uptake and transport of L-[¹⁴C]-threonine, 48-h-old cultures grown on cellophane were transferred to Petri plates containing 10 ml liquid medium and 4 mm glass beads (80 per plate) to support the cellophane. Labelled threonine (specific activity 10 mCi mol⁻¹; The Radiochemical Centre, Amersham) was used at a final concentration in the growth medium of 0.1 µCi ml⁻¹ and the cultures were incubated for 2 h. The mycelium was then separated from the cellophane membrane with tweezers and washed with 0.1 M-phosphate buffer pH 7.0 until no radioactivity could be detected in the buffer. The washed mycelium was dried at 80 °C for 24 h and weighed, and its radioactivity was measured in a Packard Tri-Carb scintillation spectrometer (model 3330).

Carbon dioxide uptake and transport. For studies of the uptake and transport of ¹⁴CO₂, 48-h-old cultures grown on cellophane were transferred for 6 h to BBL anaerobic jars (no. 32160-10) containing 1 % (v/v) CO₂ supplemented with ¹⁴CO₂ [2 mCi (3 l air)⁻¹]. Labelled CO₂ was produced from sodium [¹⁴C]carbonate (specific activity 60 mCi mmol⁻¹; The Radiochemical Centre, Amersham) by addition of conc. HCl. After incubation, the mycelium was separated from the cellophane, washed, dried, weighed and its radioactivity was measured as described above.

Fig. 1. Radial growth rates of *S. rolfsii* in the presence of different CO₂ concentrations when grown on SM (○) and on SM plus 10 mM-L-threonine (△). Results are expressed as percentages of the growth rates in air (0.03 % CO₂). Points represent the mean of 12 determinations from three independent experiments; all S.E.M. lie within the symbols.

Fig. 2. Growth of *S. rolfsii* mycelium (dry wt) in the presence of different CO₂ concentrations on SM (○) and on SM plus 10 mM-L-threonine (△). Results are expressed as percentages of the mycelium growth in air (0.03 % CO₂). Points represent the mean of 12 determinations from three independent experiments; all S.E.M. lie within the symbols.

RESULTS AND DISCUSSION

Sclerotium rolfsii was grown in atmospheres containing different amounts of CO₂. After 10 h incubation in an atmosphere enriched with 0.5 to 2 % (v/v) CO₂, mycelium growth rates had increased up to 400 % compared with cultures grown in air (Fig. 1). The average growth rate of cultures grown in air on SM was 0.78 mm h⁻¹ compared with 0.22 mm h⁻¹ for cultures grown in air on SM plus L-threonine. With 1 % CO₂ the growth rates increased to 1.57 mm h⁻¹ and 0.63 mm h⁻¹, respectively. Increasing the amount of CO₂ also induced more branched aerial mycelium. The presence of 10 mM-L-threonine increases branching of lateral hyphae and enhances sclerotium formation (Henis, Okon & Chet, 1973; Kritzman *et al.*, 1976). Our present findings confirm earlier observations on the lack of correlation between hyphal growth rate and sclerotium formation (Chet & Henis, 1968).

The mass of *S. rolfsii* mycelium increased significantly in the presence of 0.5 to 2.5 % CO₂, with or without L-threonine, and sharply declined in the presence of 7.5 % CO₂.

Fig. 3. Linear growth rate of *S. rolfsii* on SM (○—○); SM in the presence of 1 % CO₂ (○---○); SM plus 10 mM-L-threonine (△—△); SM plus 10 mM-L-threonine in the presence of 1 % CO₂ (△---△). Sclerotium formation is indicated by ▲ or ●. Points represent the mean of 15 determinations from three independent experiments; all s.e.m. lie within the symbols.

(Fig. 2). When cultures grown in air were transferred to atmospheres containing 0.3 % or more CO₂ at any time before the appearance of sclerotial initials, sclerotium formation was totally inhibited (Fig. 3). This indicates that CO₂ inhibits sclerotium formation even in the presence of L-threonine.

Mycelium of *S. rolfsii* grown either under an atmosphere enriched with 1 % CO₂ or in the presence of carboxin, with or without L-threonine, showed reduced succinate dehydrogenase activity (Table 1). Cell-free extracts of *S. rolfsii* mycelium, grown on SM or on SM plus L-threonine in the presence of 1 % CO₂, had, respectively, 38 % and 65 % of the succinate dehydrogenase and 147 % and 208 % of the isocitrate dehydrogenase activities of similar preparations from air-grown fungus. Similar results were obtained with mycelium grown in air on carboxin-supplemented media.

