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Thiamine (Vitamin B_1) Plays a Critical Role on Sugar Utilization by the Phytopathogenic Fungus, *Ustilago esculenta*

[1,2]Kuang-Ren Chung and [1]Dean D. Tzeng
[1]Department of Plant Pathology, National Chung-Hsing University,
250 Kuo-Kuang Road, Taichung, 402, Taiwan
[2]Citrus Research and Education Center, University of Florida, IFAS,
700 Experiment Station Road, Lake Alfred, FL 33850-2299, USA

Abstract: *Ustilago esculenta*, inducing edible galls in its host *Zizania latifolia*, exhibits an obligate requirement for thiamine (vitamin B_1) in axenic culture. The function of thiamine for growth in *U. esculenta* was investigated and compared with two closely related species, *U. maydis* (corn smut) and *U. scitaminea* (sugarcane smut). Sucrose was readily broken into glucose and fructose, independent of thiamine, by all three fungal species tested. Growth of *U. maydis* and *U. scitaminea* was apparently not affected by thiamine when glucose or fructose was used as the sole carbon source. By contrast, *U. esculenta* was incapable of utilizing glucose and fructose in the absence of thiamine. Addition of thiamine into a synthetic medium drastically enhanced the growth of *U. esculenta*. In all cases, *Ustilago* species preferentially utilized glucose prior to fructose. Fructose uptake in *U. esculenta* exhibited a saturated kinetic, indicative of carrier protein-mediated process. The uptake of fructose by *U. esculenta* was highly influenced by the amounts of glucose, and was likely via., a noncompetitive mode. Taken together, the results strongly indicate that thiamine plays a key role for glucose and fructose metabolisms and energy production by *U. esculenta*.

Key words: Gall, glucose, pathogen, smut fungi, water bamboo, wild rice

INTRODUCTION

Ustilago esculenta is a basidiomycetous parasite of the aquatic grass, *Zizania latifolia* (Griseb.) Turcz. The fungus completely suppresses inflorescence and seed production of the host and causes a smut gall on the culms (Chan and Thrower, 1980; Terrel and Batra, 1982; Chung and Tzeng, 2004a, b). With their tenderness and delicacy, gall tissues are edible and are usually consumed as a vegetable in Southern Asia. During August and September, tropical storms often cause disastrous damage to most vegetables. However, the galls in *Z. latifolia* grown in flooded paddies are not affected and can be used to supplement the shortage of other vegetables. The association also represents a unique and rare case in which a plant disease caused by a phytopathogenic fungus ironically results in food production. However, the interactions between the causal fungus and the perennial aquatic grass has been investigated very few. A prior study revealed that IAA levels were much higher in galls and *U. esculenta* was able to synthesize IAA in culture (Chung and Tzeng, 2004a), suggesting that hormones are likely involved in gall formation.

Three cultivars of *Z. latifolia* (green, white and red), based on the color of the outer sheath of the gall are commonly infected by *U. esculenta* in Taiwan (Yang and Leu, 1978; Chung and

Corresponding Author: Kuang-Ren Chung, Citrus Research and Education Center, University of Florida, IFAS, 700 Experiment Station Road, Lake Alfred, FL 33850-2299, USA
Tel: (863) 956-1151/1369 Fax: (863) 956-4631

Tzeng, 2004a, b). The green and white cultivars produce galls twice a year (sparsely during February-April and abundantly in September), whereas the red cultivar produces gall only in the late October. The crop is maintained in flooding water and usually rotated with rice plant. The temperature of flooding water is crucial for gall formation and gall qualities since unsuitable temperature restricts the gall development and also results in early production of teliospores. The fungus is transmitted through vegetative rhizomes to new grass seedlings in the field. Due to human selections, all *Z. latifolia* grasses in the field are associated with *U. esculenta* and the fungal isolates are highly clonal (Chung and Tzeng, 2004b). Occasionally, some plants escape from infection and the healthy plants produce inflorescence, but the seeds are sterile.

U. esculenta mycelium grows inter- and intra-cellularly in the host vegetative tissues, mostly near the apical meristems, but never found in leaf sheaths and root tissues (Yang and Leu, 1978; Chung and Tzeng, 2004a). The fungus grows as a yeast-like sporidium on the cultural medium. The nutritional requirements in cultures have been intensively investigated (Chan and Thrower, 1980; Lin, 1981; Chung and Tzeng, 2004b) and the optimum cultural conditions have been established. A prior study revealed that thiamine was able to enhance *U. esculenta* growth and the fungus was auxotrophic for thiamine (Chung and Tzeng, 2004b). However, the role of thiamine in fungal growth has not been yet determined. In this study, we report the pivotal role of thiamine in the utilization of monosaccharide by the fungus *U. esculenta*.

