The Effect of Carbon Dioxide on Fruiting and on the Degradation of a Cell-wall Glucan in *Schizophyllum commune*

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A concentration of 5 % carbon dioxide in air prevents fruiting in *Schizophyllum commune* and at the same time prevents the breakdown of R-glucan [(1→3)-β, (1→6)-β-glucan] in the wall which normally occurs during growth of the pilei. R-glucanase is produced as in cultures grown in air but cultures grown in CO₂-enriched air form more of a water-soluble (1→3)-β, (1→6)-β-glucan (mucilage) and less R-glucan. This R-glucan is more resistant to enzymic hydrolysis which may explain the decreased breakdown of R-glucan *in vivo*. Since R-glucan degradation is required for growth of the pilei to occur, CO₂ must indirectly inhibit growth of the pilei by changing the composition of the walls in the previously formed mycelium.

INTRODUCTION

In basidiomycetes an increase in the CO₂ content of the ambient air impairs fruit body formation (Taber, 1966). The effect has been especially well studied in *Agaricus bisporus* (Tschierpe & Sindén, 1964) where CO₂ concentrations as low as about 0.1 % (v/v) cause a retardation of the onset of fruiting and a decrease in the number of primordia formed. In *Schizophyllum commune*, 5 % CO₂ in air does not affect mycelial growth and mating but completely inhibits fruiting (Niederpruem, 1963). The biochemical basis of these CO₂ effects remains to be established. For *A. bisporus* it has been suggested (Rast & Bachofen, 1967; Bachofen & Rast, 1968) that inhibition of fruiting by CO₂ is correlated with an impairment of the Krebs cycle activity produced in much the same way as was later proposed for self-inhibition of spore germination in this fungus (Rast *et al.*, 1976*a, b*), i.e. inhibition of succinate dehydrogenase by the carboxylation product oxaloacetate, thus creating a metabolic situation in which the carbon flow through the glycolytic and Krebs cycle pathways becomes restricted.

In dikaryons of *S. commune*, growth of the pilei takes place after exhaustion of the glucose in the medium and is accompanied by a decrease in the wall polysaccharide R-glucan in the vegetative mycelium and stunted fruit bodies (Wessels, 1965). The R-glucan, a (1→3)-β, (1→6)-β-linked alkali-insoluble glucan that occurs in close association with chitin in the wall (Sietsma & Wessels, 1977), is degraded by R-glucanase, the activity of which increases after glucose exhaustion (Wessels, 1966; Wessels & Niederpruem, 1967). Mobilization of endogenous reserves during fruit body expansion, including degradation of wall polysaccharides, has also been demonstrated in the agaric *Flammulina velutipes* (Kitamoto & Gruen, 1976).

The present study was undertaken to see if the effect of CO₂ on fruiting in *S. commune* is related to an inhibition of R-glucan degradation.

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METHODS

Organism and growth conditions. A dikaryon of *Schizophyllum commune* was constituted by mating strains 699 (A41 B41) and 845 (A51 B51). A macerate of the dikaryon was inoculated into 1 l Erlenmeyer flasks containing 100 g sand and 30 ml minimal medium (Wessels, 1965). The cultures, in triplicate, were incubated at 24 °C under continuous illumination and were continuously flushed with air or air containing 5 % CO₂ at 40 l h⁻¹. In some experiments, designed to study the effect of CO₂ on morphology and not involving chemical determinations, agar plates were inoculated with a macerate of the dikaryon and incubated in an atmosphere of CO₂-free air (KOH trap) or air containing 5 % CO₂.

Chemical analysis. Mycelium and medium were collected from each culture by carefully decanting the mycelium from the sand on a filter and washing both the mycelium and the sand with water. The washwater containing the medium and excreted materials was made up to a known volume and used for the determination of residual glucose (with the glucostat reagent; Worthington Biochemical Corp.), polysaccharide (as anthrone-positive material minus the glucose content) and R-glucanase activity (see below). The washed mycelium was fragmented by passing it through an X-press (Biotec, Stockholm, Sweden) and walls were prepared as described previously (Wessels & Marchant, 1974). The mucilage and the various wall components (Wessels *et al.*, 1972; Sietsma & Wessels, 1977) were then extracted and quantified.

Mucilage, a (1→3)- β -(1→6)- β -glucan excreted into the medium, was determined as the sum of the glucan excreted into the medium and the glucan dissolving when the hyphae were fragmented with the X-press. Thus these measurements also included other water-soluble glucans, e.g. glycogen. However, the amount of glycogen was small since β -amylase [free of (1→3)- β -glucanase] digested less than 1 % of the water-soluble glucans.

S-glucan [(1→3)- α -glucan] was determined as the glucan extracted from the wall fraction with 1 M-KOH (20 min, 60 °C) and precipitated by adding acetic acid to the extract to pH 5.

