THE EFFECT OF NUTRIENTS AND ENVIRONMENTAL FACTORS ON GROWTH OF UROCYSTIS COLCHICI (SCHLECHT) RABENH

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ABSTRACT

THE EFFECT OF NUTRIENTS AND ENVIRONMENTAL FACTORS ON GROWTH OF UROCYSTIS COLCHICI (SCHLECHT.) RABENH.

by Roberta Louise Dow

Malt extract and phytone were the best single nutrient sources for growth of Urocystis colchici in liquid culture. Growth increased linearly with all phytone concentrations tested. Sucrose, glucose, or mannose were superior to other simple sugars. A concentration of 30 g/liter was optimum for sucrose while malt extract was best at 10 g/liter.

Small quantities of malt extract, phytone, or peptone stimulated growth when used with simple sugars. Ten grams per liter was the optimum amount of phytone when used with 30 g sucrose/liter.

More growth was obtained with organic nitrogen sources than with inorganic nitrogen sources. Of nine amino acids and four inorganic nitrogen sources added at a concentration of 0.3 g N/liter to sucrose and mineral salts, L-asparagine and L-aspartic acid gave the best growth. Asparagine at 1 g N/liter gave the best growth of any nitrogen source.

Optimum initial pH was studied using two buffer systems (citrate and phosphate) spanning the pH range 3.0 to 8.0. The optimum initial pH range for growth was 5.8 to 6.4.

Growth was studied at five temperatures (12, 16, 20, 24, and 28°C) using three different media: sucrose-asparagine, malt extract-$NaNO_3$, and phytone. The optimum temperature with sucrose-asparagine was 24°C. Growth was equally good at 16, 20, and 24°C in malt extract-$NaNO_3$ medium.

Growth in standing culture was equal to that in shake culture. Three weeks was the optimum time for harvest.

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By

Roberta Louise Dow

A THESIS

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TO MY FAMILY

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INTRODUCTION

Smut diseases have played an important role in the history of plant pathology. Spores of Tilletia caries, incitant of stinking smut or bunt of wheat, were used by Tillet (48) to establish the contagious nature of disease for the first time. It was also with stinking smut that Prévost (36) discovered that fungal spores were the "seeds" of microscopic plants, which could germinate and give rise to new individuals. These early empirical studies on smut diseases served as models for later scientific work in the nature and causes of plant diseases.

Smut was one of the first diseases to be controlled by chemicals (26). In the late 1700's European farmers used various seed treatments collectively known as le Chaulage, involving chemicals such as lye, ammonia, or copper sulphate for the control of stinking smut. The first control methods for onion smut were developed in the early 19th century. Then, as now, control consisted of either seed protection or in-row soil fumigation. The earliest control consisted of treating onion seeds with sulphur and lime (45). The first effective control was developed by Selby (42) using a formaldehyde solution dripped in the row near the seed. The formaldehyde vapors

diffused through the soil, inhibited the fungus, and allowed the onion to develop beyond the short susceptible period. This method with modification is still used today. More recently, thiram has come into use as an effective seed protectant (27). Due to the longevity of the fungus spores in the soil, this disease cannot be economically eradicated and therefore remains important in many onion growing regions despite effective control measures (52).

The taxonomic position of the onion smut fungus is controversial. Early workers used the binomial Urocystis cepulae Frost because it attacked the common onion Allium cepa, distinguishing it from U. colchici found on autumn-crocus (Colchicum autumnale L.) (45, 52). Fischer and Shaw (14) have chosen to combine Urocystis cepulae, U. allii and U. magica with the earlier described species U. colchici, based on morphological similarity of their teliospores. The host range of U. colchici as described by Fischer and Shaw is limited to members of the lily family. They felt that smut fungi of similar morphology and symptomatology, and parasitizing members of the same host family, should all be considered one species, whereas a smut fungus similar in morphology but attacking only members of a different family should be regarded as a distinct species. The writer agrees with this concept and uses Urocystis colchici (Schlecht.) Rabenh. for the onion smut fungus in this work.

The onion smut fungus is a soil borne plant pathogen (1, 3, 45, 52, 55). It overwinters as teliospores, often incorrectly referred to as chlamydospores, in the soil. The thick walled teliospore contains a single diploid nucleus and is surrounded by small sterile cells (1, 3, 45, 52). Prior to germination of the teliospore, the nucleus is believed to undergo meiosis (3). The haploid nuclei migrate into the branching promycelium, one nucleus passing into each mycelial branch. Hyphae developing from these branches directly penetrate the cotyledon of the onion (52). Systemic infection is apparently associated with penetration of the mycelium to the meristematic region. The fungus ramifies through the onion tissue, ultimately forming teliospores in the cotyledon, bulb scales, or leaves. Environmental conditions affect the period of susceptibility and amount of infection (54). Infection is low in warm climates where mean soil temperatures often exceed 25°C. Under these conditions the onion seedling develops rapidly, shortening the period during which it is susceptible to infection (54), and the fungus appears to be inhibited directly by temperatures above 25°C (53).

Little definitive work has been done on the physiology of U. colchici. The objectives of this investigation were: 1) to investigate nutritional and environmental requirements for growth; 2) to develop a defined medium yielding good growth; and 3) to compare nutritional requirements for somatic growth and spore germination.

LITERATURE REVIEW

Early workers believed that smut fungi were obligate parasites. Brefeld (5) was the first to demonstrate that they could be grown and maintained on man-made media.

Natural Media

Culture media were first made from natural products that early investigators found readily available. These extracts or wastes of plant or animal materials served as carbon, nitrogen, mineral, and vitamin sources with varying efficacy. Brefeld (5, 6, 7) demonstrated that soil extracts, dung decoctions, natural fruits and vegetables, fruit and vegetable juices, meat extracts and malt extract could be used to culture fungi. Most of these media have since been used to culture the smut fungi.

Dung and soil decoction.--Sartoris (39) found horse-dung decoction to be an inadequate medium for smut fungi, supporting little or no growth of Ustilago hordei, U. heufleri, U. maydis, U. tritici, Tilletia tritici, and T. foetans. Maire (32) reported that the growth of Ustilago maydis on horse-dung decoction was made up exclusively of yeast-like cells. Anderson (1) attempted to grow Urocystis

colchici, the onion smut fungus, on dung decoction agar but obtained very poor growth. Soil decoction agar supported even less growth.

Fruits, vegetables, and their juices.--Urocystis colchici has been grown on sterile beans, carrots, potatoes, and fresh onions (3). Only beans and potatoes were found to be good substrates. Onion decoction and onion agar have been used by several workers to culture the onion smut fungus (1, 3, 13, 45, 53). Onion decoction supported poor growth according to Anderson (1), whereas the fungus grew well on onion agar. Potato agar was also used by Anderson with only fair results. Ling (31) reported that Urocystis occulta grew poorly on sugar beet juice, dahlia tuber juice, potato or rye plant juice alone or in combination with a synthetic medium. Carrot agar was used by Potter (35) for culturing Sphacelotheca reiliana (Sorosporium reilianum). Ranker (37) felt that liquid carrot decoction was such a good medium for culture of Ustilago maydis that he used it as his control in experiments to develop a synthetic medium yielding growth equal to that of a natural medium. Ustilago maydis was found by Maire (32) to grow better on carrots than on potatoes. Cider agar was used by Bauch (2) to germinate and culture Ustilago bromivora and U. grandis.

Meat extracts.--Nutrient media consisting of meat extracts have been very popular in liquid and solid culture media of the smut fungi. Ustilago avenae, U. bromivora,

U. grandis, U. maydis, and U. striiformis develop well on this type of substrate (2, 9, 19, 20, 28, 32). Anderson (1) found beef-broth agar to be a very unfavorable medium for Urocystis colchici.

Malt extract.--One of the most widely used natural substrates for fungi is malt extract. Potter (35) was one of the first to use malt extract in the culture of the smut fungi. He found that Sphacelotheca reiliana developed luxuriant mycelium on malt extract agar. Hanna in 1929 confirmed this result (17). Thirumalachar (46) used malt extract agar for culturing Urocystis agropyri. Ling (31) utilized this rich medium for culture of the slow-growing smut fungus, Urocystis occulta. Kniep (21) found 0.5% malt extract agar supported rich growth of Urocystis anemones. Other workers to report optimum growth of smut fungi on this medium are: Thren (47) working with Ustilago nuda, Leach et al (28) studying U. striiformis, Lange de la Camp (24), studying U. tritici, and Zscheile (57) working with Tilletia caries.

