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Increase in glucan formation by *Botrytis cinerea* and analysis of the adherent glucan

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Summary. Glucan production by *Botrytis cinerea* increased from 1 g/l to 3 g/l when KNO_3 or urea replaced asparagine as the nitrogen source. A further enhancement up to 5 g/l was obtained with nitrogen-limited medium or non-growing cells. Under these conditions an extracellular glucan layer was attached to the mycelium. The adherent glucan made up 60% of the total amount of glucan produced and thus increased the total glucan yield to 13 g/l. An enzymatic analysis of the adherent glucan indicated that only about every fifth molecule of the main chain was substituted by a glucose unit. In contrast, in the free glucan of culture filtrates glucose units were distributed at approximately every second to third residue of the main chain.

Introduction

The fungus *Botrytis cinerea* produces an extracellular β-D-glucan (Montant and Thomas 1977, 1978). Its accumulation results in highly viscous broths responsible for clarification difficulties in wine technology (Dubourdieu et al. 1978a, b). The structure of this polysaccharide is similar to that of scleroglucan, consisting of a β-1,3-linked glucose backbone to which single glucose units are attached at approximately every second to third residue of the main chain by β-1,6-linkages (Dubourdieu et al. 1981a). Since these β-linked glucan polymers have been evaluated for potential industrial usage (Sutherland 1983), especially for enhanced oil recovery (Holzwarth 1985), the glucan produced by *B. cinerea* might be a commercially interesting product. Leal et al. (1979) studied the effect of carbon and nitrogen sources on the production of this polysaccharide. A maximal yield of 1 g/l glucan was obtained when asparagine instead of casamino acids or yeast extract was used as the nitrogen source. Additionally, enhanced cell dry weights occurred in nitrogen-limited medium, which has also been noted in other extracellular-glucan producing fungi, for example *Glomerella cingulata* (Sarkar et al. 1985), *Paecilomyces lilacinus* (Sarkar 1986) or *Moniliella pollinis* (Sarkar et al. 1986).

In this paper we describe the enhancement of glucan production by *B. cinerea* with different nitrogen sources associated with high amounts of glucan tightly attached to the mycelium, which is responsible for enhanced cell dry weights. Additionally, an enzymatic analysis of this glucan was performed, indicating a different distribution of the side-chains from that determined for the glucan of culture filtrates.

Materials and methods

Cultivation and preparation of [^{14}C]glucan. For growth and glucan production *B. cinerea* Pers:Fr. was cultivated in a mineral salt medium (MM) containing the following (per litre): 1.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 mg $FeSO_4 \cdot 7H_2O$, 0.5 mg $ZnSO_4 \cdot 7H_2O$, 0.02 mg $CuSO_4 \cdot 5H_2O$, 0.02 mg $MnCl_2 \cdot 4H_2O$. Unless otherwise stated the D-glucose concentration was 25 g/l, and 1.5 g/l KNO_3 was used as nitrogen source. The pH was adjusted to 5.0 with 1 *N* HCl before sterilization. Glucose was autoclaved separately. One-hundred-millilitre cultures in 500-ml erlenmeyer flasks were inoculated with 1 ml spore suspension containing 10^7 spores and incubated at 22° C and 100 rpm on a rotary shaker (Type IRC-1-T, Kühner, Basel, Switzerland).

Mycelium dry weights were obtained after centrifugation at 10000 *g* for 30 min, followed by three washes with distilled water and collection on cellulose filters (Schleicher and Schüll, Dassel, FRG), which were dried for 18 h at 60° C.

For the preparation of [^{14}C]labelled glucan 1.11 MBq (2.4 μmol) [U-^{14}C]glucose was added to 10 ml MM without any nitrogen source. The medium was inoculated with 1/10 of 3-day-old mycelium, which had been grown under standard conditions, and washed three times with 0.9% NaCl. After incubation for 24 h in 5-ml shake flasks cultures were harvested by centrifugation. For determination of [^{14}C]glucan, mycelia and culture filtrates were dialysed against 10 m*M* sodium acetate buffer containing cold 0.1 *M* glucose, and two further times against the buffer without glucose.

