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PHYSIOLOGY OF AN EDIBLE SMUT, *USTILAGO ESCULENTA*: GROWTH REQUIREMENTS, UTILIZATION OF AMINO ACIDS, AND CELLULAR COMPOSITION

FU-KUEN LIN*

Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, S.C. 29403

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Abstract

A minimal medium for the growth of the yeastlike culture of ***Ustilago esculenta*** has been developed. The optimal pH for growth is about 6.5 and 7.0. As tested by auxanographic technique, the fungus has a growth requirement for thiamine, inositol and pantothenate, and has no requirement for biotin, *p*-aminobenzoic acid, pyridoxine HCl, riboflavin, nicotinic acid, ascorbic acid, folic acid, choline chloride, ergosterol and cholesterol. With the 22 naturally occurring L-amino acids tested as sole nitrogen source for growth, all except methionine, cystine (both are sulfur-containing amino acids) and hydroxyproline could support the growth of the fungus. Of the eight D-amino acids tested as sole nitrogen source, only D-valine should support the growth; alanine, aspartic acid, cystine, glutamic acid, methionine, threonine and tyrosine could not. The content of DNA, RNA and protein on the basis of percent dry weight cells was 0.22, 4.12, and 16%, respectively. However, the dikaryotic mycelium phase of ***U. esculenta*** showed little growth in thiamine containing medium. The replacement of thiamine by a mixture of vitamins, and the addition of RNA hydrolysate and cholesterol or ergosterol also failed to improve its growth.

Introduction

'Kah-peh-sung' is a popular, tender, delicious vegetable in the summer and the fall in Taiwan. This vegetable is the hypertrophied culm resulted from the infection of *Zizania latifolia* by *Ustilago esculenta* P. Henn. Since the first recording in 1895 by P. Hennings, there has been only few studies on this fungus. The fungus parasitic within the plant is the dikaryotic mycelium phase, which grows intercellularly and intracellularly. The hypertrophy of the infected stem is probably resulted from growth-promoting substance(s)

* Former Associate Research Fellow at the Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan, Republic of China.

secreted by the fungus and indoleacetic acid has been implicated (Yu and Wu, 1967).

In an attempt to elucidate the physiology of this interesting fungus, studies on growth promoting requirements, utilization of amino acids and cellular composition were first undertaken.

Materials and Methods

Organism

Two culture types of *Ustilago esculenta* P. Henn were used in this investigation: (a) dikaryotic mycelium culture, isolated from young tissue of 'Kah-peh-sung' (*Zizania latifolia*) grown in Nankang, Taiwan and (b) yeastlike culture, arising spontaneously from a dikaryotic mycelium culture also isolated from the plant grown in Nankang, Taiwan (Fig. 1a and 1b).

Media

PDA was used for the regular maintenance of the culture and a temperature of 28°C was employed for all the experiments unless otherwise stated. The synthetic medium, glucose-asparagine-thiamine medium (GAB_1) was used for chemical tests. The GAB_1 has the following composition: glucose 20 g, DL-asparagine monohydrate 2 g, thiamine hydrochloride 0.5 mg, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 5 mg, $ZnSO_4 \cdot 7H_2O$ 4.4 mg, $MnSO_4 \cdot H_2O$ 2.75 mg, $CuSO_4 \cdot 5H_2O$ 0.4 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 1.8 mg, $CaCl_2$ 4.5 mg, NaCl 2.6 mg, and distilled water to a liter. This is a modification of the medium described previously (Lin and Gottlieb, 1974). The pH of the medium was adjusted to 6.5 with NaOH unless otherwise stated and was sterilized at 121°C for 10 min. Thiamine hydrochloride was sterilized by filtration through Millipore filter paper (0.45 μm pore diam) and was added to the sterilized medium just before its use. The solid medium contained 1.7% agar.

Growth requirement tests for the yeastlike culture

A) Growth factors. GAB_1 agar with the omission of thiamine was used. An aliquot (0.2 ml) of dilute yeastlike culture suspended in water was pipetted onto the agar plate and spread. The agar plate was kept at 28°C overnight before the addition of test compounds by auxanography (Perkins, 1944). The following growth factors were tested: *p*-aminobenzoic acid, biotin, pyridoxine HCl, riboflavin, nicotinic acid, ascorbic acid, calcium pantothenate, folic acid, myo-inositol, thiamine · HCl, choline chloride, ergosterol and cholesterol. Ergosterol and cholesterol, recrystallized before use, were dissolved in N, N′-dimethylformamide and all the other growth factors were dissolved in distilled water and sterilized by Millipore paper filtration. The test compounds were

absorbed into the filter paper disc (0.6 cm diam. from Schleicher and Schull Co.). B) Utilization of amino acids. GAB_1 agar with the omission of asparagine was used and the amino acids were examined by auxanography. The following L-amino acids were tested: alanine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and glycine. Also studied were the D-forms of alanine, aspartic acid, cystine, glutamic acid, methionine, threonine, tyrosine and valine. The test compounds were added to the agar plate as a powder. C) Hydrogen ion concentration. The effect of pH values on the growth was conducted in GAB_1 liquid medium at 28–29°C in a gyratory shaker.