Supplemented natural media.--Blizzard (3) obtained much better growth of Urocystis colchici on onion decoction agar plus sugar, than on onion decoction agar alone. Likewise potato decoction enriched with sucrose increased the growth of this organism (1). Tachibana (44) found potato dextrose agar (PDA) to be a satisfactory medium for growth

of the onion smut fungus. Urocystis occulta and Urocystis agropyri also produced good mycelial growth on PDA (31, 46). Investigators studying Ustilago maydis, U. striiformis, U. nuda (U. tritici), Tilletia caries, T. controversa, and Sphacelotheca reiliana routinely used potato sucrose agar (PSA) or PDA for culture, maintenance, and spore formation (9, 17, 22, 24, 28, 37, 47, 50, 57).

Halbsguth (16) found that peptone, an amino acid-rich, carbohydrate-free substance, added to PSA increased the growth of Tilletia tritici. Sartoris (39) recommended malt extract enriched with peptone for culturing several smut fungi.

Synthetic Media

As work continued with culturing fungi and interest increased in fungal nutrition, more definitive media were required. Halbsguth (16) stressed the importance of defining the technique of media preparation. He demonstrated that decoctions of uncut potatoes, peeled potatoes, and of potato peelings varied in their ability to support growth of Tilletia tritici. Decoctions of uncut potatoes were unable to support any growth whereas the other two supported good growth. Ranker (37) objected to the wide usage of the various infusions, decoctions, and extracts for determining physiologic specialization. He reported that the inability to standardize media made from complex natural substances

prevented duplication of research. One of the first synthetic media for fungi was developed by Brefeld (7). It consisted of glucose, $(NH_4)_2SO_4$ and NH_4NO_3 in water solution, poured over cigar ashes.

Polysaccharides.--Polysaccharides must first be broken down into simple sugars before they can be utilized by an organism. This hydrolysis requires special enzymes which some organisms do not possess. Soluble starch was reported by Zscheile (57) to be utilized by Tilletia caries, while wheat and potato starches gave little growth. The results of studies by Chung and Trione (10) showed that only minimum growth of T. controversa occurred on starch. Oatmeal agar, which contains a high quantity of starch, was reported by Sartoris (39) to be an excellent medium for culture of several fungi including T. tritici, T. foetans, and Ustilago maydis. Glycerine, dextrin and soluble starch were indicated by Volkonsky (51) to support growth of U. maydis. He reported that the enzymes of this fungus strongly attack a large number of glycosides. Wolf (56), however, found that U. maydis would not grow on soluble starch. Glycerine, starch, and dextrin were reported by Blumer (4) to give better growth of U. violaceae than simple sugars.

Mono-and disaccharides.--The simple sugars are the most thoroughly investigated carbon sources for fungi. Sucrose, glucose, and maltose appear to be the most effective for smut fungi (35, 37, 39, 40, 50, 57). Xylose,

glucose, fructose, galactose, maltose, and sucrose were able to support good growth of Ustilago maydis (51). In another study, U. maydis grew best on glucose, fructose, mannose, sucrose, maltose, and trehalose of twenty carbon sources tested (56).

Halbsguth's data showed sucrose or glucose to be better sources of carbon than crystallized maltose for growth of Tilletia tritici haplonts (16). Galactose and lactose were unable to support any growth of this organism. Anderson (1) reports favorable growth of Urocystis colchici with cane sugar (mostly sucrose) in Czapek's agar.

After reviewing the literature on carbon nutrition one cannot help but concur with Cochrane (11) who stated: "the most important single facet of carbon nutrition is the high degree of specificity; differences between species and strains are the rule in fungi."

Organic nitrogen.--Few workers have studied nitrogen sources as thoroughly as carbon sources. The widely used natural compounds usually contained enough amino acids to fulfill the nitrogen requirements of the test fungus. In most cases where organic and inorganic nitrogen sources have been tested, organic sources have been superior to the inorganic forms (4, 10, 25). In all the literature reviewed, asparagine ranked among the best nitrogen sources for growth (4, 10, 16, 41, 56, 57). Halbsguth (16),

studying T. tritici, found alanine, allantoin, and asparagine superior to all other nitrogen sources tested.

Blumer (4) tested fourteen nitrogen sources on the growth of Ustilago violaceae and found fibrin to be superior to others tested. Peptone was also found to be an excellent nitrogen source for U. violaceae. Asparagine and urea gave good growth while nucleic acids were inadequate. Further work with this fungus demonstrated that DL-serine, DL-phenylalanine, L-arginine, DL-aspartic acid, L-asparagine, and L-oxyproline were the best of 36 amino acids tested (41). Amino acid isomers of the D form were poorly utilized.

Inorganic nitrogen.--Schopfer and Blumer (41) found that $(NH_4)_2SO_4$ and ammonium citrate were as good as asparagine for growth of Ustilago violaceae. However, this was only true when vitamins were added to the media. Ranker (37) found that U. maydis could grow well on $NaNO_3$ and KNO_3 but maximum growth was obtained on a medium containing NH_4NO_3. Lange de la Camp (25) found that T. tritici grew poorly on all inorganic nitrogen sources tested.

Other Factors

The effect of hydrogen ion concentration in media on mycelial production of the smut fungi has been investigated by some authors. Most of the smuts grew best in slightly acid media (39). Using media adjusted with KOH

or HCL, crude studies were conducted by Sartoris (39) to determine the optimum initial reaction for mycelial development of four species of Ustilago. The results of his narrow range study indicate that pH 6.8 to 7.1 were best. Ranker's (37) synthetic medium which supported excellent growth of U. maydis had a pH of 5.6. Wolf (56) reports that pH 6.0 was good for growth of U. maydis. Preliminary studies by Blumer (4) indicated that U. violaceae had a strong tolerance to pH. This organism was able to develop an average amount of mycelium in media of pH 3 to 8.3. He conducted most experiments at pH 5.6 which happened to be the pH after sterilization of his unbuffered media. Trione (50) used agar media adjusted to pH 6.0 for culturing Tilletia caries and T. controversa.

Temperature.--Different species of smut fungi appear to favor different temperatures with the majority growing best around 20°C. This temperature produced well-developed cultures of T. tritici and T. foetans (39). When cultures of these fungi were placed at 1 and 2°C for one week they would die; 25°C also inhibited the mycelium which would then disintegrate. An incubation temperature of 19 to 20°C was used for T. caries by Zscheile (57) who reported that growth decreased at 15 and 25°C.

While studying four species of Ustilago, Sartoris (39) found that the optimum temperature varied with species. U. heufleri grew best at approximately 20°C. The maximum

temperature that it could tolerate was around 36 to 37°C. Haskins (19) also noted that U. maydis grew most rapidly at 30°C. Ustilago violaceae was unable to grow at 28°C. Optimum growth for this species of smut fungi occurred between 18 and 22.5°C (41). Ling (31) tested the effect of temperatures ranging from 5 to 25°C on the growth of Urocystis occulta on PDA and malt extract agar. The optimum temperature was near 20°C on both media. Growth occurred at all temperatures with mycelium produced at 25°C equal to that produced at 10°C. Walker and Wellman (53) report 18°C to be optimum for mycelial growth of Urocystis colchici.

In summary, little is known about the nutritional requirements of Urocystis colchici. It grew poorly on sterile carrots, sterile onions, onion decoction, meat extract, dung decoction and soil decoction (1, 3, 13, 45). Fair growth was obtained with sterile beans and potatoes. Onion agar, onion sucrose agar, and potato dextrose agar have been most widely used for culturing this slow growing fungus (1, 3, 13, 23, 45, 53). It has rarely been cultured on defined media.

Eighteen degrees centigrade was reported optimum for growth on onion decoction agar (53).

MATERIALS AND METHODS

Urocystis colchici teliospores were aseptically collected from sori in the leaf lacunae of infected onions (Allium cepa 'Downings Yellow Globe') using the method described by Lacy (23). The spores were placed on malt extract agar plates and allowed to germinate in an incubator at 24°C. Mass transfers were made from these colonies to potato dextrose agar (PDA) or potato sucrose agar (PSA) plates, and were transferred every three weeks so that mycelia used as inoculum would be in an actively growing condition.

Unless otherwise indicated, the basal medium contained (grams per liter): KH_2PO_4, 1.0; K_2HPO_4, 0.46; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0.01. Carbon and nitrogen sources were added as described for each experiment in the Results. All chemicals used were of reagent grade. The medium was buffered at pH 6.4 with phosphate buffer unless stated otherwise. Buffer was used initially at 0.1 M concentration and later lowered to 0.05 M or 0.01 M. Forty ml of liquid media were placed in 125 ml Erlenmeyer flasks; the flasks were plugged with cotton and autoclaved for twenty minutes at 15 psi, 121°C.

