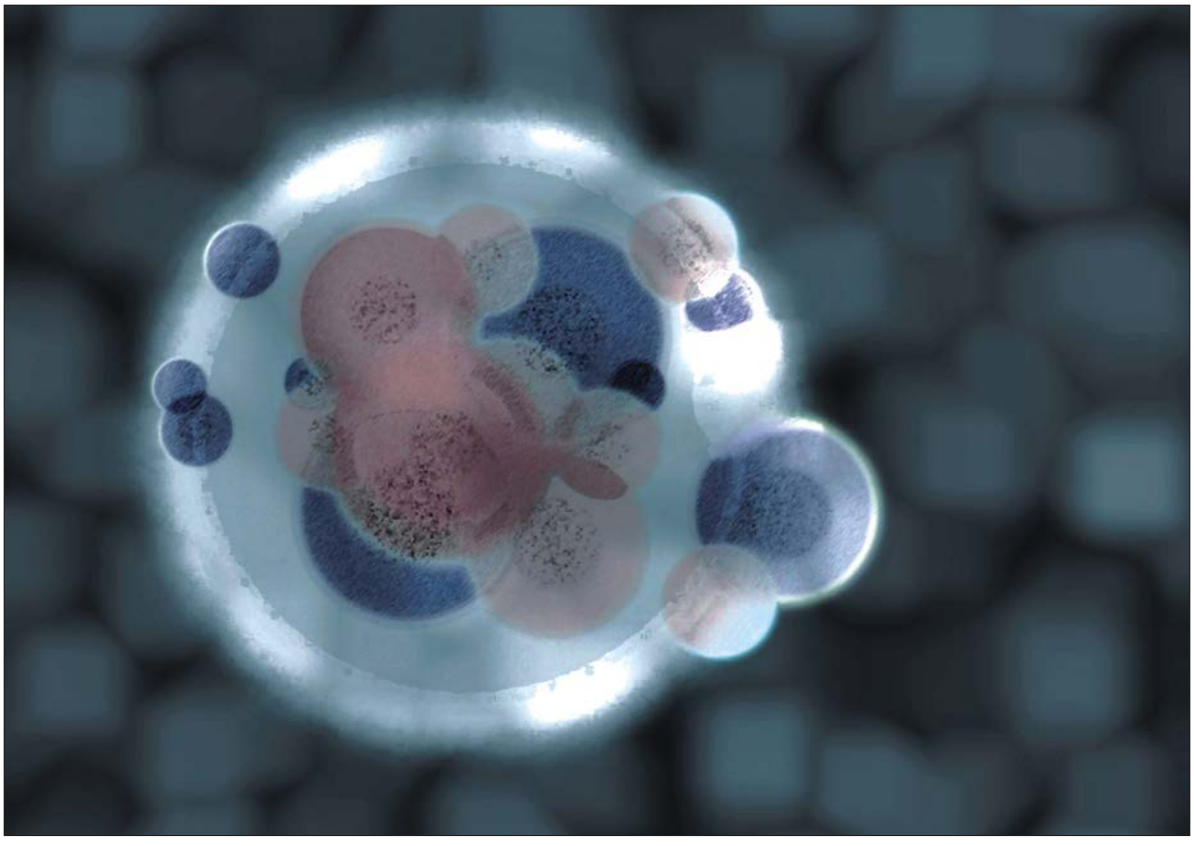

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 4 (2): 246-252, 2004
ISSN 1727-3048

Nutritional Requirements of the Edible Gall-producing Fungus *Ustilago esculenta*

[1]Kuang-Ren Chung and Dean D. Tzeng
Department of Plant Pathology, National Chung-Hsing University, Taichung, 402, Taiwan, Republic of China
[1]University of Florida, Citrus Research and Education Center, 700 Experiment Station Road,
Lake Alfred, FL 33850-2299, USA