Enzymatic degradation of glucan. For enzymatic hydrolyses the glucanase preparation "Novozym" SP-116 ("glucanex"; Novo Ferment, Basel, Switzerland) was used. This preparation contained polysaccharides which were degraded by preincubation of

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dissolved enzymes (30 units (U)/ml 10m*M* sodium acetate buffer, pH 5) at 37° C for 12 h. The preparation was then filtered through a P6DG column (Bio-Rad, Munich, FRG) to remove free sugars, and stored at −18° C. Hydrolyses were performed by mixing 1 mg polysaccharide in culture filtrates or 1 g mycelium (wet weight) with 0.3 U glucanase or laminarinase (from mollusc, Sigma, Munich, FRG) and incubating at 37° C. Reactions were stopped by heating at 100° C for 20 min, then centrifuged at 13000 *g* for 10 min. Sedimented mycelia were washed with 10 m*M* sodium acetate buffer, pH 5, and dried.

Carbohydrate determinations. When the glucose had been consumed, glucan in the culture filtrates was determined as total sugars with anthrone reagent according to Herbert et al. (1971). Culture filtrates of the growth phase were additionally dialysed against water to remove free glucose.

For the analysis of degradation products aliquots were applied to HPTLC silica gel 60 plates (Merck, Darmstadt, FRG). Chromatograms were developed ascendingly in butanol/acetone/water (4:5:2 by vol). After drying, the sugars were detected by spraying with 50% H_2SO_4. For determination of ^{14}C-labelled degradation products, segments corresponding to detected sugars charred on a parallel run and the unstained areas subdivided into sections of equal sizes were scraped out, mixed with Instant Scintillation Gel (Packard Instruments, Zurich, Switzerland) and counted in a Tricarb liquid scintillation counter (Packard Instruments). The count rates were corrected with internal standards to give disintegration rates. Additionally, unlabelled sugars were routinely separated in the form of their borate complexes via anion exchange chromatography (Biotronik LC 2000, Maintal, FRG) and reacted for detection with Cu^{2+}-bicinchoninate reagent as previously described (Schimz et al. 1985).

Glucose was assayed enzymatically with hexokinase and glucose-6-P dehydrogenase as instructed by Boehringer (Mannheim, FRG). Carbon and nitrogen was determined automatically in a microelement analyser (Perkin Elmer CHN 240 B, Überlingen, FRG) with washed and lyophilized mycelia.

Results and discussion

Effect of nitrogen sources on glucan formation

In studies on the effect of different nitrogen sources on glucan production and growth of *B. cinerea*, readily metabolizable nitrogen sources according to Pateman and Kinghorn (1976) were tested in the standard medium (MM). The most pronounced effect (Table 1) was observed with nitrate and urea: both increased glucan production threefold. As expected from the results of Leal et al. (1979), glucan yield with asparagine was approximately 1 g/l. The same results were obtained with ammonium salts, probably due to an inhibitory effect of free NH_4^+ as assumed for pullulan production by *Aureobasidium pullulans* (Seviour and Kristiansen 1983). Nitrite as the nitrogen source had an intermediate effect on glucan production.

Table 1. Growth and glucan production of *Botrytis cinerea* with different nitrogen sources

Nitrogen source (0.2 g/l *N*)	Incubation time (days)	Mycelium dry weight (g/l)	Glucan conc (g/l)
L-Asparagine	10	13	0.7
NH_4Cl	11	9	0.9
NH_4NO_3	10	12	1.4
$(NH_4)_2SO_4$	10	8	1.1
$NaNO_2$	14	7	2.1
KNO_3	12	10	2.8
$NaNO_3$	11	9	2.8
$H_2N\text{-}CO\text{-}NH_2$	10	10	3.4