MATERIALS AND METHODS

Fungal Cultures and Media

U. esculenta P. Henn., *U. maydis* (DC.) Corda and *U. scitaminea* Syd. and P. Syd. isolates were maintained on Potato Sucrose Agar (PSA) plates as described earlier by Chung and Tzeng (2004a). The modified Czapek's basal medium used for fungal growth and carbohydrate utilization, the source of inoculum, growth conditions and growth measurement were as previously described by Chung and Tzeng (2004b).

Detection of Carbohydrates in Liquid Cultures

Each of 5 mL culture solutions was collected by low speed centrifugation (8000 g for 10 min), then the supernatants were filtrated through a 0.2 μM pore size of Millipore membrane (Millipore, Bedford, MA, USA). The amount of sucrose, glucose and fructose in solution was analyzed using a model 1330 High Performance Liquid Chromatography (HPLC) (Bio-Rad, Richmond, CA, USA). The carbohydrates were separated on a Bio-Rad Aminex CarbonhydeHpx-87-c column (300×7.8 mm, 9 μm), using the glass double distilled water as a mobile phase at 70°C. The presence of carbohydrates was detected using a model 1305A Bio-Rad RI monitor. All carbohydrates were verified by analysis of the authentically commercial product (Merck Chemical Co., Germany) at the same conditions. Each treatment was with 3 replicates and all experiments were repeated at least twice.

Repression of Fructose Uptake

U. esculenta was grown in the modified Czapek's solution by replacing sucrose with the equivalent carbon content of fructose at 25°C for 7 days. Cultures were separated by centrifugation (6500 g for 15 min), then washed with distilled water. Fungal sporidia were suspended in 20 mL of sodium phosphate (50 mM, pH 5.7) containing 10 μM thiamine in a 125 mL flask. For competition studies, fructose plus the competing glucose with the concentration at 5 and 10 mM were filtrated and added to the solutions. Controls were solutions with no glucose. Assays were performed on a rotary shaker at 25°C and the sporidia were counted with a hemocytometer. In all cases, 5 mL solutions were removed periodically, rapidly separated by centrifugation and washed with distilled water. Fructose uptake during the depression time courses was assayed at the original concentration at 0.4, 1, 2 and

4 mM. The amounts of fructose retained after incubation were detected by HPLC as described. Results of fructose uptake are indicated as picomoles of fructose accumulated per minute per sporidium of *U. esculenta*.

RESULTS

U. esculenta grew poorly in Czapek's minimum solution. Fungal growth was greatly enhanced while thiamine was added (Fig. 1a). The change of sucrose, glucose and fructose in solutions was examined with one-day interval of incubation. In the presence of thiamine, the amount of sucrose originally amended was decreased sharply and was not detected after 4 days (Fig. 1b). The amounts of glucose and fructose were simultaneously increased and reached the maximum after 2 days while sucrose was almost depleted. At the first three days, growth of *U. esculenta* did not show significant increase in the presence of thiamine (lag phase). The fungus grew exponentially after 4 days (Fig. 1a). The amount of glucose was dramatically decreased in parallel with the fungal growth. The amount of fructose was decreased 2 days later than that of glucose. The glucose was completely depleted after 6 days and fructose was completely consumed after 8 days. Fungal growth then reached a stationary phase due probably to the lack of carbon sources. In the basal medium, sucrose was also converted into glucose and fructose without thiamine (Fig. 1c). In contrast, the amount of glucose or fructose remained unchanged throughout the whole incubation periods. Fungus did not gain significant growth in the absence of thiamine (Fig. 1a). The results strongly indicated that thiamine is not required for sucrose conversion, but absolutely needed for dissimilation of glucose and fructose and for fungal growth in *U. esculenta*.