Abstract: The parasitism of *Ustilago esculenta* P. Henn. in the perennial aquatic grass, *Zizania latifolia* (Griseb.) Turcz. results in the development of edible smut gall, "Kah-Peh-Sung" which has long been extensively cultivated as a vegetable in Taiwan and southern China. The nutritional requirements of *U. esculenta* were investigated in a semi-defined liquid medium to explore further studies of the causative fungus and its interaction with host plant. The fungus grew as yeast-like sporidia *in vitro* and grew very poorly in the Czapek's medium. Of the 13 vitamins and growth factors tested for growth stimulation, thiamine was found to contribute the most for fungal growth in culture. Among 14 carbohydrates tested in the presence of thiamine, the most favorable in order of effectiveness were sucrose, raffinose, fructose, glucose, maltose and galactose. The fungus utilized most of the amino acids tested. The most suitable inorganic nitrogen sources in order were potassium nitrate, sodium nitrate and ammonium phosphate. Organic amino nitrogen sources were preferred to inorganic ones. Fungal growth was correlated with the increase of C/N ratio. The optimum temperature for fungal growth ranged from 20 to 28°C and the optimum pH ranged from 4 to 7. The maximum growth was reached in 7 d under the optimum conditions.

Key words: Growth factors, nutrition, smut, thiamine, *Zizania* grass

INTRODUCTION

Ustilago esculenta P. Henn is a basidiomycetous, parasite of the perennial aquatic grass, *Zizania latifolia* (Griseb.) Turcz. The fungus has very restricted host range and by far *Z. latifolia* is the only known host. The fungus stimulates enlargement of the culms of the host grass and results in an edible gall (Fig. 1). For several centuries, the swollen, infected culms have been cultivated as a vegetable, commonly called "Kah-Peh-Sung" or "Gau-Sun" in Taiwan and southern China. Based on the color of the outer sheath of the gall, three cultivars of *Z. latifolia* (green, red, white) are commonly infected with *U. esculenta* in Taiwan. The green cultivar is planted in late December and the edible galls are usually harvested sparsely during February-May and abundantly in September. The red and white cultivars are planted later and produce galls only in October and November. In unfavorable conditions, galls are usually full of dark teliospores and are not suitable for consumption. Because of its unique flavor and delicacy, this type of smut gall became very popular in Taiwan and southern China. Moreover, the production of gall occurs during the typhoon (tropical storm) season in which most of vegetables are affected and thus the galls usually provide an alternative for consumers. This fungus therefore is highly beneficial and economically important.

U. esculenta spends its entire life cycle in the host plant (Fig. 2). The fungus grows inter- and intracellularly in the stem tissues, especially near the apical meristem and is not found in leaf and root tissues[1]. The fungus completely prevents the grass host from producing inflorescences and incites gall development at the node regions beneath the apical meristem. The edible galls (ca 3-4 cm in width and 15-20 cm in length) usually are formed within 10-15 d despite the fact that the plants have been planted in the soil for over 6-8 months. At this stage the inner tissues of gall appear white and are full of fungal hyphae. The fungal hyphae further develop to form dark teliospores in the gall tissues (Fig. 1A). Temperature higher than 28°C promotes the formation of teliospores. The fungus overwinters as mycelium and teliospores in grass rhizomes. The infected grass in the field is exclusively propagated using an asexual rhizome. Therefore, the fungus is transmitted through the vegetative tissues of the grass host. Alternatively, the teliospores retained in the field may initiate infection, but this has not been yet experimentally demonstrated.

Corresponding Author: Kuang-Ren Chung, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850-2299, USA

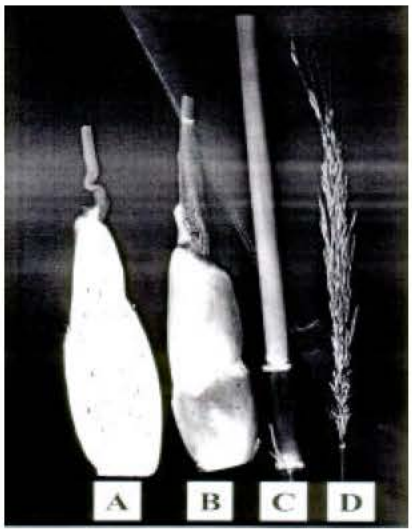

Fig. 1: Edible gall in the perennial aquatic grass, *Zizania latifolia*, is incited by a phytopathogen *Ustilago esculenta* (A, B) and the healthy, uninfected grass © which produces inflorescences (D). The gall is formed at the region of the third to fourth nodes beneath the apical meristem and is *ca.* 3 to 4 cm in width and 15 to 20 cm in length. The gall may contain numerous dark fungal teliospores as shown in the cross section (A)