Since the teliospores of U. colchici germinate poorly or not at all in liquid media (1) it was necessary to use mycelial fragments as inoculum. Mycelium free of agar was aseptically scraped from the surface of PDA plates with a stainless steel spatula. Mycelia were washed 3 times by centrifugation in sterile glass-distilled water then resuspended in sterile, glass-distilled water. A sample of the hyphae was pipetted onto PDA or PSA plates to assay for possible fungal or bacterial contamination before using the inoculum. Meanwhile, the rest of the inoculum was maintained in glass-distilled water at 2°C (44). Uncontaminated inoculum was fragmented in sterile distilled water with a Sorvall Omnimixer. After the first 30 second period of fragmentation, the size of the hyphal fragments was microscopically examined. If the length of the fragments averaged more than 45 μ, the inoculum was fragmented 30 seconds longer. Concentration of the hyphal fragments was determined with a hemacytometer and adjusted by dilution to 4×10^6 fragments per ml. One ml of the inoculum suspension was aseptically transferred to each flask of the medium under a Microvoid hood, surface-disinfested with Chlorox. The lip of each flask was flamed before entry of the pipet and after delivery of the inoculum. All flasks were incubated for three weeks at 22°C unless otherwise indicated. In experiments investigating

nitrogen sources, all glassware was acid-washed with concentrated HCl and rinsed in tap and then distilled water.

Growth of the fungus was measured by dry weight of the mycelium. The fungus was collected by filtration on oven-dried Reeve-Angel, glass-fiber filter papers. The combined filtrates of all flasks of a treatment were collected and the pH was measured to determine any change from the original pH.

All experiments were repeated one to three times, and the data were analyzed statistically.

RESULTS

Effect of carbon sources on growth.--Experiments were designed to determine which carbon sources were capable of supporting good growth. Three complex carbon sources, one polysaccharide, two disaccharides, and four monosaccharides were added at the rate of 10 g/liter to the basal medium containing $NaNO_3$ at 2 g/liter as the nitrogen source. The control consisted of the basal medium plus $NaNO_3$. Only D-isomers of the sugars were used. The results (Fig. 1) show malt extract (rich in carbohydrates, amino acids, and vitamins) and phytone (papaic digest of soybean meal containing amino acids, vitamins, and carbohydrates) superior to all others tested. Sucrose, D-mannose, and D-glucose were not significantly different from each other but were significantly better than the five other carbon sources. Dextrin, peptone (carbohydrate-free protein hydrolysate containing vitamins and amino acids), maltose, and D-fructose yielded little or no growth. Growth with maltose and D-fructose was not significantly different from that of the control.

Effect of oxygen on growth.--To determine if aeration enhanced growth of this fungus, inoculated flasks

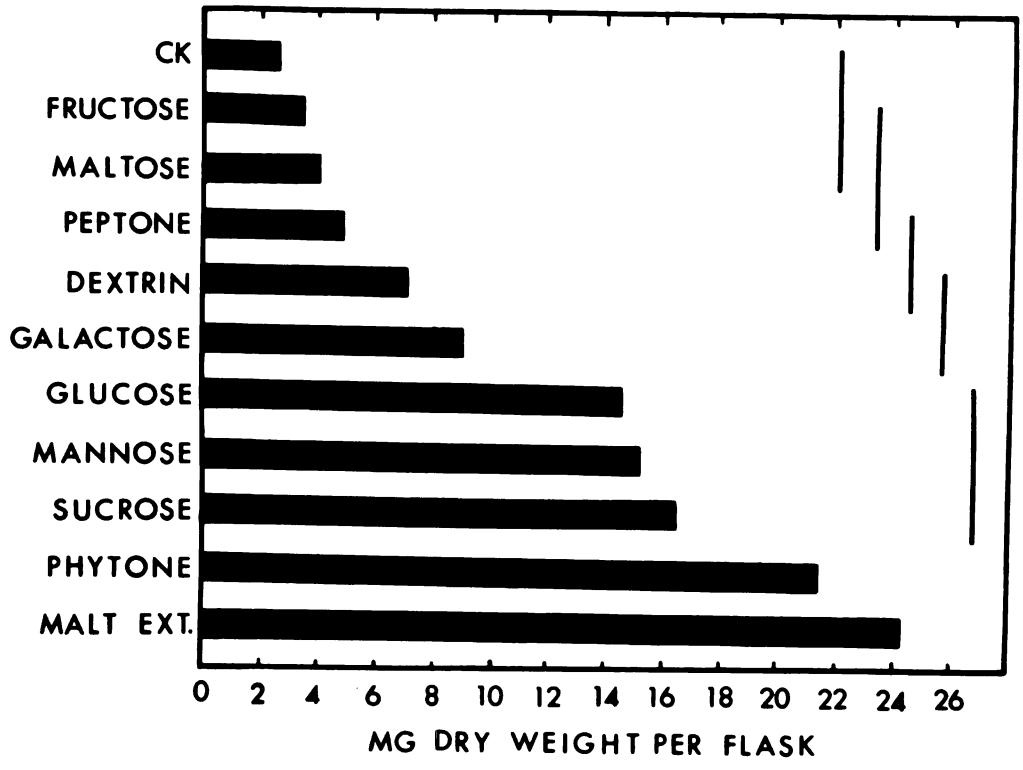

Figure 1.--Effect of carbon sources on growth of Urocystis colchici. Carbon sources were added at 10 g/liter to a basal mineral salts-$NaNO_3$ medium. Bars joined by a common line did not differ significantly at the 5% level.

containing the basal medium with malt extract, 10 g/liter, and $NaNO_3$, 2 g/liter, were placed on a reciprocating shaker set at 90 strokes/minute. Other flasks were placed on a shelf in the same room. After three weeks, shaken flasks yielded an average of 25.3 mg and standing flasks 25.1 mg mycelial dry weight. Growth in the shaken flasks was in the form of submerged pellicles while that in the standing flasks was made up of both surface mycelium and submerged, loosely-organized clumps. Apparently aeration of the medium is not required for maximum growth of this organism.

Effect of time on amount of growth.--In nutritional studies it is necessary to measure growth at the time when maximum growth has occurred and prior to autolysis. Malt extract and sucrose were added at the rate of 10 g/liter to the basal medium containing $NaNO_3$ at 2 g/liter. Dry weight measurements were made at weekly intervals. Within one week good growth was obtained with the malt extract medium (Fig. 2). It supported maximum growth after three weeks. Growth began more slowly in the sucrose medium and was at the highest level after four weeks.

Later studies using phytone and sucrose-phytone also showed that 3 weeks was optimum for growth. On the basis of these results it was felt that a three week period was most suitable for obtaining optimum growth.

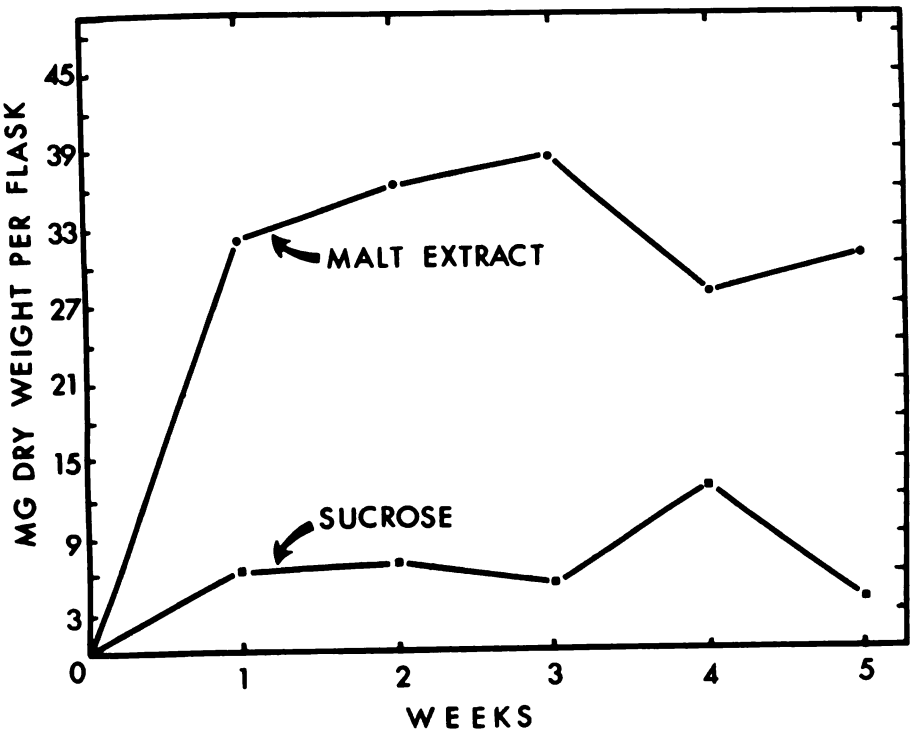

Figure 2.--Effect of time on growth of Urocystis colchici. Ten grams malt extract or sucrose were added per liter of basal mineral salts medium containing 2 g $NaNO_3$.

Effect of concentration of malt extract on growth.--Malt extract was added at concentrations of 0.1, 0.5, 1.0, 5.0, 10.0, 15.0, and 20.0 g/liter to the basal mineral salts solution containing $NaNO_3$ (2 g/liter). After autoclaving, the medium was filtered through Whatman number 1 filter paper to remove any precipitate. The medium was then dispensed and reautoclaved for 15 minutes.