Glucose concentration was 20 g/l

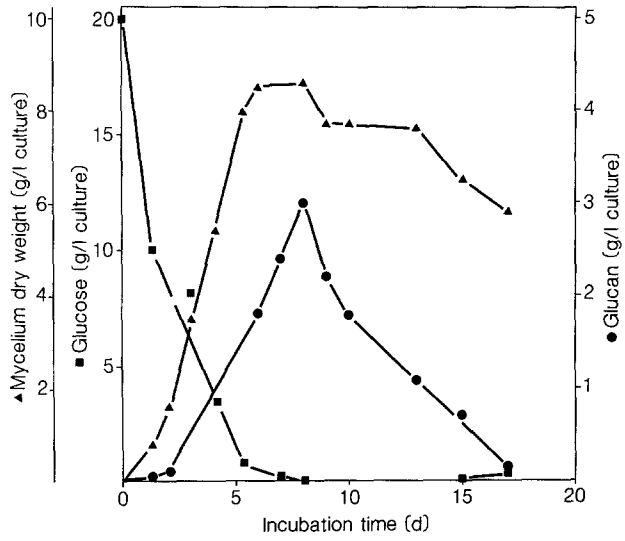

Fig. 1. Growth, glucose consumption and glucan formation in batch cultures of *Botrytis cinerea*

Glucan formation ceased when glucose was consumed (Fig. 1). In contrast to the findings of Leal et al. (1979) glucan was degraded with further incubation time, and glucanase activity was present as well (data not shown). These results indicate that extracellular glucan is synthesized as a storage compound and utilized by the organism under starvation conditions.

Enzymatic determination of glucan

In a culture medium supplemented with asparagine (20 g/l) and the vitamins B_1 and B_2 (10^{-6} *M*) *B. cinerea* not only formed the homopolysaccharide glucan but also a heteropolysaccharide (Kamoen and Jamart 1973; Kamoen et al. 1980). In order to investigate whether under optimized conditions a heteropolysaccharide was also synthesized, the total sugar content of the culture filtrate (measured with anthrone reagent) was compared with the amount of glucose resulting from the enzymatic treatment of the polysaccharide. The β-1,3-glucanase laminarinase failed to degrade *B. cinerea* glucan, indicating that the enzyme cannot bypass the β-1,6-linkages of the side chains. Therefore the glucanase preparation "Novozym" was used.

Kinetic analysis revealed that for times up to 1 h only glucose and the β-1,6-glucose disaccharide gentiobiose were formed; this was assayed by HPTLC-analysis (Fig. 2, sample 2) and confirmed by borate complex chromatography. As shown by Dubourdieu et al. (1981b, 1985) these degradation products are characteristic of the *B. cinerea* glucan. After prolonged incuba-