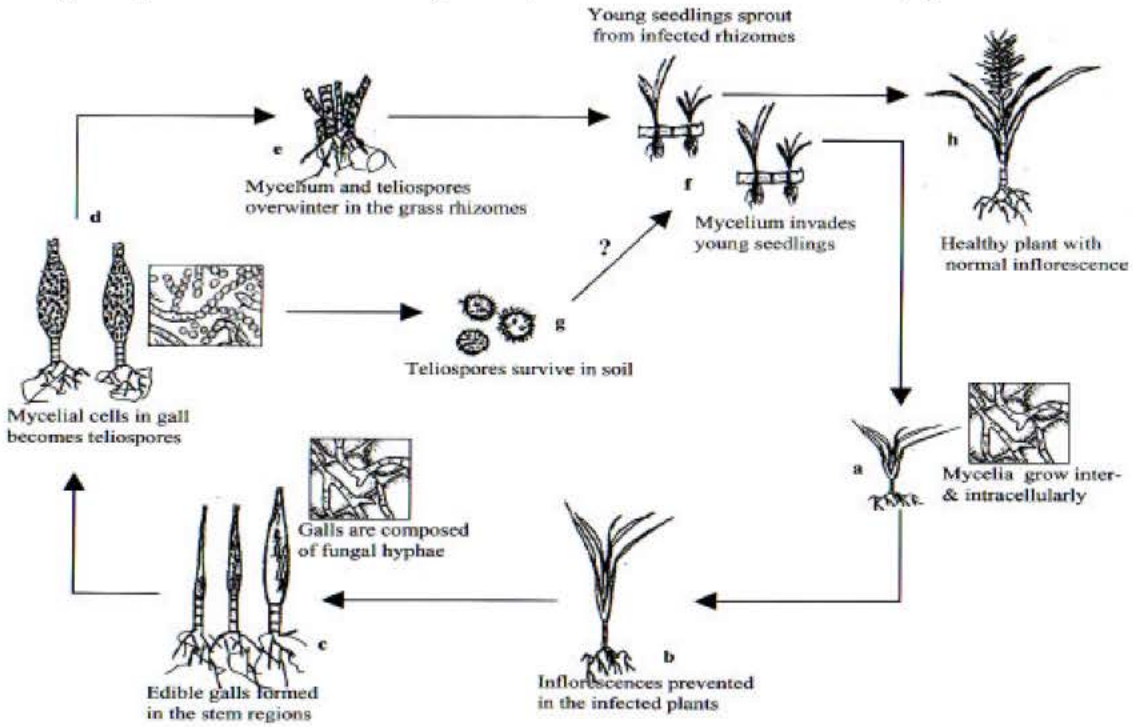

Fig. 2: Life cycle of *Ustilago esculenta* in *Zizania latifolia*. Fungal structures are shown in boxes. The fungus spends its entire life cycle in the host plant and produces edible galls. The fungus grows inter and intracellularly in the stem tissues near the apical meristem (a) and is not found in root and leaf. The fungus prevents inflorescence production of the host (b) and incites gall development at the basal stem, in which dikaryotic hyphae are abundant (c). The fungal hyphae further develop to form black teliospores in gall tissues (d). *U. esculenta* overwinters as mycelium and teliospores in the grass rhizomes (e) and is transmitted into new shoots through asexual propagation (f). Alternatively, the teliospores decomposed from galls survive in soil (g). The ability of teliospores in soil to infect new plants has not yet been experimentally documented. In a few cases, the rhizomes escape infection and the plants produce normal inflorescences, but the seeds are sterile (h)

Occasionally, some of the rhizomes are free of the fungus and the new growing grasses are uninfected. The uninfected plants grow slightly higher than the infected ones and are able to produce inflorescences but with sterile seeds. The uninfected healthy plants are nonproductive and usually removed by growers. Thus all grass hosts in the field are infected with *U. esculenta*. Due to vegetative propagation, the fungal lineages are highly clonal. The crop is grown in flooded paddies and usually rotated with rice. The temperature of the flooding water (usually kept under 25°C) is critical for gall development and prevention of teliospore formation. Unsuitable temperature limits gall size and tends to induce early production of dark, sandy teliospores.

Due to the lack of fundamental research on the causal fungus and the grass host, knowledge of crop management depends solely on the growers' experiences. The yield, gall quality and cropping systems all remain to be improved. It will be imperative to develop a cultural system to further explore the physiological, molecular and genetic basis of fungal production of plant growth regulators and their relevance to gall development. Although nutritional requirements have been investigated[2-4], apparent discrepancies exist between different studies. The nutritional requirements for *in vitro* growth of *U. esculenta* were conducted to establish reliable cultural conditions for further studies.