Concentrations of 0.1, 0.5, and 1.0 g malt extract/liter showed no significant difference in growth (Table 1). Five grams/liter yielded nearly five times more growth than 1.0 g/liter and 10.0 g/liter nearly twice as much as 5 g/liter. Ten and 15 g/liter were best but not significantly different from each other. A further increase in the concentration decreased the amount of growth.

Effect of adding a complex nutrient source to a simple sugar.--To determine whether unknown nutritional factors in malt extract might have an effect on the utilization of simple sugars, 0.1 g or 1.0 g malt extract were added to one liter basal mineral salts medium containing 2.0 g $NaNO_3$ and simple sugars, each at 10 g/liter. Dry weight yields were greater on media containing sucrose, glucose, or mannose plus 1.0 g malt extract (Table 2) than with the same sugars alone. Therefore it appeared that nutritional factors in relatively small amounts of malt extract could have considerable effect on growth in the

presence of some simple sugars. However, 0.1 g malt extract per liter added to 9 carbohydrate sources showed no effect on the utilization of simple sugars by U. colchici.

Table 1.--Effect of concentration of malt extract on growth of Urocystis colchici in a basal mineral salts--$NaNO_3$ medium.

Malt extract g/liter	Dry weight[a] mg
0.1	1.5 a
0.5	3.5 a
1.0	2.7 a
5.0	13.9 b
10.0	26.1 cd
15.0	27.3 d
20.0	22.7 c

[a]Based on Duncan's multiple range test for significance at the 5% level. Means which have letters in common are not significantly different.

In other experiments, phytone and peptone (0.1 or 1.0 g/liter) were added to 7 carbohydrate sources, each used at a concentration of 10.0 g/liter (Table 3). The basal medium contained 2.0 g $NaNO_3$/liter. Controls consisting of the basal medium without a carbon source and the basal medium plus the two concentrations of phytone and peptone without additional carbon source were included.

The data show that in all cases 1.0 g phytone/liter added to a simple sugar significantly increased growth. Peptone at 1.0 g/liter gave significantly better growth when added to simple sugars except in the case of galactose. Growth with malt extract was not improved by addition of phytone or peptone.

Table 2.--Effect of simple sugars with malt extract on growth of Urocystis colchici.

Carbohydrate, 10 g/liter	Dry weight, mg	
	Alone	Plus malt extract at 1.0 g/liter
Malt extract	24.0	22.5
Maltose	9.0	9.0
Sucrose	8.3	46.5
Glucose	10.0	42.5
Mannose	9.0	26.6

Effect of sucrose concentration on growth.--Sucrose at 0.1, 5.0, 10.0, 20.0, 30.0, or 50.0 g/liter was added to the basal medium containing 2 g $NaNO_3$/liter. Fig. 3 shows that dry weight increased as the concentration increased up to 30.0 g/liter, but growth with 50.0 g/liter was not significantly better.

Table 3.--Growth of Urocystis colchici on carbohydrate sources alone and with peptone or phytone.

	Carbohydrate source (10 g/liter)							
	Ck (no carbon)	Malt extract	Maltose	Sucrose	Glucose	Galactose	Mannose	Fructose
No supplement	2.6[a]	20.2	7.3	15.6	11.9	9.0	14.8	7.8
Peptone, 0.1 g/liter	2.7	26.8	7.0	23.4	30.0	17.8	23.8	13.8
Peptone, 1.0 g/liter	5.9	19.9	11.8	26.9	28.1	10.2	26.7	31.2
Phytone, 0.1 g/liter	2.4	17.7	8.4	29.1	30.2	19.8	25.1	8.9
Phytone, 1.0 g/liter	5.4	22.0	14.2	73.4	61.1	14.6	26.6	24.7

[a]mean mg dry weight of six replicates.

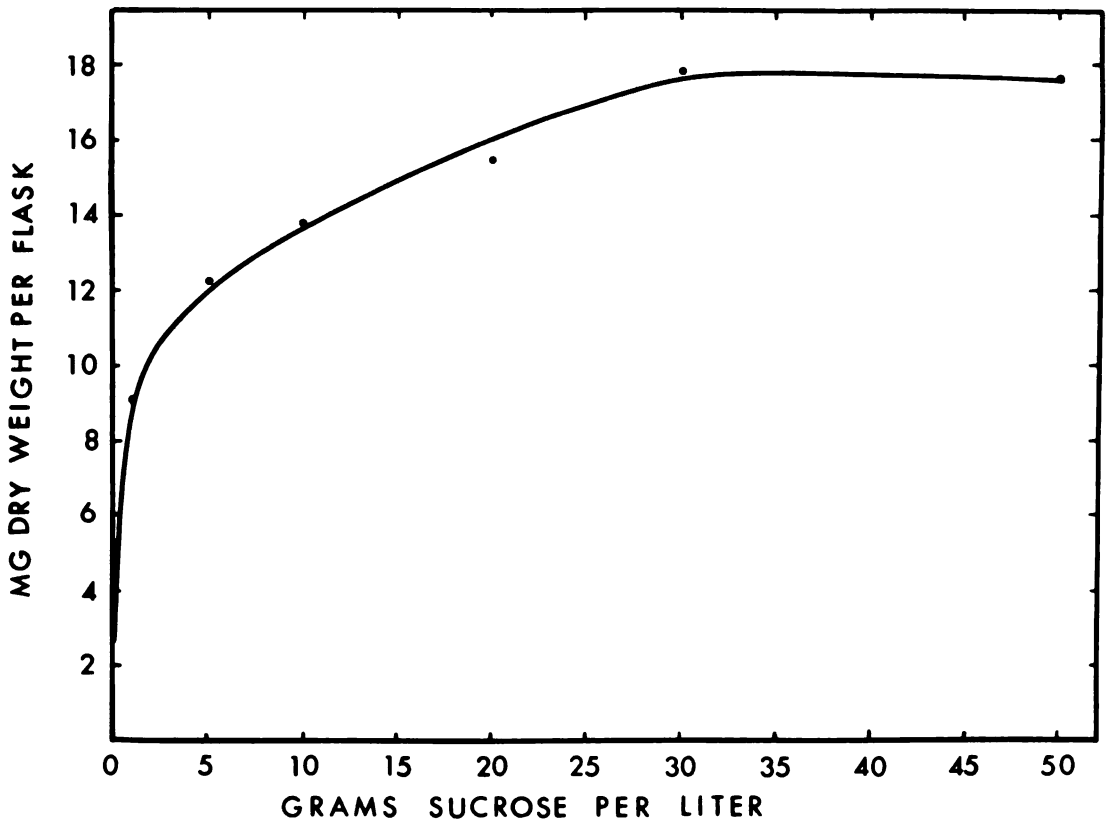

Figure 3.--Effect of concentration of sucrose on Urocystis colchici growing on a basal mineral salts-$NaNO_3$ medium.

Effect of phytone concentration on growth.--Phytone at six concentrations (0.1, 5.0, 10.0, 20.0, or 30.0 g/liter) was added to the basal medium. The data of Fig. 4 show a direct correlation between increase in growth and increase in phytone concentration. The best growth was obtained with 30.0 g/liter.

Effect of concentration of phytone on growth in media containing the optimum sucrose concentration.--Phytone at 0.1, 5.0, 10.0, 20.0, or 30.0 g/liter was added to the basal medium containing 30.0 g sucrose/liter. Controls containing 30.0 g sucrose/liter without phytone, and the basal medium without a carbon or nitrogen source were included. Fig. 5 shows that 5.0, 10.0, and 20.0 g phytone/liter greatly increased growth. The control containing sucrose without phytone was not significantly different from the control without carbon and nitrogen. Sucrose plus 0.1 g phytone supported poor growth but it was significantly better than the control.

Effect of various nitrogen sources on growth.--Nine amino acids were added at a concentration calculated to give 0.3 g N/liter. This amount of nitrogen was selected since it corresponds with that contained in 2.0 g $NaNO_3$/liter which was used in previous experiments. Sucrose at 10.0 g/liter was added as the carbohydrate source (at this time it was not known that the optimum concentration of sucrose was 30.0 g/liter).