No differences in isocitrate lyase activity were detected in any of the cell-free extracts of *S. rolfsii* grown on SM, SM plus CO₂ or SM plus L-threonine. However, the activity in extracts of the fungus grown on SM plus L-threonine and incubated under an atmosphere containing 1 % CO₂ was 1.9 times higher than that in extracts of the fungus grown in air. The addition of CO₂, L-threonine or both caused a reduction in the glyoxylate content of the fungal mycelium, but significantly increased the activity of malate synthase and malate dehydrogenase. The activity of these two enzymes was similarly increased in mycelium grown on a carboxin-supplemented medium (Table 1).

The activity of glyoxylate dehydrogenase and the amount of oxalate produced were much reduced after 10 h incubation in the presence of 1 % CO₂ (Table 1). There was a correlation of $r = 0.99$ between glyoxylate dehydrogenase activity and the rate of oxalate production [$\mu\text{g h}^{-1} (\text{mg mycelium dry wt})^{-1}$], $y = 0.0667 + 6.3168x$.

Kritzman *et al.* (1976) suggested that hyphal morphogenesis and formation of sclerotia in *S. rolfsii* require an increased supply of carbohydrate intermediates and energy and that these are mainly supplied by the glyoxylate pathway. Spore formation and fruiting in other fungi are also affected by atmospheres with increased amounts of CO₂ (Tabak & Cooke, 1968). According to Plunkett (1954, 1956), CO₂ accumulation inhibits sporophore development in *Collybia velutipes*. Niederpruem (1963) found an increase in the vegetative growth

Table 1. *Effect of supplementation with CO₂ (1 %, v/v), carboxin (0.05 µg ml⁻¹) and L-threonine (10 mM) on the activity of some enzymes and products of the tricarboxylic acid and glyoxylate cycles*

Results are the mean of five independent experiments, each carried out with three replicates of the same preparation.

Supplement	Succinate dehydrogenase		Isocitrate dehydrogenase		Isocitrate lyase		Malate synthase		Malate dehydrogenase		Glyoxylate dehydrogenase		Glyoxylate		Oxalate	
	Units ^a	%	Units ^b	%	Units ^c	%	Units ^d	%	Units ^e	%	Units ^f	%	Units ^g	%	Units ^h	%
None	533.9	100	0.059	100	0.867	100	0.079	100	0.072	100	0.278	100	128.1	100	1.70	100
CO ₂	202.2	37.9	0.087	147	0.878	101.2	0.160	202.5	0.127	176.3	0.099	35.6	86.3	67.3	0.65	38.2
Carboxin	375.5	70.3	0.103	174.5	—	—	0.163	206.3	0.113	156.9	0.095	34.1	—	—	0.64	37.6
CO ₂ + carboxin	278.8	52.2	0.205	347.4	—	—	0.488	617.7	0.146	202.7	0.097	34.8	—	—	0.58	34.1
L-Threonine	516.4	100	0.125	100	0.975	100	1.083	100	0.075	100	0.027	100	73.6	100	0.13	100
L-Threonine + CO ₂	338.1	65.4	0.260	208	1.875	192.3	1.252	115.7	0.161	214.6	0.047	174.0	64.3	87.3	0.23	176.9
L-Threonine + carboxin	202.4	39.2	0.195	156	—	—	0.972	89	0.148	197.3	0.042	155.5	—	—	0.20	153.8
L-Threonine + CO ₂ + carboxin	182.4	35.3	0.238	190.4	—	—	0.859	79.3	0.157	209.3	0.046	170.3	—	—	0.25	192.3

a, µg succinate (mg protein)⁻¹ min⁻¹ (S.E.M. ± 0.15); *b*, absorbance at 340 nm (mg protein)⁻¹ min⁻¹ (S.E.M. ± 0.009); *c*, µg glyoxylate (mg protein)⁻¹ min⁻¹ (S.E.M. ± 0.02); *d*, absorbance at 412 nm (mg protein)⁻¹ min⁻¹ (S.E.M. ± 0.003); *e*, µmol malate (mg protein)⁻¹ min⁻¹ (S.E.M. ± 0.005); *f*, absorbance at 340 nm (mg protein)⁻¹ min⁻¹ (S.E.M. ± 0.009); *g*, µg glyoxylate (mg protein)⁻¹ (S.E.M. ± 0.4); *h*, µg oxalate h⁻¹ (mg dry wt mycelium)⁻¹ (S.E.M. ± 0.005).

Table 2. *Effect of CO₂ (1 %, w/v) on the amounts of cell wall, protein, glycogen, free glucose and lipids in S. rolfsii grown on SM with or without L-threonine (10 mM)*

Results are the mean of five independent experiments, each carried out with three replicates of the same preparation.