MATERIALS AND METHODS

Fungal isolates and basal medium: Fungal cultures of *Ustilago esculenta* were isolated from the infected gall tissues and routinely maintained on potato sucrose agar (PSA) plates. As a source of inoculum for growth media, sporidia from a fresh culture were suspended in sterile water with vigorously shaking. The concentration of the resultant aqueous suspension was adjusted to 10^6 sporidia mL^{-1} with a hemocytometer, then 1 mL of suspension was added to 125 mL Erlenmeyer flasks containing 24 mL basal medium. The basal medium used in the nutritional studies was modified from Czapek's solution by supplement 10 μM thiamine or 0.5% yeast extract with 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4$-$7H_2O$, 0.05% KCl, 0.01% $FeSO_4$-$7H_2O$, 3% sucrose. The original pH was adjusted to 6.5 unless otherwise indicated. In carbon utilization studies, the basal medium was supplemented with 90 mM of the carbon source, equivalent carbon content to that of sucrose. Polysaccharides (pectin, starch) were added at a final concentration of 5 mg mL^{-1}. In nitrogen utilization experiments, the basal medium was amended with 24 mM of the nitrogen source, equivalent nitrogen content to that of sodium nitrate. Each 10 μM vitamin or growth promoting substance was added in the medium. Amino acids, vitamins, growth-promoting substances were purchased from Sigma Chemical Co. (St. Louis, Mo, USA), while all other chemicals or reagents were from Merck Chemical Co. (Germany). All the chemicals used in this study were reagent grade or equivalent in purity. Glass double distilled water was used for preparation of all media.

Growth measurement: For fungal growth, cultures were incubated at 25°C unless otherwise stated on a rotary shaker. At the end of the incubation period, the contents of each 25 ml Erlenmeyer flask were filtered through a pre-dried and pre-weighed Whatman No.1 filter paper (Maidstone, England) and washed with deionized water. The filters were dried at 90°C for 24-36 h, then cooled down in a vacuum dessicator to obtain a constant dry weight. Each nutrient variable was run in 4 replicates and all experiments were repeated at least twice.

Statistical analysis: The significance of treatments was determined by analysis of variance and treatment means were separated by the Waller-Duncan k ratio t test ($p<0.01$).

RESULTS AND DISCUSSION

U. esculenta barely grew in the liquid Czapek's medium (Fig. 3). The growth was greatly stimulated in potato sucrose broth (PSB), or by supplementing with 0.5% yeast extract or 10 μM thiamine (Fig. 3). However, 0.5% malt extract or peptone only slightly supported fungal growth (data not shown). Among the 13 vitamins or growth factors tested for growth promotion of *U. esculenta*, only thiamine (vitamin B1) significantly and pyridoxine (vitamin B6) slightly stimulated fungal growth in the Czapek's solution (Fig. 4). The growth stimulated by thiamine was comparable to that obtained by supplementing the medium with yeast extract. The obligate requirement of thiamine for growth suggests that *U. esculenta* is auxotrophic for thiamine. This result is in agreement with the findings of other groups[2-4]. No significant stimulation of fungal growth was observed with nicotinic acid, riboflavine, biotin, α-tocopherol, folic acid, ascorbic acid, β-carotene, inositol, cyanocobalamin, aminobenzoic acid, or D-pantothenate. Yeast extract by itself supported significant growth and served as sources of vitamins and growth factors in carbon and nitrogen utilization. Thiamine appeared to be one of the pivotal components in yeast extract. There was no significant difference between various amounts of thiamine for