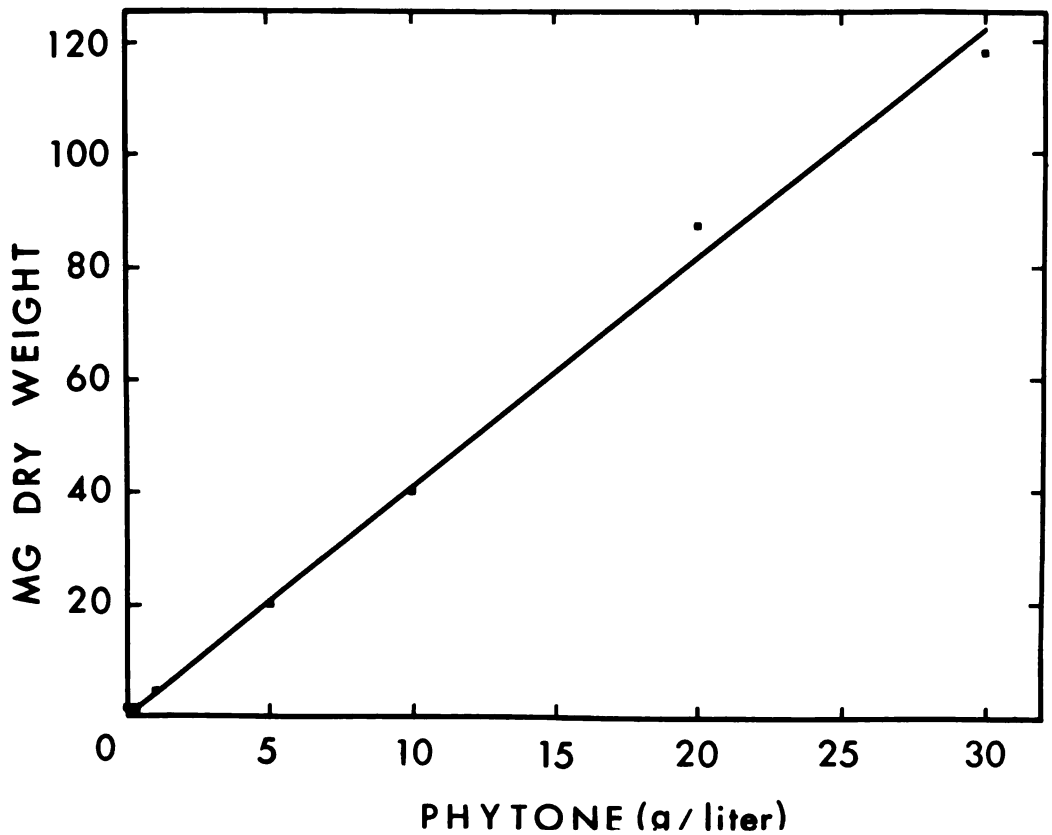

Figure 4.--Effect of concentration of phytone on Urocystis colchici growing in a basal mineral salts-$NaNO_3$ medium.

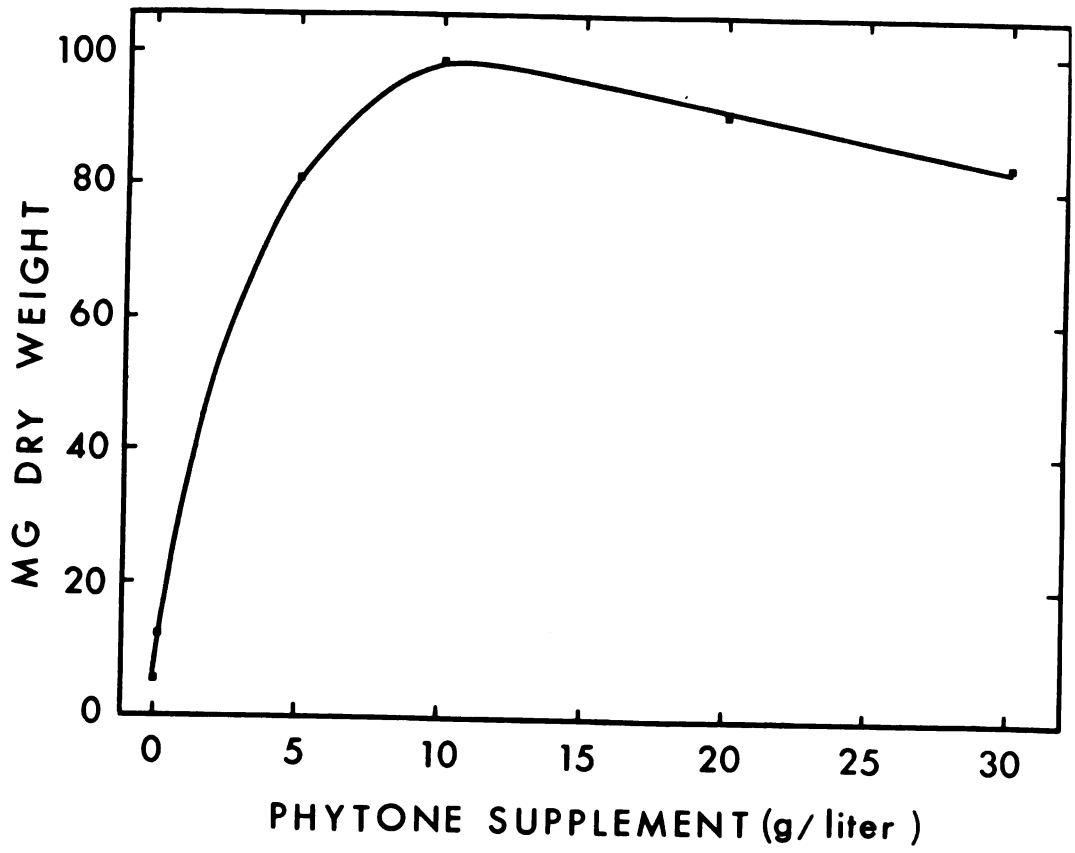

Figure 5.--Effect of concentration of phytone on Urocystis colchici growing in a sucrose (30 g/liter)-$NaNO_3$ (2 g/liter) medium.

The best growth was obtained with L-aspartic acid and L-asparagine (Fig. 6). L-glutamine and L-glutamic acid also gave good growth. L-histidine gave the least amount of growth but this was still significantly greater than the sucrose alone.

Four inorganic nitrogen sources were added at a concentration of 0.3 g N/liter to the basal medium containing sucrose at 10.0 g/liter. All the inorganic nitrogen sources were inferior to the best organic nitrogen sources (Figs. 6 and 7). Potassium nitrate and NH_4NO_3 were significantly better than the others. There was no difference between $NaNO_3$ and $(NH_4)_2SO_4$ but they were better than the control without nitrogen (Fig. 7). The pH of the NH_4NO_3 medium dropped to 5.7 and that of $(NH_4)_2SO_4$ dropped to 5.9 while the others remained at 6.4.

Effect of temperature on growth.--Three media were used in separate experiments to test the effect of temperature on growth. They were: 1) phytone, 10 g/liter; 2) malt extract, 10 g/liter, plus $NaNO_3$, 2 g/liter; and 3) sucrose, 10 g/liter, plus L-asparagine, 0.3 g N/liter. Flasks were placed in unlighted incubators set at 12, 16, 20, 24, and 28°C. Fig. 8 shows that 24°C was optimum with all media except phytone which had maximum growth at 20°C. The fungus grew very poorly in all media at 28°C. No significant difference was found in growth on malt extract media at 16, 20, or 24°C.

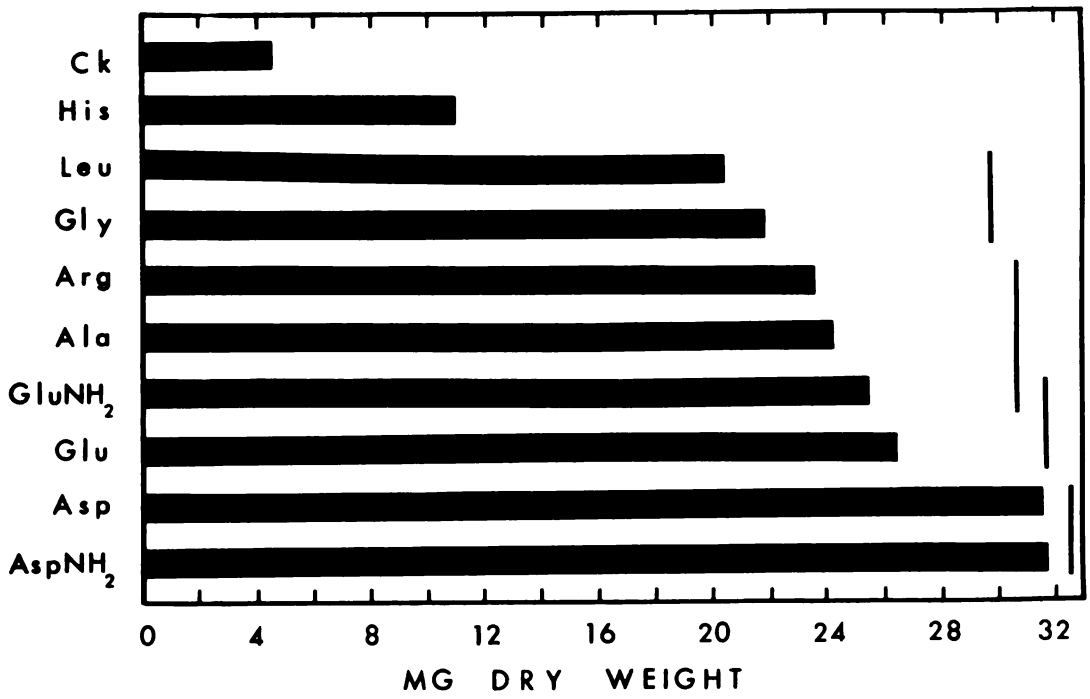

Figure 6.--Effect of amino acids on growth of Urocystis colchici. Amino acids were added at a concentration of .3 g N/liter to the basal mineral salts medium containing 10.0 g sucrose/liter. Ck = no nitrogen source; His = L-histidine; Leu = L-liucine; Gly = glycine; Arg = L-arginine; Ala = L-alanine; Glu NH_2 = L-glutamine; Glu = L-glutamic acid; Asp = L-aspartic acid; and $AspNH_2$ = L-asparagine. Bars joined by a common line did not differ significantly at the 5% level.