Growth requirement tests for the dikaryotic mycelium

The following agar media were used: GAB_1, GAB_1 + inositol (1 g/liter), GAB_1 + nicotinic acid (1 g/liter), GAB_1 + RNA hydrolysate, GA + vitamin mixture + RNA hydrolysate, GAB_1 + cholesterol or ergosterol (0, 12.5, 25, 50, 100 μg/ml). The vitamin mixture (Lin and Gottlieb, 1974) contained in each liter of agar medium: thiamine·HCl, pyridoxine·HCl, calcium pantothenate, *p*-aminobenzoic acid, nicotinic acid, 0.5 mg each; myo-inositol 5 mg, biotin 5 μg, and riboflavin 0.25 mg. The mixture was sterilized by Millipore filter paper filtration and was added aseptically to the medium just before use. RNA hydrolysate was prepared as follows. To 5 g of RNA was added 25 ml 1 N NaOH and hydrolyzed at 121°C for 10 min. The pH of the hydrolysate was adjusted to 6.0; the volume to 50 ml. The preparation was then centrifuged to remove the insoluble and sterilized by Millipore filter paper filtration. An aliquot equivalent to 0.2 g of RNA was added to a liter of agar medium. Cholesterol and ergosterol were recrystallized from alcohol dissolved in N, N′-dimethylformamide and added to the medium aseptically just before use.

Fractionation of the cells

The yeastlike culture cultivated in GAB_1 liquid medium was harvested at log phase of growth and washed twice with 0.9% NaCl. The cells were lyophilized and dried to constant weight at 75°C. The dried cells was fractionated according to a modification of the procedures described by Gottlieb and Van Etten: a) Powdered dried cells (80 mg) were suspended in 5 ml of 80% ethanol. After 30 min, the supernatant fluid was again extracted with another 5 ml of 80% ethanol for 5 min and the supernatant fluid removed by centrifugation. b) The residue from step(a) was suspended in 5 ml of ethanol: ether (3:1, v/v), and the extraction were repeated as above. c) The residue from step (b) was suspended in 5 ml of diethyl ether and the extraction and the centrifugation were repeated as above. d) The residue from step (c) was

suspended in 5 ml of ice-cold 5% trichloroacetic acid (TCA) for 30 min and then for 5 min as above. e) The residue from step (d) was suspended in 5% TCA and hydrolyzed at 70°C for 30 min. The supernatant fluid from this step contained nucleic acids. This fraction was analyzed for DNA and RNA. f) The residue from step (e) was suspended in 5 ml ether twice to remove TCA. The dried residue was then suspended in 8 ml of 0.2 N NaOH and hydrolyzed at 121°C for 15 min. The supernatant fraction obtained after centrifugation was assayed for protein.

DNA was estimated by the indole method (Hubbard *et al.*, 1970), RNA by orcinol test (Ashwell, 1957), and protein by a modification of Lowry's method (Miller, 1959), using calf thymus DNA, yeast RNA and bovine albumin as standards, respectively.

Results

Minimal medium

The yeastlike culture of *U. esculenta* showed negligible growth, if any, on glucose-asparagine agar during the test periods of 3 to 4 days. This medium was therefore used as the minimal medium for testing growth requirement and utilization of amino acids.

Growth factors

When the yeastlike culture of *U. esculenta* was tested for growth factors individually by auxanography, the fungus grew vigorously with thiamine and slightly with pantothenate or inositol. No response was observed with *p*-aminobenzoic acid, biotin, pyridoxine·HCl, riboflavin, nicotinic acid, ascorbic acid, folic acid, choline chloride, ergosterol and cholesterol. Thus the fungus has a growth requirement for at least thiamine, inositol and pantothenate but not for the other growth factors tested.

Hydrogen-ion concentration effect

The pH of the GAB_1 liquid medium was adjusted with NaOH to 5.0, 5.5, 6.0, 6.5, and 7.0 before autoclaving and the growth of the fungus measured by turbidity at 650 nm or by cell number counting with hemocytometer (Table 1 and Fig. 2). The growth of the yeastlike culture was best at pH 6.5 and 7.0.

Utilization of amino acids

Of the 22 L-amino acids tested as the sole nitrogen source for the growth of the fungus in the GB_1 agar, only L-methionine, L-cystine and hydroxy-L-proline were unable to support the growth. In contrast, of the eight D-amino acids, only D-valine supported growth; inactive were D-alanine, D-aspartic acid, D-cystine, D-glutamic acid, D-methionine, D-threonine and D-tyrosine.