R-glucan [(1→3)- β -(1→6)- β -glucan] was extracted from the wall residue after S-glucan extraction with 0.5 M-HCl (100 °C, 1 h) followed by 1 M-KOH (20 min, 60 °C). All glucan fractions were quantified with the anthrone reagent (Fairbairn, 1953).

Chitin was determined by hydrolysis of the residue remaining after S-glucan and R-glucan extraction with 6 M-HCl (120 °C, 2 h) and estimating the glucosamine content of the hydrolysate using the method of Johnson (1971).

Enzymic analysis. A crude R-glucanase preparation was isolated from the culture filtrate of *S. commune* strain k8 as described by Wessels (1969). Exo-(1→3)- β -glucanase was purified from the culture filtrate of the basidiomycete QM806 (Sietsma & Wessels, 1977). The culture filtrate of *S. commune* was tested for R-glucanase activity using an R-glucan/chitin preparation from strain 699 as a substrate. The R-glucan from strain 699 is very similar to that of the dikaryon 699 × 845 (Sietsma & Wessels, 1977).

The enzymic assays were done in 1 ml 0.05 M-sodium acetate buffer pH 5.8 containing either 0.5 mg enzyme or 0.5 ml culture filtrate and 10 mg R-glucan (added as R-glucan/chitin or as cell walls); incubation was at 35 °C for 1 h. The reaction was stopped by adding 1 ml 0.15 M-Ba(OH)₂ and 1 ml 5 % (w/v) ZnSO₄. The precipitate was sedimented by centrifuging and the supernatant was tested for solubilized anthrone-positive materials.

RESULTS

When *S. commune* 699 × 845 was grown in air, numerous primordia were produced after 6 days growth. At that time only 1 to 2 % of the glucose in the medium was left (Table 2). When the cultivation time was extended to 12 days some of the fruit body primordia expanded, that is the pilei attained their final size and form by unilateral growth of the hymenial surface. However, when the organism was grown in air containing 5 % CO₂, a thick mycelial mat was formed with aerial hyphae obscuring any fruit body primordia. Full-grown fruit bodies were not observed. Again, after 6 days growth the glucose in the medium was virtually exhausted (Table 2). Wessels (1965, 1966) has shown that one of the prerequisites for pileus formation is a depletion of the carbon source in the medium; he also established a close relationship between normal pileus formation and R-glucan degradation by the enzyme R-glucanase. Table 1 shows that less R-glucan but more mucilage was formed when cultures were grown in CO₂-enriched air. During glucose starvation, the amount of mucilage decreased both in CO₂-grown and in air-grown cultures but the amount of R-glucan only decreased in the air-grown cultures. In both types of cultures the amounts of S-glucan and chitin hardly changed during starvation.

Table 1. *Carbohydrate material produced by Schizophyllum commune strain 699 × 845 during growth in an atmosphere of air or air plus 5 % CO₂*

The glucans are expressed as mg anhydroglucose per culture and chitin as mg anhydro-acetylglucosamine per culture.

Culture conditions	Carbohydrate material			
	Mucilage	S-glucan	R-glucan	Chitin
6 days, air	77.9	27.3	57.6	4.8
6 days, air + 5 % CO ₂	146.1	19.7	24.8	5.3
12 days, air	24.4	27.2	19.2	5.0
12 days, air + 5 % CO ₂	56.7	21.7	22.9	5.2

Table 2. *Residual glucose and R-glucanase activity in the culture filtrate of Schizophyllum commune strain 699 × 845 after cultivation under the specified conditions*

R-glucanase activity is expressed as μg glucose equivalents liberated from R-glucan/chitin of strain 699 h⁻¹ (ml medium)⁻¹.

Culture conditions	Residual glucose (%)	R-glucanase activity
6 days, air	1.25	10
6 days, air + 5 % CO ₂	1.58	12
12 days, air	0.02	168
12 days, air + 5 % CO ₂	0.02	212

Table 3. *Activity of R-glucanase from Schizophyllum commune K8 and (1 → 3)- β -glucanase from QM806 on cell walls and R-glucans isolated from Schizophyllum commune 699 × 845 cultivated under the specified conditions.*

All incubation mixtures, whether containing cell walls or the R-glucan derived from these walls, contained the same amount of R-glucan (10 mg ml⁻¹). The susceptibility is expressed as μg glucose equivalents released by the enzyme preparations (see Methods).

Culture conditions	S-glucan/ R-glucan ratio in the wall	Susceptibility to:			
		R-glucanase		(1 → 3)- β -glucanase	
		Walls	R-glucan	Walls	R-glucan
6 days, air	0.47	204	451	127	521
6 days, air + 5 % CO ₂	0.80	76	211	47	286
12 days, air	1.42	38	187	61	311
12 days, air + 5 % CO ₂	0.95	34	237	54	399

In both cultures the enzyme R-glucanase was produced after the glucose in the medium had been exhausted (Table 2). This means that the higher CO₂ pressure did not suppress R-glucanase production, thus excluding the possibility that the lack of R-glucan degradation *in vivo* was caused by absence of the enzyme. Another possibility was that the R-glucan in the walls of CO₂-grown cultures was less susceptible to enzymic breakdown. This is suggested by the data of Table 3 which show that walls and R-glucan isolated from 6-day-old CO₂-grown cultures were less sensitive to both R-glucanase and (1 → 3)- β -glucanase than were walls and R-glucan from 6-day-old cultures grown in air. However, at 12 days the susceptibilities of walls and R-glucans from air-grown cultures and from CO₂-grown cultures were both low.