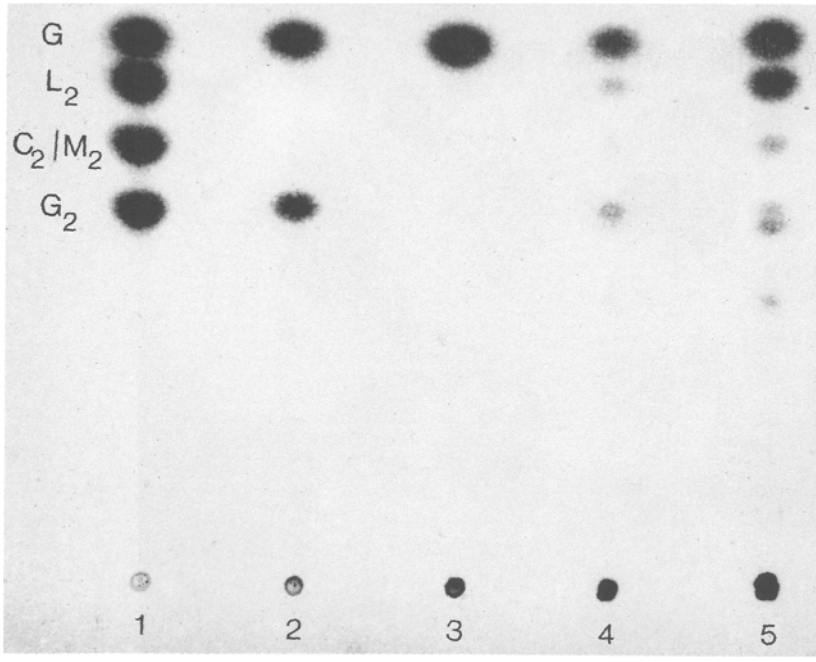

Fig. 2. Degradation products of glucan after hydrolysis with glucanase: *1*, standards containing 10 µg each of glucose (*G*), laminaribiose (L_2), cellobiose (C_2) or maltose (M_2), and gentiobiose (G_2); *2*, 5 µl culture filtrate incubated for 1 h; *3*, as *2* but for 5 h; *4*, 5 µl supernatant of mycelium pretreated with glucanase for 60 min and repeatedly incubated for 15 min; *5*, as *4* but for 30 min

tion of up to 5 h gentiobiose was also cleaved to glucose (Fig. 2, sample 3), thus making quantitative comparison possible. After complete enzymatic hydrolysis the quantitative determination of glucose yielded the same amounts of glucan as measured for total sugars with the anthrone reagent. It is evident from these results that polysaccharides other than glucan are not synthesized by *B. cinerea* in the standard medium.

Effect of high C/N ratios on glucan formation and cell dry weights

The production of *B. cinerea* glucan is promoted by high C/N ratios (Leal et al. 1979). Thus, glucan yields increased with increasing glucose concentration or decreasing KNO_3 concentration, respectively (Table 2). However, it was remarkable that mycelium dry weights also increased when low nitrogen contents of the medium should restrict fungal growth. The same effect was observed when glucan formation was investigated with resting cells: mycelia grown for 3 days in MM were harvested, washed several times to remove any remaining nitrogen and transferred to the MM lacking any nitrogen source. After 8 days glucose was consumed and 5 g/l glucan was produced. Although a nitrogen-free medium was used, mycelium dry weights nevertheless increased from 3.5 to 16 g/l.

Table 2. Effect of glucose and KNO_3 concentrations on glucan production and on C/N ratios of the mycelium of *B. cinerea*

Glucose conc[a] (g/l)	Incubation time (days)	Glucan yield (g/l)	Mycelium	
			Dry weight (g/l)	C/N ratios
20	7	2.2	8.3	17.4
25	7	2.8	8.8	19.9
30	8	3.5	12.3	22.7
KNO_3 conc[b] (g/l)				
10	8	2.7	7.4	11.8
5	8	2.6	9.6	16.4
1	7	4.5	14.0	29.3
0.5	18	6.0	14.5	26.8

[a] KNO_3 1.5 g/l
[b] Glucose 20 g/l

From these results it was evident that increased mycelium dry weights did not correlate with fungal growth, as was additionally confirmed by the fact that C/N ratios of the mycelia increased with increasing C/N ratios of the medium (Table 2). This was due to extracellular glucan still adhering to the washed mycelium, as demonstrated by Indian ink preparations (Fig. 3). The adherent polymer could not be recovered by subsequent washes: the first supernatant still contained approximately 0.7 g/l, the second about 0.3 g/l, and the third supernatant only <0.1 g/l glucan. Neither homogenization of the mycelial aggregates with an Ultra-Turrax TP 18–10 (Janke and Kunkel, Staufen, FRG) nor heating to 70°–80° C, as described for the preparation of the extracellular glucan of fleshy fungi (Davis et al. 1965) and also commercial glucan preparations (Rogers 1973), enhanced the polymer content of the supernantants significantly. Therefore enzymatic analysis was performed to determine the adherent glucan.