Table 1. *Cell number of the yeastlike culture of Ustilago esculenta at various pH value of the medium after four days of cultivation*

	Cell number/ml cultnre
pH 5.0	1.29×10^6
pH 5.5	1.42×10^6
pH 6.0	2.93×10^6
pH 6.5	8.04×10^6

The RNA hydrolysate was also an excellent sole nitrogen source for the growth of the yeastlike culture.

Growth requirement studies on dikaryotic mycelium

The addition of inositol, nicotinic acid and RNA hydrolysate to GAB_1 agar did not increase the growth rate of the fungus over that on the GAB_1 agar, (on which the diameter of the colony after 30 days of incubation was about 1.8 cm.). Similarly, the replacement of thiamine by the vitamine mixture failed to promote growth. Supplementing the medium with cholesterol or ergosterol, at concentration ranging from 12.5 to 100 μg/ml, to GAB_1 agar also did not enhance the growth rate of the fungus.

Content of DNA, RNA, and protein

In the yeastlike culture, these three important metabolic substances were analyzed. Their content, on the basis of % dry weight of cells, were 0.217 ± 0, 4.12 ± 0.25, and 16.03 ± 0.95 for DNA, RNA and protein, respectively.

Discussion

Ustilago esculenta can exist in two forms (dimorphism), mycelial and yeastlike. The dikaryotic mycelium isolated from the young tissue of 'kah-peh-sung' (*Zizania latifolia*) developed normal dikaryotic mycelium colony on PDA plate. However, after long incubation periods, yeastlike colonies tend to appear within the mycelium colony. The same phenomenon occurred when the mycelium was cultivated in the GAB_1 liquid medium. In an attempt to elucidate the physiology of the fungus the growth requirements of both types of culture were studied.

U. esculenta has been reported to require thiamine for good growth of yeastlike culture (Su, 1961). The present study showed that the yeastlike culture not only has a requirement for thiamine, but also for inositol and pantothenate. The growth was excellent with thiamine, but only to a small

extent with either inositol or pantothenate alone. The growth requirement for thiamine, inositol and pantothenate has also been reported in yeasts. The association of inositol heterotrophy with thiamine has been quite common in fungi (Fries, 1965).

The yeast type culture of the fungus has a pH optimum around 6.5 to 7.0. Although the growth at pH 7.0 was slightly better than that at 6.5, the medium at pH 7.0 would turn yellow and produce precipitates after sterilization. Thus pH 6.5 was chosen for the medium.

With the 22 naturally occurring L-amino acids tested as the sole nitrogen source for growth, only three amino acids—methionine, cystine (both sulfur-containing amino acids), and hydroxy-L-proline failed to sustain the growth. The results suggest that the fungus probably has a defect in transport and/or catabolism system for these amino acids to obtain the nitrogen which is necessary for the building of cellular constituents for growth. Inhibition of growth by L-methionine and hydroxy-L-proline has been reported in some fungi (Fries, 1949; Robbins and McVeigh, 1946; Schopfer and Blumer, 1943; Stockdale, 1953). The ability of this smut to utilize L-proline but not hydroxy-L-proline is intriguing in that hydroxylation of an amino acid results in the loss of utilizability of that amino acid. This phenomenon points out the high specificity of the transport and/or catabolism system for these amino acids.

With the eight D-amino acids examined as sole nitrogen source, only D-valine could support the growth of the fungus. D-alanine, D-aspartic acid, D-cystine, D-glutamic acid, D-methionine, D-threonine as well as D-tyrosine could not, although the L-form of alanine, aspartic acid, glutamic acid, threonine and tyrosine could. This difference again indicates the great specificity of the transport and/or catabolism system of these amino acids in this fungus. The ability of *Ustilago* sp. to utilize D-valine has been reported before (Blumer and Schopfer, 1940).

The growth of the dikaryotic mycelium phase of this smut in the synthetic media has been relatively slow, with a colony diameter of less than 2.0 cm after 30 days of incubation. The supplements of vitamin mixture, inositol, nicotinic acid, RNA hydrolysate, or sterols did not enhance the growth. The growth of this dikaryotic mycelium on PDA was better than that on synthetic media. The dikaryotic mycelium phase of *Ustilago* sp., which is parasitic on the plant, in general is unable to or very difficult to grow *in vitro*. The search for improved growth of the dikaryotic mycelium *in vitro* is still in progress.

The content of DNA, RNA, and protein, the three important metabolic substances vital to cellular growth, was 0.2, 4.1 and 16% of dry weight of cells. They are in the range generally found for fungi (Lin and Gottlieb, 1974).

