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ESTIMATION OF MYCELIAL GROWTH OF BASIDIOMYCETES BY MEANS OF CHITIN DETERMINATION

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Key Word Index—Chitin; basidiomycetes; growth of mycelium.

Abstract—After hydrolysis of chitin in 6 M HCl, the glucosamine produced was assayed colorimetrically. The pH of the hydrolysate was adjusted to a value close to three by addition of Na acetate; this procedure avoids the elimination of excess acid by evaporation under reduced pressure or freeze-drying. Under these conditions, the amount of glucosamine determined by the assay represented an average of 90% of the amount which would result from a total hydrolysis of the chitin. The method was used to assay the chitin in the mycelia of basidomycetes obtained *in vitro*. The measured amount of glucosamine was proportional to the mycelial biomass and allowed the estimation of fungal growth.

INTRODUCTION

The walls of the filamentous fungi, particularly those of the basidomycetes, contain some chitin which, together with cellulose, make up the microfibrillar skeleton [1–4]. A specific assay of the fungal chitin therefore estimates the amount of mycelium from a saprophyte fungus, whether a parasite or a symbiont, which is present in the tissues of a plant host. This method has already been used to estimate the mycelial biomass of a basidiomycete growing on wood tissue [5] to evaluate the degree of infection of plants with fungal pathogen parasites [6–8] and to estimate the amount of mycelium in vesicular–arbuscular mycorrhiza [9–11].

The methods of assay employed differ according to the type of hydrolysis chosen to solubilize the polymer, and by the colorimetric assay of the residues obtained. The solubilization of the chitin may be obtained by enzymic hydrolysis with chitinase [6] or by chemical hydrolysis in the presence of a concentrated base or a strong acid [5–11]. The colorimetric assay may be carried out on *N*-acetylglucosamine residues [12, 13] or on glucosamine residues [14, 15]; the latter method is more sensitive and accurate.

After hydrolysis in acid, we have tried to simplify the conditions of the colorimetric assay of glucosamine [14, 15] so as to establish an assay of the chitin which may be used to make a rapid estimation of the mycelial biomass.

RESULTS

Assay of pure chitin

The establishment of the assay was carried out on purified chitin. The hydrolysis of chitin in 6 M HCl (16 hr at 80°) is almost total and yields acetyl and glucosamine residues [5]. An aqueous solution of glucosamine is assayed by the colorimetric method of Tsuji *et al.* [14, 15]. The deamination by nitrous acid (HNO_2) transforms the glucosamine residues into 2,5-anhydromannose with a free aldehydic function on carbon 1; this compound reacts with the 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) producing, in the presence of ferric chloride solution, an intense blue colour. The measurement of absorbance is made at 653 nm. Extreme pH values do not permit the development of the coloured reaction ($pH \geq 10$) or attenuate it considerably ($pH < 1$). Thus, it is essential to eliminate excess acid after hydrolysis of the chitin. Freeze-drying [6] or evaporation under reduced pressure [5] are methods that are too lengthy for a series of assays.

We carried out the colorimetric assay on part of the hydrolysate in the presence of Na acetate. 1 ml of the hydrolysate contains *ca* 6 mmol of protons. The addition of 5 ml 1.25 M NaOAc brings the pH to a value close to three which allows a good development of the coloured reaction. With glucosamine solutions in 6 M HCl (concentrations between 1 and 30 μg/ml) the *A* values are linear with concentration, and they enable us to determine the molar extinction coefficient $\epsilon M = 3.187 \times 10^4$, a value close to that quoted by Tsuji *et al.* [15]. In addition, using the same solution of glucosamine (15 μg/ml), the assays of nine aliquots gave the values of 14.95 ± 0.35 μg/ml ($P = 0.01$).

The total hydrolysis of a weight p of chitin would produce n molecules of glucosamine (and n molecules of acetic acid) and the formation of a weight p of polymer from these n molecules of acetylglucosamine causes the loss of $n-1$ water molecules, thus: $p = n \cdot 221 - (n-1) \cdot 18$ (221 = MW acetylglucosamine; 18 = MW water); thus:

$$n = p - 18/203.$$

n is therefore the number of glucosamine residues which would be produced by total hydrolysis, and it

corresponds to a mass of glucosamine, $x = n \cdot 179$ (179 = MW glucosamine). If x' is the mass of glucosamine given by the assay, the efficiency of the hydrolysis will be $R = x'/x$.

For 20 samples of *ca* 2 mg of chitin weighed exactly, the hydrolysis in 6 M HCl [ratio wt (in mg)/vol. (in ml) equal to 0.1] gave an average yield $R = 90.9 \pm 4.9\%$ ($P = 0.05$). By varying the ratio wt of chitin (in mg)/vol. of acid (in ml), we obtained an average value of 89.2%: the absorbance values are proportional to the weights of chitin.