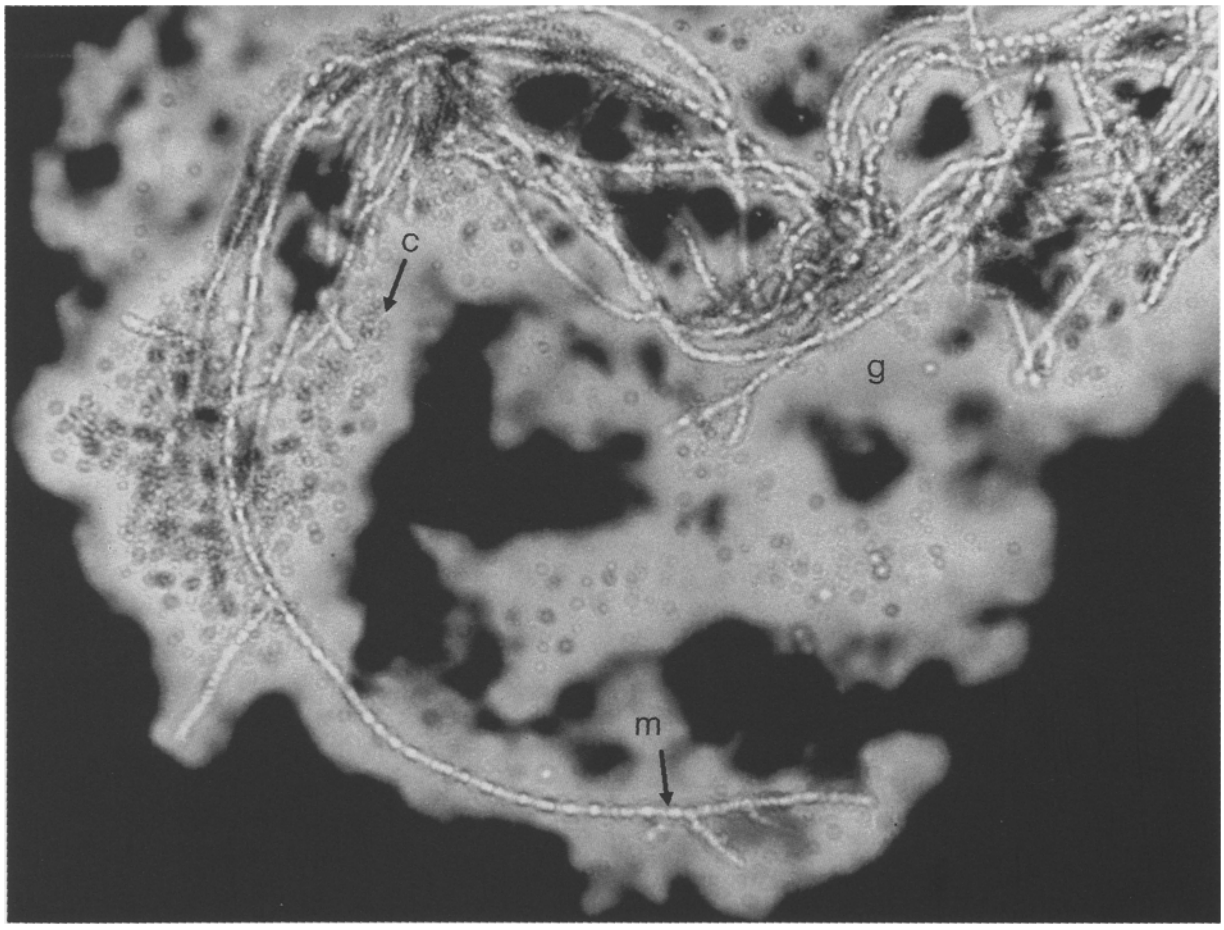

Fig. 3. Extracellular glucan attached to washed mycelium of *B. cinerea* as visualized by Indian ink: *m*, mycelium; *g*, glucan; *c*, conidia

Analysis of the glucan attached to the mycelia

For quantitative determination of glucan attached to the cells it was first hydrolysed with glucanase. Since also cell-wall and thus cytoplasmic components could be degraded, a time-dependent hydrolysis was performed. During periods of up to 1 h glucose and gentiobiose was formed, as expected, from the degradation products of free glucan in culture filtrates. Unexpectedly, the β-1,3-glucose disaccharide laminaribiose was also found, arising from either the cell-wall or the attached glucan. However, Indian ink preparations confirmed that the mycelia were still enclosed in a glucan layer. Also, after an incubation time of 1 h 15 min, no other components apart from glucose, laminaribiose and gentiobiose were detected (Fig. 2, sample 4). Only after prolonged incubation (1 h 30 min) were degradation products distinct from the β-glucan characteristic components found (Fig. 2, sample 5), indicating that the cell-wall components had degraded resulting in cell lysis. Thus, it was likely that the laminaribiose found was a component of the extracellular glucan layer.