Two closely related species, *U. maydis* and *U. scitaminea*, were also investigated on thiamine requirement for growth stimulation and its effect on the carbohydrate utilization. In contrast to *U. esculenta*, *U. maydis* exhibited a good growth in Czapek's basal solution with or without thiamine

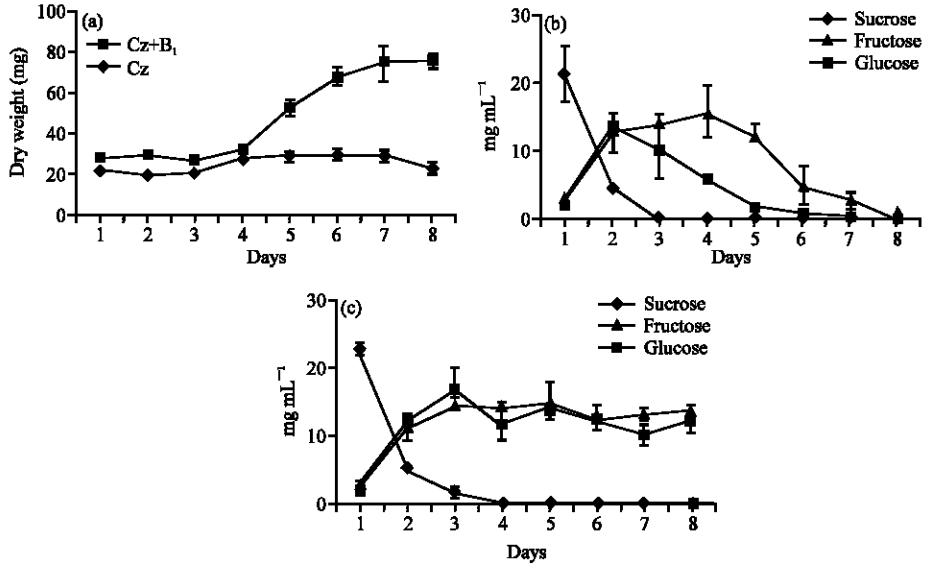

Fig. 1: Requirements of thiamine on fungal growth (a), glucose (b) and fructose (c) dissimilation in *Ustilago esculenta*. Fungal isolate was grown in the Czapek's solution with ($Cz+B_1$) or without (Cz) thiamine under continuous shaking at 25°C

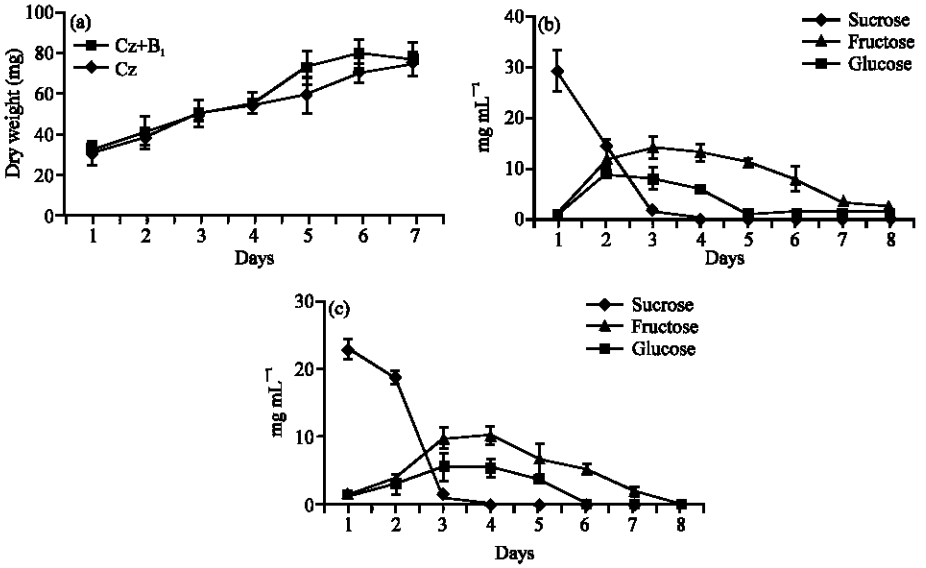

Fig. 2: Effect of thiamine on fungal growth (a), glucose (b) and fructose (c) utilization in *Ustilago maydis* grown in liquid solutions containing thiamine ($Cz+B_1$) and without adding thiamine (Cz)