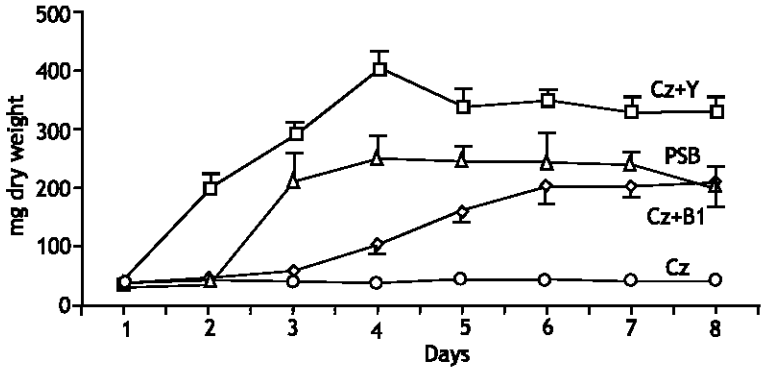

Fig. 3: Growth curves of *Ustilago esculenta* in Czapek's basal medium (Cz), Czpake's solution supplemented 10 μM thiamine (Cz+B1), or 0.5% yeast extract (Cz+Y) and potato sucrose broth (PSB). Media with pH #6.5 were inoculated with a sporidial suspension, then incubated on a rotary shaker at room temperature. At the end of incubation period, the fungal spores were harvested and the dry weight was measured as described in the text

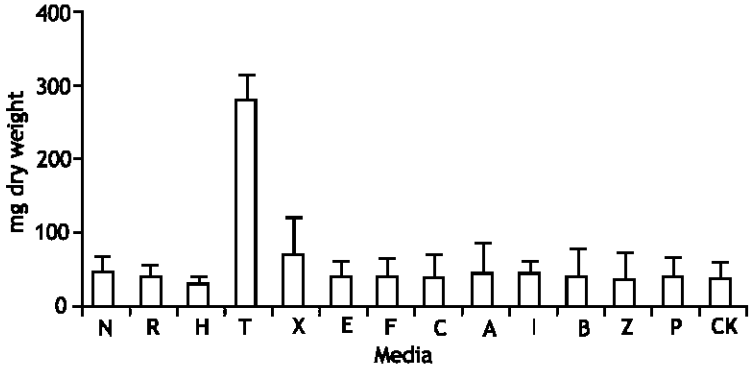

Fig. 4: Effect of various vitamins and growth factors on the growth of *Ustilago esculenta* in Czapek's solution. Growth factors (N, nicotinic acid; R, riboflavin; H, biotine; T, thiamine; X, pyridoxine; E, á-tocopherol; F, folic acid; C, ascorbic acid; A, â-carotene; I, inositol; B, cyanocobalamin; Z, aminobenzoic acid; P, D-pantothenic acid; CK, no vitamin supplement) were sterilized by Millipore filtration, then added into media. Sporidial suspensions were added to media and incubated at room temperature. Growth was measured after 7 days

Table 1: Effect of hydrogen-ion (pH) concentrations on the growth of *Ustilago esculenta* on three different media

	Dry weight±SD (mg)[a]		
pH	Cz[b] + thiamine	Cz + yeast extract	PSB[c]
2.0	nd[d]	31.3±2.7c	nd
3.0	47.2±3.2c	52.2±5.9c	nd
4.0	248.9±17.4a	289.6±21.4a	248.6±19.5a
5.0	253.2±13.3a	302.6±18.8a	190.2±8.6ab
6.0	191.3±12.7b	308.7±9.4a	234.6±13.1a
7.0	140.8±.5b	176.3±11.7b	159.3±15.4ab
8.0	51.3±7.9c	69.6±4.1c	228.7±23.8a
9.0	29.8±5.6cd	65.2±8.3c	nd

[a]Dry weights (mg 25 mL^{-1} medium) are indicated as means±standard deviations (SD) of two different experiments.
[b]Czapek's basal medium [c]potato sucrose broth [d]no data

promoting fungal growth (data not shown). The maximum growth was obtained about 7 days after inoculation in the medium containing 10 μM thiamine (Fig. 3).

All fungi require vitamins for growth or development. Thiamine had no effect on the morphology of *U. esculenta* wherein the fungus grew as sporidia. Thiamine consists of a pyrimidine moiety and a thiazole group and is required by supplement for growth in many fungi[5-11] Thiamine and its biologically active form thiamine-pyrophosphate have a wide variety of biochemical effects in orgamsms. They usually serve as cofactors for many enzymes in TCA cycle including pyruvate decarboxylase and pyruvate dehydrogenase. They are important components for carbon metabolism and energy production. Thiamine has also been demonstrated to increase gene expression in yeast[12]. Furthermore, thiamine is able to affect the intracellular level of other metabolites such as amino acids, lipids and organic acids in many fungi[13-15]. Interestingly, pigment formation of teliospore is inhibited by thiamine in *U. hordei*[16].

The hydrogen-ion (pH) concentrations in Czapek's solution with thiamine suitable for fungal growth were from 4.0 to 7.0 with the optimum of pH 5.0 (Table 1).