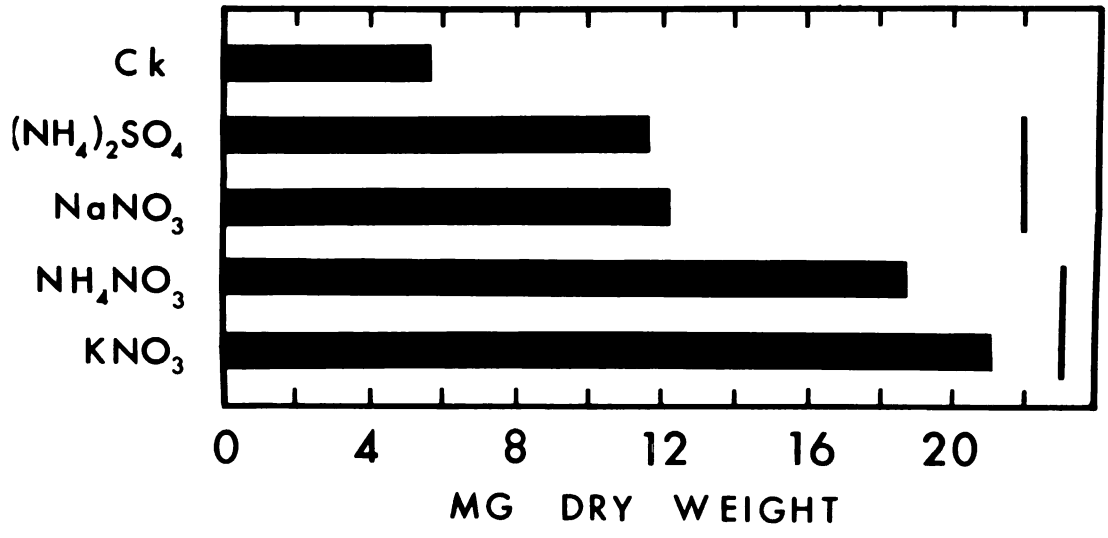

Figure 7.--Effect of inorganic nitrogen sources on growth of Urocystis colchici. Nitrogen sources were added at 0.3 g N/liter to the basal mineral salts medium containing 10 g sucrose/liter. Bars joined by a common line did not differ significantly at the 5% level.

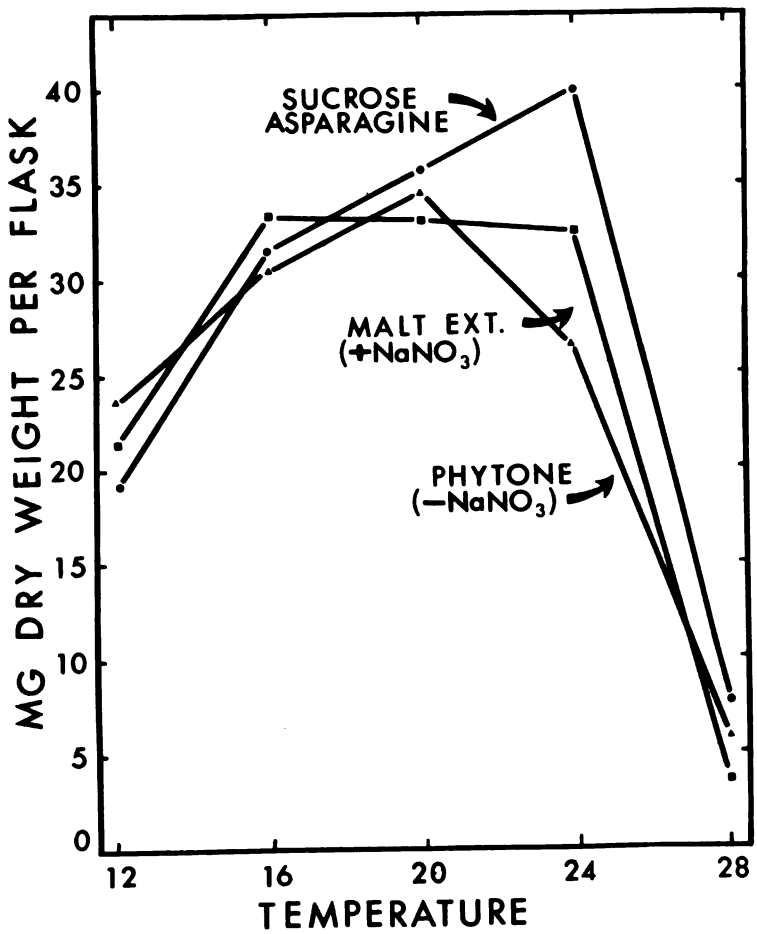

Figure 8.--Effect of temperature on growth of Urocystis colchici in three different media. Malt extract, sucrose, and phytone were used at 10 g/liter. Asparagine and $NaNO_3$ were used at 0.3 g N/liter.

Effect of initial pH on growth.--Two buffer systems were used to span the range of pH 3 to 8. The initial pH was measured after autoclaving to insure that it was unaltered by heat. Final pH measurements were made after three weeks growth.

Citrate buffers of pH 3 to 6.4 were made using combinations of citric acid and sodium citrate to give a final concentration in the medium of 0.01 M (12). Sucrose, 10 g/liter, and L-asparagine, 0.3 g N/liter, were added to the buffered basal medium. Growth at initial pH values of 6.0 to 6.4 were best (Fig. 9). Growth at pH 5.4 was less than at either 6.0 or 6.4 but was significantly more than at any of the lower pH values. There was no significant difference between the effect of initial pH 3.0 through 5.0. The final pH of all treatments varied less than 0.2 pH units from the original with the exception of pH 3.0 and 3.4 which changed to 3.4 and 3.9 respectively.

Monobasic potassium phosphate and dibasic potassium phosphate were used in different combinations to obtain pH values of 5.8 to 8.0 (12). Increments of 0.2 pH units were used in one set of experiments while 0.4 and 0.6 unit increments were used in another. The basal medium contained the buffer at 0.01 M concentration. Ten grams sucrose/liter and 2 g $NaNO_3$/liter were added to the buffered basal medium in one experiment while 30 g sucrose/liter and 0.3 g N/liter as asparagine were added to the buffered basal medium in