Supplement	Cell wall ^a	Protein ^b	Glycogen ^c	Free glucose ^d	Lipid ^e
None	68.29	7.87	0.238	0.0073	0.04
CO ₂	46.80	8.72	0.069	0.0032	0.03
L-Threonine	73.33	8.78	0.113	0.0066	0.08
L-Threonine + CO ₂	68.88	9.08	0.053	0.0011	0.037

^a, Dry wt, as a percentage of dry wt mycelium (S.E.M. $\pm$ 0.05); ^b, percentage of dry wt mycelium (S.E.M. $\pm$ 0.01); ^c, mg glucose (mg dry wt mycelium)⁻¹ (S.E.M. $\pm$ 0.003); ^d, mg glucose (mg dry wt mycelium)⁻¹ (S.E.M. $\pm$ 0.0001); ^e, mg lipid (mg dry wt mycelium)⁻¹ (S.E.M. $\pm$ 0.005).

of *Schizophyllum commune* in sealed chambers, but an inhibition of fruiting: this inhibition was reversed on aeration.

Bettelheim & Gay (1963) suggested that, in yeast, sporulation increased when a high level of glyoxylate was formed. Acetate alone, however, did not supply enough energy and intermediates to meet the level required for sporulation. We have found that the presence of carbon dioxide also caused a reduction in the amounts of mycelial glucose, reserve materials (glycogen, lipids) and wall material, but had no effect on the protein content (Table 2). There were no qualitative or quantitative differences in the chemical constituents (carbohydrates, proteins, lipids and ash) detected in walls of *S. rolfsii* grown in 1 % CO₂ or in air. The relatively high amount of lipid present in *S. rolfsii* grown on L-threonine can be explained by the direct incorporation of L-threonine into the lipid fraction. This was demonstrated by the uptake of labelled threonine into the lipid fraction (2319 c.p.m. mg⁻¹) during 2 h incubation.

When incubated in an atmosphere containing 1 % CO₂ supplemented with labelled CO₂, *S. rolfsii* mycelium incorporated 734 c.p.m. (mg dry wt mycelium)⁻¹ h⁻¹ on SM and 2135 c.p.m. (mg dry wt mycelium)⁻¹ h⁻¹ on SM plus L-threonine. The distribution of radioactivity in the SM-grown mycelial fractions [c.p.m. (mg dry wt mycelium)⁻¹] was as follows: wall, 76; material insoluble in trichloroacetic acid, 137; material soluble in trichloroacetic acid, 1975. The radioactivities of the oxalic acid fractions excreted by *S. rolfsii* grown on SM and SM plus L-threonine in the presence of ¹⁴CO₂ were 1078 and 354 c.p.m. (mg dry wt mycelium)⁻¹ respectively. When grown on SM in 1 % CO₂, *S. rolfsii* produced 2.8 times more oxalic acid than when grown on SM plus L-threonine (Table 1). Similarly, the radioactivity of the oxalic acid produced in the presence of ¹⁴CO₂ on SM was three times that produced on L-threonine.

A scheme based on the data presented, which would explain the biochemical basis of the morphogenetic effect of CO₂ on *S. rolfsii*, is shown in Fig. 4. According to this scheme, CO₂ is not only incorporated into cell constituents but also inhibits the tricarboxylic acid cycle, thus affecting both the type and amounts of carbohydrate intermediates. The fungus overcomes the partial inhibition of the tricarboxylic acid cycle by operating the alternative glyoxylate pathway. In this respect, the effect of CO₂ on the tricarboxylic acid and glyoxylate cycles is similar to that of carboxin which inhibits succinate:2,6-dichlorophenolindophenol reductase (Ulrich & Mathre, 1972) and sclerotium formation. Only one of the two possible metabolic pathways was operating (glyoxylate shunt, Fig. 4), and no sclerotia were formed in the presence of high CO₂ concentrations. A few sclerotia were formed on SM and, in that case, only one pathway was operating (the tricarboxylic acid cycle, Fig. 4). Increasing

Fig. 4. A metabolic scheme summarizing the tricarboxylic acid and glyoxylate cycles in *S. rolfsii* as operated under the conditions indicated.

numbers of sclerotia were formed in the presence of L-threonine which activates both the glyoxylate and the tricarboxylic acid cycles, while inhibiting the production and release of oxalic acid by this fungus (Kritzman *et al.*, 1976).

The data indicate that the simultaneous operation of the tricarboxylic acid and glyoxylate cycles in *S. rolfii* supplies increased levels of energy and carbohydrate intermediates at a balance which may be essential for sclerotium formation.

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