Assay of chitin in the mycelia of basidiomycete fungi

Hebeloma cylindrosporum Romagn. mycelia were grown in Petri dishes on a sheet of sterilized cellophane placed over a 1.5% agar culture medium. Table 1 indicates the results of the chitin assay on samples harvested after 2 months growth: the previous preparation of the tissues does not result in any significant differences.

Samples of varying wts (1–30 mg dry matter) were hydrolysed in 5 ml of 6 M HCl; 1 ml of the hydrolysate was then diluted in 5 ml of 1.25 M NaOAc to adjust the pH. A new dilution in a mixture 6 M HCl, 1.25 M NaOAc (ratio vol./vol., 1:5) enabled us to bring the expected concentrations of glucosamine to a value compatible with the range of the working curve (1–30 μg of glucosamine/ml). Figure 1 indicates that the amount of glucosamine is proportional to the wt of mycelium.

Measurement of growth of a basidiomycete mycelium using the chitin assay

Suillus luteus (L. *ex* Fr.) S. F. Gray mycelia were grown on a liquid culture medium with the sole source of nitrogen being either NH_4Cl or KNO_3 (7.5 mM). Samples were harvested after different growth times. The amounts of glucosamine assayed in the mycelia increased in proportion to the wts of fresh matter as for the dry matter (Fig. 2). Fig. 3 represents the growth curves for the fungus as a function of time. The amounts of glucosamine assayed at each sampling coincide with the masses of fresh matter.

The chitin assay enabled us to make a correct evaluation of the mass of mycelial tissue and the growth of the fungus. The amount of chitin in the mycelium grown on a solution containing nitrate is always smaller than for a fungus grown on a solution containing ammonium: 44.8 and 69.8 μg glucosamine/mg dry tissue, respectively. This amount tends to increase with the age of the mycelium in the case of ammonium nutrition, but it remains stable for nitrate nutrition.

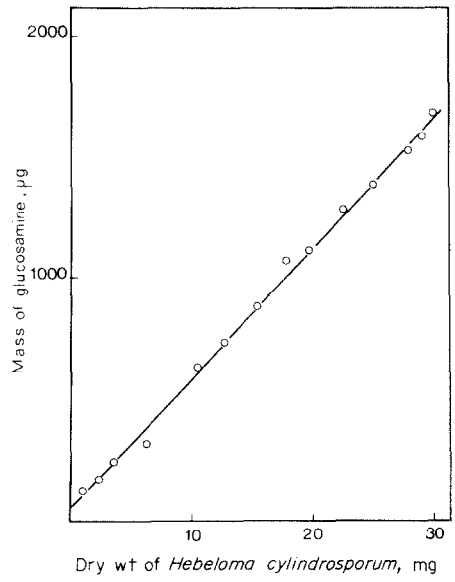

Fig. 1. Correlation and linear regression between the mass of glucosamine and the amount of mycelium of *Hebeloma cylindrosporum* (hydrolysis in 6 M HCl —coloured reaction according to Tsuji *et al.* [14, 15]. Linear regression $Y = 39.64 + 54.26\,x$. Correlation coefficient $r = 0.997$.

DISCUSSION

It is possible to evaluate the mass of a basidiomycete mycelium and to follow its growth by means of the assay of the chitin, a specific constituent of the walls of these fungi. The assay of glucosamine by the colorimetric method used [14, 15] is specific, interferes little with the sugars and is very sensitive (working curve of 1–30 μg/ml); the acid hydrolysis yield is high, *ca* 90% whereas alkaline hydrolysis only produces 50% of glucosamine residues. Moreover,

Table 1. Glucosamine yield, after acid hydrolysis, from the mycelium of *Hebeloma cylindrosporum* prepared in different ways

	Treatments			
Parameters	I	II	III	IV
n = size of sample	10	8	9	8
mg of dry matter	4.64 ± 0.22 ($P = 0.05$)	4.74 ± 0.46 ($P = 0.05$)	5.55	5.55
μg of glucosamine/ mg of dry matter	74.24 ± 3.03 ($P = 0.05$)	73.5 ± 4.24 ($P = 0.05$)	67.9 ± 4.45 ($P = 0.05$)	75.47 ± 3.04 ($P = 0.05$)

I: weighing of x mg of intact dry mycelium.
II: weighing of x mg of dry mycelium ground in a mortar.
III: 1 ml of a suspension with coarsely ground mycelium (mycelium ground in a mortar).
IV: 1 ml of a suspension finely ground (mycelium homogenized in a Potter grinder).