Table 2: Growth comparison of *Ustilago esculenta* on the basal medium supplemented with 10 μM thiamine, sodium nitrate and various carbon sources

Carbon source		Dry weight±SD (mg)[a]
Pentoses	D-arabinose	32.3±0.7de
	D-xylose	55.3±1.4d
Hexoses	D-fructose	143.9±4.7b
	D-galactose	107.7±4.8bc
	D-mannose	115.4±6.2bc
	D-glucose	123.1±8.2b
Disaccharides	Maltose	55.4±2.1d
	Lactose	47.7±5.6d
	Sucrose	292.3±18.4a
Oligosaccharides	Raffinose	161.5±14.7b
	Soluble starch	46.2±6.3d
	Pectin	47.5±3.9d
Polyols	D-mannitol	55.4±7.4d
	Ethanol	40.8±3.6d
	Control[c]	15.4±1.4e

[a] Dry weights (mg 25 mL^{-1} medium) are indicated as means±standard deviations (SD) of two different experiments

[c]Controls that did not contain any carbon source, but contained all other nutrients

Table 3: Effect of various nitrogen sources on the growth of *Ustilago esculenta* on the basal medium supplemented with sucrose and 10 μM thiamine

Nitrogen source	Dry weight ±SD (mg)[a]	Nitrogen source	Dry weight ±SD (mg)[a]
Calcium nitrate	47.0±3.4ef	L-cysteine	102.3±11.6d
Potassium nitrate	253.5±21.2b	Valine	286.7±26.1a
Sodium nitrate	215.4±18.7bc	L-histidine	301.2±19.7a
Ammonium sulfate	58.1±5.2ef	L-tryptophan	206.2±13.9b
Ammonium chloride	77.8±2.9de	L-phenylalanine	290.6±21.5a
Ammonium phosphate	182.1±12.5c	L-proline	323.9±17.8a
Ammonium nitrate	70.1±6.2de	L-methionine	43.5±11.2ef
Urea	42.7±3.6f	Threonine	129.4±9.6c
L-glutamic acid	270.7±17.6ab	Serine	323.6±29.6a
L-glutamine	221.7±14.9bc	Lysine	129.5±12.1c
L-aspartic acid	210.9±15.2bc	Arginine	223.7±17.3b
L-asparagine	298.2±23.6a	Oruithine	182.6±14.7c
â-alanine	296.1±19.3a	Citruline	331.5±23.8a
Glycine	79.4±7.3de	Tylosine	324.6±19.9a
L-leucine	300.5±18.4a	Control[b]	37.7±5.6f

[a]Dry weights (mg 25 mL^{-1}) are indicated as means±standard deviations (SD) of two different experiments

[b]Controls that did not have a nitrogen source, but contained all other nutrients

Fungal growth was inhibited in the pH #3.0 and pH above 8.0. Similar patterns were observed when fungus was grown in the medium containing yeast extract or in potato sucrose broth (Table 1). At the end of the growth period, pH in all media tended to increase to 8 (data not shown) and the increase in pH appeared apparently to be the major factor for growth inhibition. The range of temperatures for fungal growth was from 16 to 28°C (Fig. 4). The fungus had prolonged lag phase at 16°C and fungal growth was heavily inhibited at 32°C.

In a defined medium the fungus exhibited an extreme requirement for thiamine. Fourteen different carbon sources were screened for growth of *U. esculenta* in the presence of thiamine (Table 2). Two pentoses, arabinose and xylose, were not utilized. Of the four hexoses tested, the fungus utilized fructose, galactose and glucose but not mannose. Of the four disaccharides screened, only maltose and sucrose supported significant growth. The fungus did not utilize lactose and cellobiose. Good growth was observed on raffinose, whereas no growth occurred on soluble starch, pectin, mannitol or ethanol. Overall, among the 14 carbon sources tested in the presence of thiamine, the most favorable in order were sucrose, raffinose, fructose, glucose, maltose and galactose. The optimum concentration of sucrose for growth was at 146 mM (~50 mg mL^{-1}). Glucose and fructose are readily metabolized through the glycolytic pathways in fungi[17]. These carbon sources were utilized only in the presence of thiamine, indicative of the major role of thiamine for sugar metabolism. Glucose and fructose are the most abundant sugars in *Z. latifolia* and sucrose is the major translocated sugar in the host plant[18], which in turn confirms that these sugars support most fungal growth *in vitro* and *in vivo*.