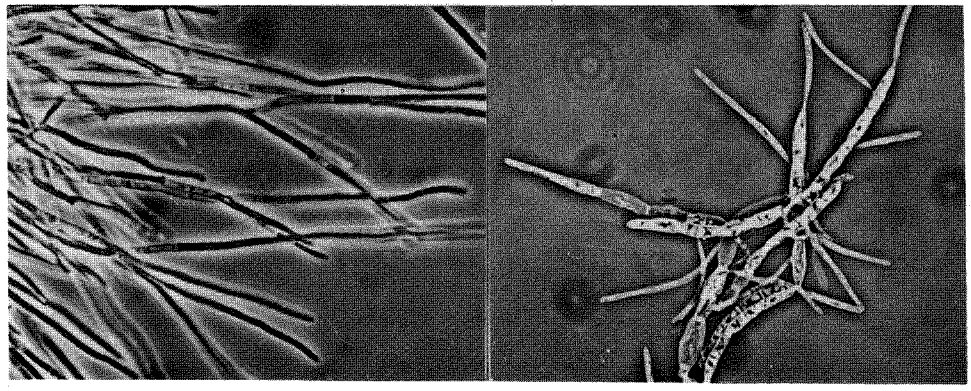

Fig. 1. Dimorphism of ***Ustilago esculenta.*** (a) The dikaryotic mycelium (×900). (b) The yeastlike culture arising spontaneouly from a dikaryotic mycelium colony (×900).

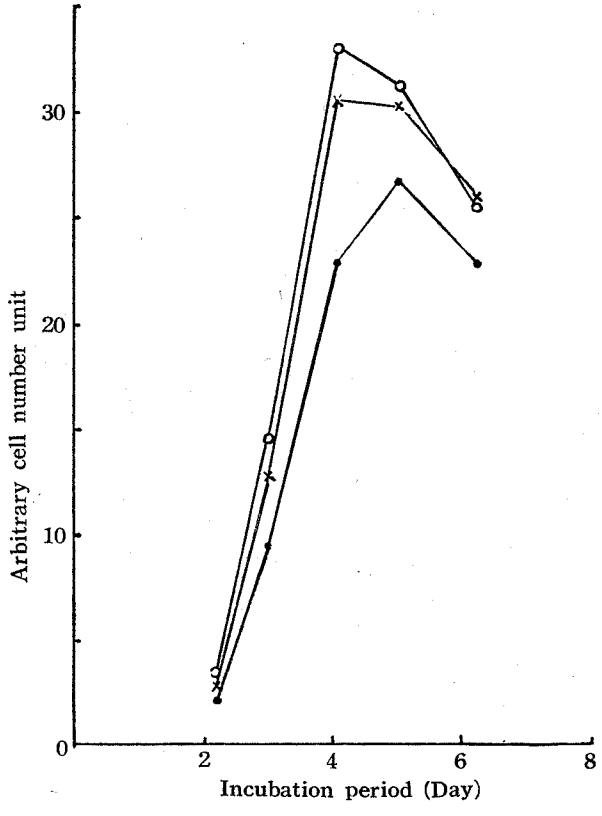

Fig. 2. Effect of pH value of the medium on the growth of the yeastlike culture of ***Ustilago esculenta.*** The cell density was measured by turbidity at 650 nm. ○—○, pH 7.0; ×—×, pH 6.5; and ●—●, pH 6.0.

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茭白筍黑穗菌，*Ustilago esculenta* 的生理：生長需求，胺基酸之利用，和細胞組成份

林 福 坤

美國南加羅林那醫學院基礎及臨床免疫及微生物系

現已發展出一種培養形態似酵母菌的茭白黑穗菌 *Ustilago esculenta* 生長，發育所需最少量培養基。其生長最適合的 pH 值大約在 6.5～7.0 之間。以細菌發育檢查技術做實驗，可知此種眞菌在發育期中需要 Thiamine, inositol, pantothenate, 而不需要 biotin, *p*-aminobenzoic acid, pyridoxine·HCl, riboflavin, nicotinic acid, ascorbic acid, folic acid, choline chloride, ergosterol 和 cholesterol。以 22 種天然存在的 L 型胺基酸做爲生長時唯一氮源所做的實驗結果可知：除了 methionine, cystine 和 hydoxyproline 外，其他 L 型胺基酸均可支持其生長。另外，以 8 種 D 型胺基酸做爲唯一氮源的實驗顯示：只有 D-valine 爲其生長發育所需，其他如 alanine, aspartic acid, cystine, glutamic acid, methionine, threonine 和 Tyrosine 均不能供給正長發育所需之營養。DNA, RNA, 蛋白質於細胞中所佔乾重量的百分比分別是 0.22%, 4.12% 和 16%，然而 *U. esculenta* 在雙核菌絲態時，在培養其中却需要加一點 Thiamine。若以混合成份維他命取代 Thiamine, 以及加入 RNA hydrolysate, 或 cholesterol, ergosterol 時，均無法使其正常生長。