If the effect of CO₂ on pileus formation is mediated by a change in the enzymic susceptibility of R-glucan in the preformed mycelium, then CO₂ must act before any fruit bodies are produced. To test this, 6-day-old agar cultures grown in air were transferred to an

atmosphere of air plus 5 % CO₂ and incubation was continued for another 6 days. The growth of the pilei was not inhibited. However, when CO₂-grown cultures were transferred after 6 days to an atmosphere without CO₂, no full-grown fruit bodies formed.

High temperature (33 °C) also inhibits fruiting in *S. commune* grown in air (Wessels, 1965). However, when agar cultures were cultivated at 33 °C in an atmosphere without any CO₂ (KOH pellets in the lid of the Petri dish), normal fruit bodies formed. Thus the effect of elevated temperature may actually be due to high CO₂ tensions in the culture as a result of increased growth rate.

DISCUSSION

If one supposes that the primary effect of CO₂ on the metabolism of Schizophyllum mycelium is basically the same as proposed for the self-inhibition of the *A. bisporus* spore by CO₂ (Rast *et al.*, 1976*a, b*), then in the cultures grown in CO₂-enriched air more of the glucose taken up should be channelled into non-glycolytic pathways, such as polysaccharide synthesis. Table 1 shows that CO₂-grown cultures do produce more glucan but this relates only to the mucilage.

The results also show that CO₂ inhibits the breakdown of R-glucan, which normally accompanies pileus formation. No effect on R-glucanase production was found, but CO₂ appears to decrease the susceptibility of R-glucan towards hydrolytic enzymes. Extraction of S-glucan greatly enhances the activity of the enzymes on the R-glucan/chitin complex, particularly when the S-glucan to R-glucan ratio is high (Table 3). However, the major effect of CO₂ does not seem to be an increase in the protection of R-glucan by other polymers but rather on the enzymic susceptibility of the R-glucan itself.

Since the total amount of glucan (R-glucan plus mucilage) broken down in the presence or absence of added CO₂ is about the same, it follows that the *in situ* degradation of the wall-bound R-glucan is crucial for the support of pileus growth. That CO₂ affects the susceptibility of R-glucan to enzymes as it is synthesized explains why CO₂ had no effect when present only during pileus growth (Schwalb, 1971; this paper).

Using another dikaryon (K8), it was previously noted that elevated temperature has the same effects as described for CO₂ in this paper, i.e. inhibition of degradation of R-glucan *in vivo*, no effect on R-glucanase formation and decreased susceptibility of the walls (Wessels, 1965, 1966). Therefore, the effect of elevated temperature may actually be due to high CO₂ tensions in the cultures arising because of increased catabolism of glucose. This contention is supported by the fact that normal fruit bodies are produced at 33 °C if the CO₂ produced metabolically is trapped in KOH (Schwalb, 1971; this paper).

The action of R-glucanase on the R-glucan releases mainly large soluble glucan fragments consisting of (1→3)-β-linked chains with side branches bearing (1→6)-β-linked glucose molecules (Wessels, 1969; Sietsma & Wessels, 1977). Exo-(1→3)-β-glucanase removes these (1→3)-β-linked chains from the R-glucan by sequential hydrolysis starting at the non-reducing end (Sietsma & Wessels, 1977). Both enzymes leave a resistant glucan core that is closely associated with chitin. This may be the reason why the residual R-glucan left after *in vivo* degradation in air-grown cultures (Table 3, 12 days in air) is much less susceptible to R-glucanase and (1→3)-β-glucanase than the non-degraded R-glucan (6 days in air). The insensitivity of R-glucan from CO₂-grown cultures to R-glucanase and (1→3)-β-glucanase may indicate that, in these cultures, the glucanase-sensitive part of R-glucan was either not synthesized or not attached to the resistant core. The latter possibility seems particularly attractive because the mucilage that is excreted more abundantly in the CO₂-grown cultures (Table 1) is structurally similar to the (1→3)-β-glucanase-sensitive part of the R-glucan (Sietsma & Wessels, 1977).

In summary, our results show that CO₂ indirectly affects the growth of the pilei of *S. commune* by changing the wall composition during the growth period. They do not

exclude the possibility that CO₂ also has an effect on earlier stages of fruit body formation. Because CO₂ promotes the formation of a thick mat of aerial hyphae it is difficult to clarify this point.

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