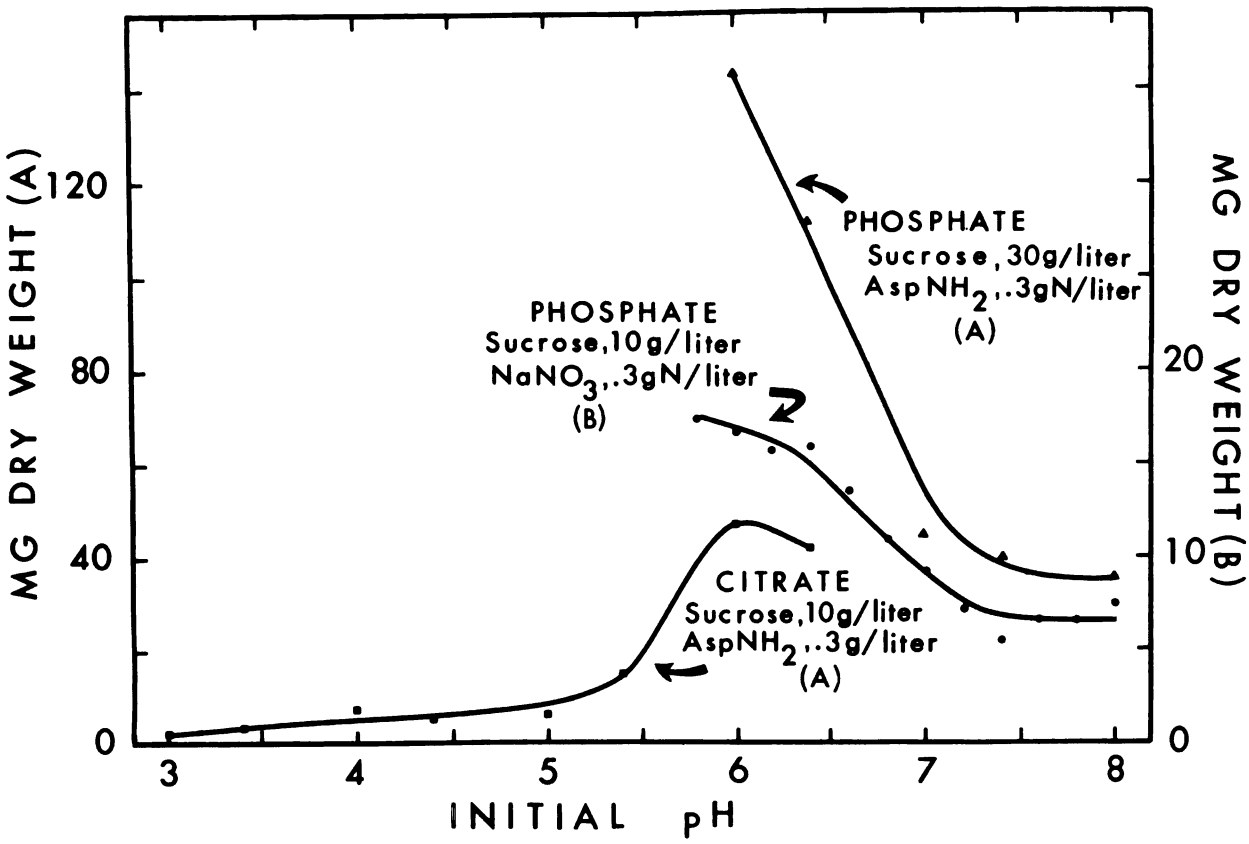

Figure 9.--Effect of initial pH on growth of Urocystis colchici. Carbon, nitrogen, and citrate or phosphate buffer were added to the basal mineral salts medium as indicated. The curves designated (A) are graphed according to the scale at the left while the curve designated (B) follows the scale at the right.

another experiment. Growth at an initial pH of 6.0 was found to be best in experiments using sucrose and asparagine (Fig. 9). A pH of 6.4 gave significantly better growth than higher pH values. Growth did not differ significantly at pH 5.8 to 6.4 in media containing sucrose and $NaNO_3$. Final pH values were within 0.2 units of the original pH value with the exception of 7.4, 7.6, 7.8, and 8.0 which were lowered by 0.4, 0.4, 0.6, and 0.6 of a pH unit, respectively.

Effect of concentration of two amino acids on growth.--L-asparagine and glycine were added at concentrations of 0.1, 0.3, 0.6, 1.0, or 3.0 g N/liter to the basal medium containing 30 g sucrose. Although the media were buffered at pH 6.4 it was necessary to adjust the pH with NaOH. At a concentration of 3.0 g N/liter as asparagine, a large amount of crystaline precipitation occurred after autoclaving. Four replicates with the least amount of precipitation were selected for inoculation. Crystals that were collected on the filter paper when harvesting these replicates were removed with forceps. The filtered mycelium was thoroughly rinsed with distilled water to dissolve any small crystals that might not have been observed.

No significant difference was found between the control and media containing 0.1, 0.3, 0.6, or 1.0 g N/liter as glycine (Fig. 10). The best growth using glycine,

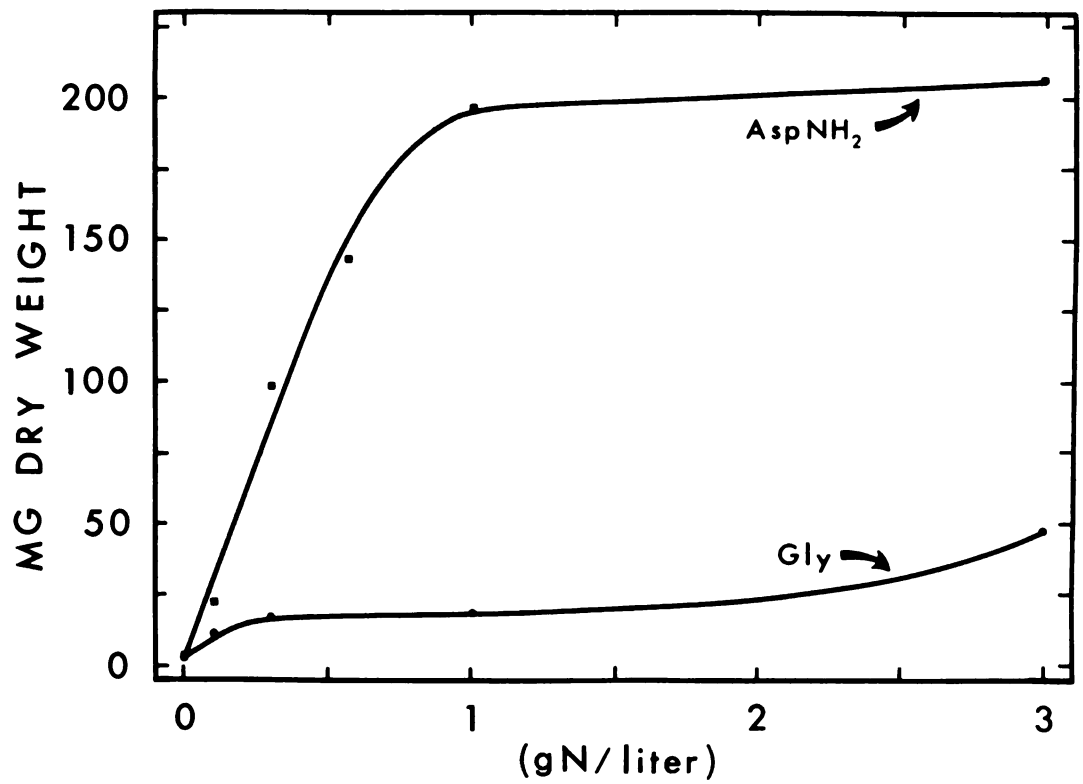

Figure 10.--Effect of concentration of two amino acids on growth of Urocystis colchici. Amino acids were added at concentrations of 0.1, 0.3, 0.6, 1.0, or 3.0 g N/liter to the basal mineral salts medium. $AspNH_2$ = L-asparagine and Gly = glycine.

although relatively low, was obtained using 3.0 g N/liter. The pH of all concentrations except 3.0 g N/liter dropped from 6.4 to 5.6.

L-asparagine, as shown earlier, was far superior to glycine at all concentrations (Fig. 10). Each increasing level of nitrogen gave a significantly greater amount of growth until an optimum concentration of 1.0 g N/liter was reached. A concentration of 3.0 g N/liter was not significantly better than 1.0 g N/liter.

DISCUSSION

Malt extract and phytone yielded more growth than any other single source of nutrients tested. One cannot, however, attribute the growth obtained with these two media to their superiority as carbon sources. Malt extract is not only an excellent source of carbohydrates, primarily maltose and glucose, but also contains 16 amino acids, riboflavin, nicotinic acid, pantothenic acid, ascorbic acid, and pyridoxal (18). Similarly, phytone consists of carbohydrates, 18 amino acids, and 9 vitamins (15).

D-glucose, a constituent of both malt extract and phytone, was one of the best of the simple sugars tested. It is utilized by virtually all fungi (11). Growth with sucrose and D-mannose were as good as with D-glucose. These results correspond with those of Wolf (56) who found Ustilago maydis grew best with these two simple sugars. Of 57 fungi studied by Lilly and Barnett (30), only four were able to utilize sucrose. Zscheile (57) also found that galactose and fructose were poor substitutes for sucrose, and that neither glucose nor fructose, nor the two combined in concentrations equal to that of sucrose, produced growth equal to that obtained with sucrose.

Possibly only the glucose moiety of sucrose was utilized by Urocystis colchici in the experiments reported here. This was suggested by the lack of growth with D-fructose, the other member of the disaccharide. D-fructose was no better than the control without a carbon source.

Ustilago heufleri was also reported by Sartoris (39) to be unable to utilize fructose, and fructose inhibited Tilletia tritici. Concentrations of 0.02 M to 0.50 M were used with poor growth at all concentrations. With the exception of these two organisms, most workers have reported good growth of smut fungi with fructose. According to Cochrane (11), D-fructose is generally equivalent to D-glucose. Fructose is known to be more rapidly degraded by autoclaving in the presence of minerals than are the other sugars (33). Since the carbon sources were autoclaved with the basal medium, the poor growth of Urocystis colchici on fructose may reflect its inability to utilize the breakdown product, 5-hydroxymethylfurfural. In experiments conducted by Chung and Trione (10) the sugars were autoclaved separately from the basal medium. They obtained excellent growth of Tilletia controversa with fructose. Ustilago maydis was reported by Wolf (56) to grow well on fructose but no mention was made of the sterilization method.