Nitrogen utilization studies reveal that *U. esculenta* was capable of utilizing a wide variety of nitrogen sources, especially organic amino acids. In the presence

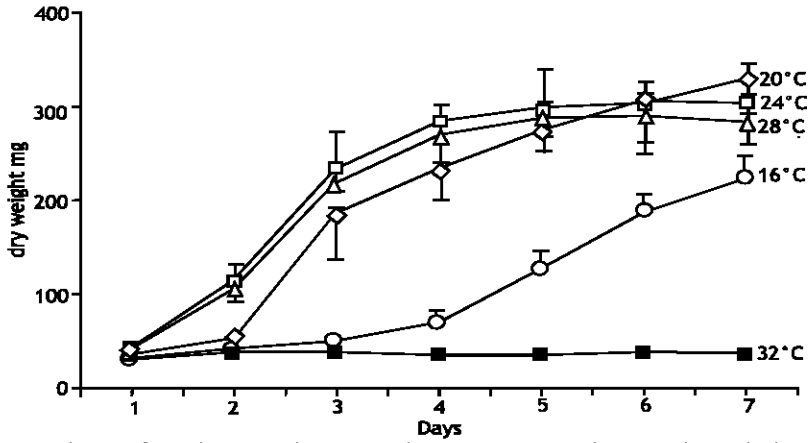

Fig. 5: Growth comparisons of *Ustilago esculenta* at various temperatures in Czapek's solution supplemented with 0.5% yeast extract. Growth measured as mg dry weight at the end of incubation

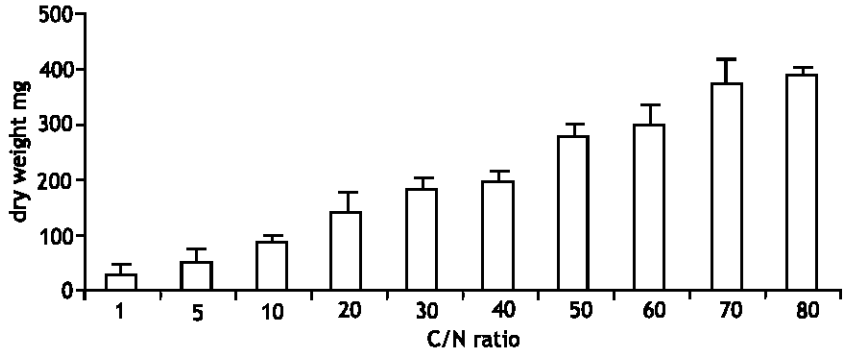

Fig. 6: Effect of carbon/nitrogen (C/N) ration on the growth of *Ustilago esculenta* in Czapek's solution containing 10 μM thiamine. Sucrose and potassium nitrate were used as the sole carbon and nitrogen sources, respectively, in Czapek's basal solution

of thiamine, the most suitable inorganic nitrogen sources in order were potassium nitrate, sodium nitrate and ammonium phosphate, whereas calcium nitrate, ammonium sulfate and ammonium nitrate supported only slight fungal growth (Table 3). The optimum concentration of potassium nitrate
and sodium nitrate was 20 mM. The apparent ability to grow on nitrate indicates that the enzymes in the nitrate reduction pathway are very active in *U. esculenta*. The fungus was unable to utilize urea as the sole nitrogen source probably due to lack of a specific transport system for urea as suggested in *Aspergillus nidulans*[19] or due to absence of urease. The fungus was able to utilize most of the 22 amino acids tested (Table 3). Glycine, threonine, lysine and sulfur-containing amino acids such as methionine and cysteine supported little fungal growth, indicating that the sulfate-containing amino acids such as methionine and cysteine apparently are toxic to the fungus.

In general, most amino acids were good nitrogen sources and organic amino nitrogens were preferred to inorganic nitrogen sources. Some amino acids such as glycine and lysine which supported the fungal growth slightly could have less efficient uptake systems, or lack one or more enzymes in the catabolic pathway. The amount of fungal growth increased with carbon/nitrogen (C/N) ratio (Fig. 6) when sucrose and potassium nitrate were used as the sole carbon and nitrogen sources, respectively, suggesting that *U. esculenta* demands high amount of carbon source for optimum growth.