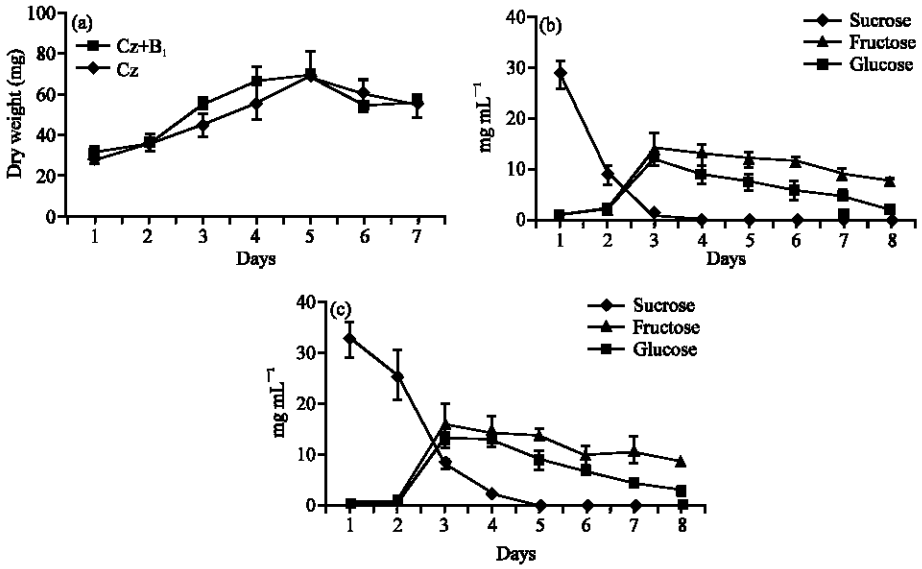

Fig. 3: Effect of thiamine on fungal growth (a) glucose (b) and fructose (c) utilization in *Ustilago scitaminea*. The fungus was grown in the Czapek's solutions with ($Cz+B_1$) or without (Cz) thiamine

(Fig. 2a). As with *U. esculenta*, sucrose was converted into glucose and fructose independent of thiamine by *U. maydis* (Fig. 2b, c). *U. maydis* also tended to utilize glucose prior to fructose for growth.

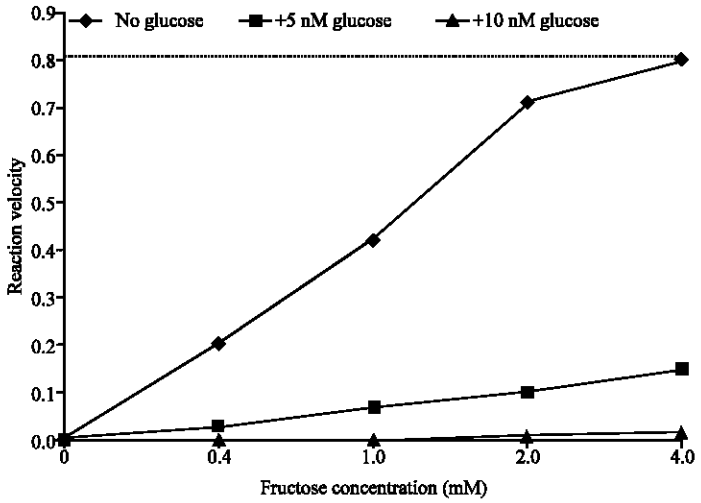

Fig. 4: Inhibition of the rate of fructose transport by glucose in *Ustilago esculenta* with or without the presence of glucose in 50 mM phosphate buffer (pH 5.7) containing 10 μM thiamine. The velocity of fructose uptake is indicated as picomole of fructose accumulated per minute per sporidium of *U. esculenta*

Regardless of the presence of thiamine, growth of *U. scitaminea* exhibited exponentially within 6 days and growth started to decrease in both media (Fig. 3a). As with other two species, the amount of sucrose in the media was converted into glucose and fructose by *U. scitaminea* within 3-4 days (Fig. 3b, c). Again, glucose was utilized prior to fructose as observed in other two species.

In this study analysis of the changes of glucose and fructose in cultures revealed that *Ustilago* species tended to utilize glucose prior to fructose in all cases. The inhibition of fructose uptake by glucose in *U. esculenta* was further investigated. In the absence of glucose, a specific fructose uptake system with K_m (ca. 3.9 mM) appeared in solution inoculated with *U. esculenta*. The velocity of fructose uptake by *U. esculenta* increased in agreement with the increased concentration of fructose, and gradually reached a plateau ca. at 3 mM (Fig. 4). Further addition of fructose did not significantly change the fructose uptake rate. When glucose was added, the rate of fructose uptake was dramatically reduced and the inhibition was correlated with the concentration of glucose. The rate of fructose uptake in the presence of 10 mM glucose was much lower than the uptake rate in the presence of 5 mM glucose (Fig. 4). Double reciprocal plot of fructose uptake with or without the presence of glucose gave lines that intersect at $-1/K_m$ (data not shown).