Maltose, like fructose, supported no more growth of U. colchici than the control. This contrasts with the many reports of maltose being one of the best carbon sources for

the smut fungi (37, 39, 40, 56). However, two other smut organisms, Tilletia controversa (10) and T. tritici (25) have been shown to grow poorly with maltose. The failure of maltose to be utilized by Urocystis colchici is especially surprising since malt extract, which contains large amounts of maltose (18), yields such excellent growth. This phenomenon may be explained in several ways. Other sugars in malt extract such as sucrose and glucose may support enough growth to induce production of maltase, allowing utilization of maltose. Formation of adaptive enzymes have been reported in Ustilago striiformis (8). Alternatively, the vitamins present in malt extract may make utilization of maltose possible. Schopfer and Blumer (41) found that thiamine moieties allowed Ustilago violaceae to grow well on poor or seemingly unutilizable nitrogen sources. A third possibility is that other carbohydrates of malt extract are utilized, and maltose simply cannot be absorbed.

Disaccharides can be utilized by fungal cells in one of two ways: by exoenzymes cleaving the compound into monosaccharides which are then transported across the cell membrane by monosaccharide carrier systems, or by movement of disaccharides by specific membrane transport systems directly into the cell (38). The inability to utilize maltose may be due either to the lack of exoenzymes (maltases) or the lack of a maltose membrane transport system.

Indirect evidence from this study suggest that sucrose is acted upon by an exoenzyme and only glucose is transported into the cell. Most fungi, however, move the entire molecule of sucrose across the membrane (38).

Peptone, a carbohydrate-free protein hydrolysate, supported poor growth of Urocystis colchici. Peptone consists of peptides, amino acids and vitamins (15). Growth with this as a carbon source indicates that U. colchici is capable of utilizing amino acids, although poorly, as an energy source. Amino acids have generally been found to be poor carbon sources for fungi (11, 29).

Comparing the effect of carbon sources on growth in this investigation with the effect on spore germination as reported by Lacy (23), it generally appears that similar metabolic mechanisms are involved. Malt extract and phytone provided the greatest amount of both growth and spore germination. Neither growth nor germination occurred with maltose. Sucrose gave the greatest amount of both growth and germination among the simple sugars tested. D-fructose supported less germination than sucrose but more than glucose, in contrast with the results of growth studies. Peptone was able to support a low level of germination as well as a small amount of growth.

Oxygen did not play an important role in the growth of Urocystis colchici under the conditions of this experiment. Aeration of the liquid medium by shaking did not

increase growth. Generally aeration increases the amount of growth in liquid culture (11). A few fungi, however, appear to have low oxygen requirements. Growth of Diplocarbon rosae, causal organism of black spot of roses, was not increased by shaking (43).

Growth curves for liquid-cultured fungi typically show three phases: 1) a phase of no apparent growth; 2) a phase of rapid, approximately linear growth; and 3) a phase of no growth or autolysis and decline in dry weight (11). To insure harvest as close as possible to the end of phase two but prior to phase three it was necessary to conduct time course growth experiments. The lag phase in these experiments was very short since fragmented mycelium was used as inoculum rather than spores. Optimum growth was obtained with three media which contained organic nitrogen and vitamins. The growth phase was lengthened when these nutrient factors were not present (Fig. 2). This was suggested also by growth experiments not reported here.

Malt extract was found to yield maximum growth at 10 g/liter. These results are similar to those of Lacy (23) who showed that 5 or 10 g malt extract was superior to 20 g/liter for spore germination. In the experiments reported here, malt extract medium appears to be saturated with nutrients at 10 g/liter and further increases in initial concentration do not increase growth. At very high concentrations one would expect the occurrence of inhibition due to toxic materials.

Growth factors and/or organic nitrogen sources in peptone were found to stimulate growth of Urocystis colchici in the presence of simple sugars. Improved utilization of nutrient sources by addition of vitamins has been noted by Schopfer and Blumer (41) with Ustilago violaceae. Peptone or phytone (0.1 g/liter) greatly stimulated growth in the presence of sucrose, mannose, and glucose, but results with fructose, maltose, and galactose (three unutilized or poorly utilized carbon sources) varied. Maltose showed no stimulation with this concentration, fructose showed some stimulation, and galactose showed more stimulation than the other two sugars.

Interestingly, no stimulation of Urocystis colchici was achieved when phytone or peptone were added to malt extract, in contrast to the results of spore germination studies (23). This suggests that different requirements for nutrients (qualitative or quantitative) occur during spore germination and somatic growth.

Thirty grams sucrose per liter (approximately .088M) was found to yield maximum growth of any concentration tested. The 10 g sucrose/liter used in previous carbon source experiments, on the basis of results with malt extract, was probably limiting. It appears that optimum carbohydrate concentration varies greatly between species of fungi (10, 16, 39, 41, 57).

Studies on the effect of concentration of phytone demonstrated a direct correlation between increases in phytone concentration and increases in growth up to the highest concentration used. Carbohydrate was possibly the limiting factor in these experiments since phytone is notably high in amino acids and other growth factors. In media containing an optimum sucrose concentration an optimum phytone level of 10 g/liter was reached.

Nitrogen studies showed amino acids were superior to inorganic nitrogen sources. This had been found to be true with many fungi (10, 11, 16, 41). L-asparagine was found to be one of the best nitrogen sources for growth of Urocystis colchici. This corresponded with the results of Halbsguth (16) for Tilletia tritici, Zscheile (57) for T. caries, Chung and Trione (10) for T. controversa, Wolf (56) for Ustilago maydis. Schopfer and Blumer (41) for U. violaceae, and Ling (31) for Urocystis occulta. L-aspartic acid, which was as good as L-asparagine in the results reported here, has not been tested as often as asparagine in nutritional studies of smut fungi. Ustilago maydis (56) and U. violaceae (41) were found to grow as well with aspartic acid as with asparagine. L-glutamic acid and L-glutamine were shown to be good nitrogen sources for Urocystis colchici. Ustilago violaceae also grew well with glutamic acid (41).

Potassium nitrate and NH_4NO_3 were the best of the four inorganic nitrogen sources tested. Ammonia of NH_4NO_3 is believed to be utilized first (34). This is presumed on the basis of the drop in pH generally indicating ammonia utilization. The pH of the NH_4NO_3 media dropped to 5.7 and that of $(NH_4)_2SO_4$ to 5.9. There was no pH change in the media containing KNO_3 and $NaNO_3$.

Growth was obtained over the range of pH 3 to 8. At low initial pH values the organism was able to raise the pH, and at high pH levels it acidified the medium. Thus, it was demonstrated that Urocystis colchici has the ability to alter its environment by changing the reaction of the medium. The optimum pH range appears to be quite narrow, i.e. pH 5.8 to 6.4, which nearly duplicates the optima of teliospore germination, i.e. pH 5.5 to 6.5 (23). The pH optimum of Urocystis occulta corresponds to that of U. colchici with the peak amount of growth at pH 6.2 (31). U. colchici appears to have a narrower pH optimum than Tilletia controversa which grew best between pH 6.0 and 8.0 (10) or T. caries which favored 6.0 to 8.6 (57).

The optimum temperature for growth of Urocystis colchici depends upon the culture medium used. Sucrose and asparagine provided the best growth at 24°C while phytone media supported better growth at 20°C than at 24°C. Malt extract media was able to support similar amounts of growth at 16, 20, and 24°C. Walker and Wellman (53) found

maximum growth of U. colchici to occur at 18°C on onion decoction agar. Cochrane (11) stated that "there is no single temperature optimum of growth. Temperature optima and ranges reported are valid only under specified conditions of time, medium, and method of measurement."

Maximum germination of teliospores of U. colchici on malt extract-phytone agar obtained by Lacy (23) occurred at 24°C while Walker and Wellman (53) report maximum germination at 15°C on onion decoction agar. Togashi (49) reports that from studies of several plant pathogens optimum temperature for spore germination is close to that for in vitro growth.

It has been determined that: 1) sucrose was one of the best simple carbon sources for growth of U. colchici; 2) thirty grams sucrose per liter was the optimum concentration; 3) L-asparagine was an excellent nitrogen source; 4) oxygen was not an important factor when using 40 ml of liquid medium in 125 ml flasks; 5) approximately three weeks growth gave maximum dry weight of the fungus; 6) the optimum temperature for growth was 20 to 24°C; 7) the optimum pH range was 5.8 to 6.4; and 8) one g N/liter as asparagine when used with 30 g sucrose/liter gave the best growth.

For the first time a defined medium had been developed for this organism. This defined medium yielded the greatest amount of growth so far obtained with U. colchici.

Information was obtained on the nutritional and environmental growth requirements of U. colchici. Comparisons were made between the requirements for somatic growth and spore germination, thus expanding our knowledge on the biology of this organism.

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