We have established a complete cultural system for this unique fungus, providing an opportunity to investigate the *U. esculenta-Z. latifolia* grass interactions. *U. esculenta* exhibited absolute requirement for thiamine (vitamin B_1) and was able to utilize a wide variety of carbon and nitrogen sources. The results from this study provide reliable conditions for future study at physiological and molecular levels of such factors as the production of indole-3-acetic acid, cytokinins and other growth regulators in culture and their relevance to fungal pathogenicity/virulence and gall formation.

ACKNOWLEDGMENTS

We thank Dr. L.W. Timmer (University of Florida) for his critical review of the manuscript. This work was supported by research grants from the National Science Council and the Council of Agriculture, Taiwan, Republic of China.

REFERENCES

1. Yan H.C. and L.S. Leu, 1978. Formation and histopathology of galls induced by *Ustilago esculenta* in *Zizania latifolia*. Phytopathol., 68: 1572-1576.
2. Su, H.J., 1961. Some cultural studies on *Ustilago esculenta*. Special Publication College Agriculture National Taiwan University, 10: 139-160.
3. Chan, Y.S. and L.B. Thrower, 1980a. The host parasite relationship between *Zizania caduciflora* Turcz. and *Ustilago esculenta* P. Henn., II. *Ustilago esculenta* in culture. New Phytologist, 85: 209-216.
4. Lin, F.K., 1981. Physiology of an edible smut, *Ustilago esculenta*: growth requirements, utilization of amino acids and cellular composition. Botany Bulletin Academia Sinica, 22: 103-111.
5. Bartnicki-Garcia, S. and W.J. Nickerson, 1961. Thiamine and nicotinic acid. Anaerobic growth factors for *Mucor rouxii*. J. Bacteriol., 82: 142-148.
6. Osborne, L.D. and L.B. Thrower, 1964. Thiamine requirement of some wood-rotting fungi and its relation to natural durability of timber. Trans. British Mycol. Soc., 47: 601-611.

7. Riding, W.H., M.E. Gallegy and V.C. Lilly, 1969. Thiamine requirements helpful in distinguishing isolates of *Pythium* from those of *Phytophthora*. Phytopathol., 59: 737-742.
8. Rawla, G.S. and S.S. Chahal, 1975. Comparative trace element and organic growth factor requirements of *Ramulispora sacchari*. Trans. British Mycol. Soc., 64: 532-535.
9. Barnett, E.A. and W.A. Ayers, 1981. Nutritional and environmental factors affecting growth and sporulation of *Sporidesmium sclerotivorum*. Canadian J. Microbiol., 27: 685-691.
10. Kulkarni, R.K. and B.D. Nielsen, 1986. Nutritional requirements for growth of a fungus endophyte of tall fescue grass. Mycologia, 78: 781-786.
11. Bertoni, M.D. and L. Levin, 1997. Nutritional requirements for growth of an endophyte: *Ceratopycnidium baccharidicola*. Review of Argentina Microbiol., 29: 131-136.
12. Ichikawa, K., Y. Shiba, M. Yamazaki and N. Serizawa, 1997. Thiamine increase expression of yeast gene. Bioscience Biotechnol. Biochem., 61: 1221-1224.
13. Rozenfeld, S.M. and E.N. Disler, 1971. Characteristics of the pool of free intracellular amino acids in the thiamine heterotrophic yeast *Candida lipolytica*. Mikrobiologiya, 40: 218-222.
14. Tachibana, S. and J. Siode, 1971. Effect of thiamine on the L-malate fermentation by *Schizophyllum commune*. J. Vitaminol., 17: 215-218.
15. Nishikawa, Y., I. Kanamura, T. Kamihari and S. Fukui, 1977. Effect of thiamine and pyridoxine on the lipids composition of *Saccharomyces carlsbergensis* 4228. Biochem. Biophys. Acta, 486: 483-489.
17. Cochrane, V.W., 1976. Glycolysis. In: The Filamentous Fungi, Vol. 2. (Eds. J.E. Smith and D.R. Berry), Edward Arnold Press, London, pp: 65-91.
18. Chan, Y.S. and L.B. Thrower, 1980b. The host parasite relationship between *Zizania caduciflora* Turcz. and *Ustilago esculenta* P. Henn., III. Carbohydrate metabolism of *Ustilago esculenta* and the host-parasite combination. New Phytologist, 85: 217-224.
19. Dunn, E. and J.A. Pateman, 1972. Urea and thiourea uptake in *Aspergillus nidulans*. Heredity, 29: 129.