To confirm this result it was investigated whether laminaribiose was also released from glucan synthesized de novo with non-growing cells. Experiments were performed with [^{14}C]glucose, which was incorporated into glucan synthesized in vivo for 24 h. After time-dependent hydroylsis of glucan with glucanase ^{14}C-labelled degradation products were determined by HPTLC analysis. As shown in Table 3 ^{14}C-labelled laminaribiose was detected. Since under the conditions used cell wall synthesis was prevented this result is again in agreement with laminaribiose being a constituent part of the mycelia-attached glucan.

The laminaribiose found indicates that the mycelia-attached glucan is different from free glucan. The degradation of free glucan in culture filtrates with glucanase resulted in radioactive ratios of glucose and gentiobiose of 3:4 (Table 3), according to a molar ratio of 3:2 as found by Dubourdieu et al. (1981a). However, from mycelia-attached glucan radioactive ratios of approximately 2:1 were determined, indicating a molar ratio of 4:1. Considering that probably only every fifth molecule of this glucan is gentiobiose the mycelia-attached glucan would structurally approach the β-1,3-glucan laminarin. However, the presence of an extracellular mixture of laminarin (from which also laminaribiose would be released by enzymatic hydrolysis) and a glucan (structurally corresponding to that of the culture filtrate) can be excluded because the β-1,3-glucanase laminarinase did not degrade the mycelia-attached polysaccharide.

For a first quantitative determination of the extracellular glucan formed the incorporation rate of glu-

Table 3. Distribution of ^{14}C-labelled degradation products of glucan on HPTLC plates after hydrolysis with glucanases

Glucan	Time (min)	^{14}C-Labelled glucan degradation products ($\times 10^5$ dpm[b]/ml culture per 24 h)				
		Glucose	Laminaribiose	Gentiobiose	Undegraded	Total
Attached to mycelium	10	0.50	0.17	0.31	1.34	2.32
	30	1.11	0.25	0.55	0.98	2.89
	60	1.66	0.27	0.69	0.35	2.97
In culture filtrates	30	0.25	n.d.[a]	0.31	0.91	1.47
	60	0.60	n.d.[a]	0.86	0.32	1.78

[a] n.d. = not detected
[b] dpm = disintegrations per minute

cose in glucan was calculated. Based on data obtained after a 60-min enzymatic hydrolysis (Table 3), an incorporation rate of 3.7 µmol glucose/ml culture per 24 h was calculated for the glucan of culture filtrates and 6.2 µmol glucose/ml culture per 24 h for the mycelia-attached glucan. Thus, 40% of the glucan produced was detected in culture filtrates whereas 60% was adherent to the mycelia.

To verify this result obtained with ^{14}C-labelled experiments the total amount of the glucan produced under standard culture conditions was determined. This was done by direct enzymatic glucose assay after complete hydrolysis of the glucan degradation products. Mycelia-attached glucan was degraded by glucanase for 60 min. Then the degradation products were removed by centrifugation and cleaved to glucose by further glucanase treatment. Glucose assays revealed that 5.3 g/l glucan was detectable in culture filtrates and 8.1 g/l on the mycelia, resulting in a total amount of 13.4 g/l glucan formed. Thus, the same distribution of the extracellular glucan as determined with ^{14}C-labelled degradation products was ascertained. Correspondingly, mycelium dry weights with adherent glucan were on average 12.3 g/l, and without glucan generally 4.6 g/l. Consequently, 65% of the mycelium dry weights was made up of glucan.

Based on these data the production of *B. cinerea* glucan is comparable with the scleroglucan production by *Sclerotium rolfsii* in a 14-l fermentor, yielding 15 g/l glucan (Griffith and Compere 1978). Assuming that the glucan production by *B. cinerea* will be optimized in scale-up fermentations and a simple method of removing the glucan from the mycelium will be developed this glucan might become attractive for commercial use.

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