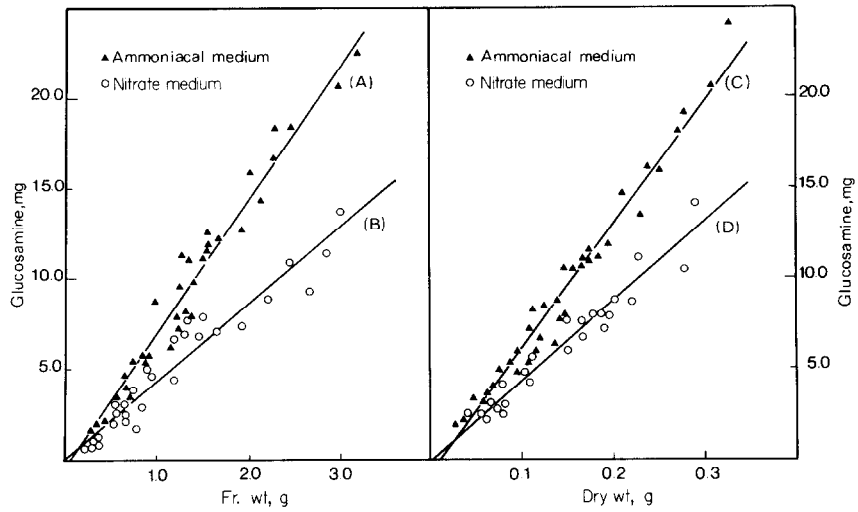

Fig. 2. Correlation and linear regression between the amounts of glucosamine assayed in the dry mycelia of *Suillus luteus* and fr. wt (A) and (B) or dry wt (C) and (D) of thalli grown on an ammonium medium (▲) or a nitrate medium (○). (A) $Y = -0.38 + 7.46\,x$; $r = 0.974$ (B) $Y = 0.024 + 4.26\,x$; $r = 0.955$. (C) $Y = -1.06 + 69.79\,x$; $r = 0.983$. (D) $Y = -0.280 + 44.84\,x$; $r = 0.993$.

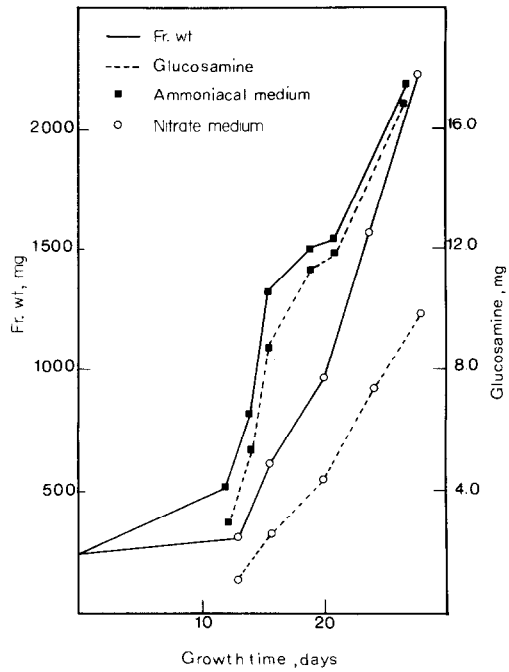

Fig. 3. Increase with time of fr. wts of, and amounts of glucosamine, in mycelia of *Suillus luteus* grown on an ammonium or nitrate medium. ——fr. wt; ---- glucosamine; ■ ammonium medium; ○ nitrate medium.

the latter method requires numerous washings to remove excess base. However, hydrolysis in 6 M HCl solubilizes the wall polysaccharides, which does not happen during alkaline hydrolysis [16]. The sugars produced may create a slight interference during the colour reaction. The chitin concentration of the fungus may thus be slightly overestimated. This disadvantage which could be avoided by using alkaline hydrolysis, appears to be largely compensated by the simplicity of the method we use.

EXPERIMENTAL

Hydrolysis was carried out in 6 M HCl for 16 hr at 80°. Each sample was weighed precisely (between 0.1 and 3 mg of chitin—chitin Sigma Cat. No. C 3641—or between 3 and 30 mg of dry fungal material) was hydrolysed in 5 ml of acid, in a glass tube closed with a screw-cap. Before closing the tubes, the material was pre-soaked in acid at room temp. for 3 hr, with the tubes open.

Adjustment of pH. To 1 ml of hydrolysate, 5 ml of 1.25 M NaOAc was added and the pH of the soln then approaches 3.

Colorimetric assay. To 1 ml of soln containing the glucosamine residues, 1 ml of 5% $KHSO_4$ and 1 ml of 5% $NaNO_2$ were added. After shaking, the mixture was allowed to stand for 15 min (deamination time). Then 1 ml of 12.5% $H_2NSO_3NH_4$ was added (destruction of excess HNO_2). After shaking (5 min) 1 ml of 0.5% MBTH was added. Reaction time was 1 hr without shaking before adding 1 ml of 0.5% $FeCl_3$. The colour was allowed to develop for 30 min; the *A* measured at 653 nm was stable for *ca* 20 hr.

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