DISCUSSION

Among the pathways for carbohydrate oxidations, thiamine and its active form are required for several biochemical reactions and have been demonstrated to stimulate growth in many fungi (Bartnicki-Garcia and Nickerson, 1961; Osborne and Thrower, 1964; Ridings *et al*., 1969; Rawla and Chahal, 1975; Kulkarni and Nielsen, 1986). They are prerequisite as cofactors for many enzymes, including pyruvate decarboxylase, pyruvate dehydrogenase of pyruvate oxidation, α-ketoglutarate dehydrogenase of citric acid cycle and transketolase of pentose phosphate pathway (Mathews and van der Holde, 1990; Pohl *et al*., 2004).

In this study, we demonstrated that thiamine was absolutely required by *U. esculenta* for growth and thiamine apparently facilitated the utilization of glucose and fructose. In many fungi, glucose and

fructose are mainly metabolized through the glycolytic pathway (Cochrane, 1976; Pohl *et al*., 2004). However, the majority of glucose is utilized via., pentose phosphate pathway and some of the intermediates may enter Embden-meyerhoff pathway in *U. maydis* (McKinsey, 1959). Finding of that thiamine is required for glucose and fructose utilization in *U. esculenta* indicated that thiamine is an important factor for carbohydrate oxidation and energy production in this fungus. Thiamine also has some other biological effects such as on inducing gene expression (Ichikawa *et al*., 1997; Kubodera *et al*., 2003) and on the biosynthesis of some apoenzymes (Witt and Neufang, 1970; Pohl *et al*., 2004). Moreover, thiamine is able to increase the level of amino acids, lipids and organic acids in fungal cells (Tachibana and Siode, 1971; Rozenfeld and Disler, 1971; Nishikawa *et al*., 1977).

This present study also revealed that three *Ustilago* species were able to breakdown sucrose into glucose and fructose, indicative of the presence of strong invertase activity in these fungi. Stimulation of host plant invertase has been reported in maize infected by *U. maydis* (Billett *et al*., 1977). However, the direct activity of invertase in *Ustilago* species remains to be determined. Low amounts of glucose and fructose were detected in solutions after 8 day incubation, indicating that *U. scitaminea* has poor transport system for glucose and fructose, or the fungus requires another cofactor such as pyridoxine for complete oxidation of glucose and fructose. Nevertheless, the requirement of thiamine for utilization of hexoses and for growth is unique in *U. esculenta*, strongly indicating that *U. esculenta* is thiamine auxotrophic isolate.

To utilize carbohydrates, fungi have to transport sugars from medium into cells (Lagunas, 1993). Two general sugar transport systems have been described in *Neurospora crassa* (Schneider and Wiley, 1971; Rand and Tatum, 1980). System I is present constitutively and is able to transport various monosaccharides, including glucose, 3-O-methyl glucose, fructose and L-sorbose. Another kinetically distinct system II is de-repressed in starving conditions and is able to transport glucose, sorbose, galactose, mannose 2-deoxyglucose and talose. In this study, the velocity of fructose uptake exhibited Michaelis-Menton saturation kinetics in the absence of glucose in *U. esculenta* (Fig. 4), indicating that fructose uptake appeared to be carrier protein-mediated process. Moreover, the rate of fructose uptake was inhibited by the presence of glucose and the inhibition was highly dependent on the concentrations of glucose. The V_{max} for inhibited uptake was different from that for uninhibited uptake, suggestive of noncompetitive inhibition as described in *N. crassa* (Schneider and Wiley, 1971). The inhibition of sugar uptake by glucose is a general phenomenon in fungi and other organisms since glucose has the highest affinity for carrier proteins (De Bruijne *et al*., 1988; Bisson *et al*., 1993; Meijer *et al*., 1996). In the system of carrier-mediated (noncompetitive) inhibition of sugar uptake, two competed sugars strive for modifying the structure of the carrier protein to favor the formation of sugar-carrier complex. Alternatively, the inhibition may be due to inhibitor suppression of the genes for carrier protein biosynthesis or by changing the shape of the protein (Garraway and Evans, 1984).

In conclusion, in this study we have elucidated the role of thiamine in the utilization of glucose and fructose in *U. esculenta*. In the presence of thiamine, glucose and fructose were quickly consumed and the fungus gained a good growth. In contrast, thiamine was apparently not required for promoting growth and sugar utilization in other two related *Ustilago* species. All three *Ustilago* species tested were capable of converting sucrose into the two corresponding hexoses and utilizing glucose prior to fructose. Fructose uptake appeared to be carrier protein-mediated process in *U. esculenta*. Inhibition of fructose uptake by glucose was dependent on glucose concentration and the mode was probably noncompetitive.

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