ORGANIC AND INORGANIC NUTRITION OF *TILLETIA CONTRAVERSA*

by

CHUEN-SHANG CHUNG

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APPROVED:

[redacted]

Assistant Professor of Botany

[redacted]

Professor of Chemistry

In Charge of Major

[redacted]

Chairman of Department of Chemistry

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

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INTRODUCTION

The filamentous fungus, *Tilletia contraversa*, has long been known to cause dwarf bunt in wheat, rye, and many grasses. Like other smut fungi, it develops its vegetative mycelium and teliospores on living hosts. The importance of these fungi lies in the fact that they are parasites of man's important food crops and the annual losses due to bunt run into millions of dollars in the United States.

Research on this disease was at first chiefly concerned with the production of resistant varieties of wheat, but in recent years more fundamental and widespread investigations have been undertaken. Chemical seed treatments and resistant wheat varieties are enabling impressive gains in the battle against common smut of wheat in the Pacific Northwest. In fact, smut incidence in the Pacific Northwest has declined sharply since 1956. However, new and more complex pathogenic races arise occasionally from genetic recombinations. The commercially grown resistant varieties of wheat serve to screen out the less virulent races and perpetuate these more virulent new races. In order to keep pace with

this changing pathogenic spectrum it is necessary to study the genetics of the host-pathogen relations, and the mechanisms by which the resistant varieties of wheat protect themselves against the smut fungi. To investigate these problems, in turn, it is necessary to grow the smut fungi in artificial media and to investigate their physiology and exact nutritional requirements.

The germination of teliospores of the smut fungi and the growth of mycelium of the non-parasitic stage in artificial or semi-artificial media have been reported by many workers (3, 12, 17, 21, 33, 34, 40, 43). Although the dwarf bunt fungus has long been cultured on media containing plant products, its exact nutritional requirements have not been thoroughly investigated. The objects of the investigations reported herein were to find better conditions for growth and to study the exact organic and inorganic nutritional requirements of mono-nucleate mycelium of Tilletia contraversa.

REVIEW OF LITERATURE

A. History

The discovery of Tilletia contraversa Kuhn (Conners, 1954) in the United States was first reported by Young (42). Fischer (6) later described this smut fungus as a new species since this species was different in morphology and life cycle from that of Tilletia caries (DC). He gave this fungus the name of Tilletia brevifaciens. Conners (2) in 1954, comparing T. brevifaciens and the type collection of T. contraversa Kuhn on Agropyron spp. in Europe, concluded that the two were synonymous and that the dwarf bunt fungus should be referred to by the earlier name T. contraversa.

Recently, in a similar comparison of the dwarf bunt fungus with other species of Tilletia on cereals and grasses, Duran and Fischer (4) came to the conclusion that Tilletia pancicii on Hordeum spp. and T. calospora on Alopecurus agrestis should be reduced to synonymity under T. contraversa.

B. Morphology and life cycle

The morphology of Tilletia contraversa has been studied by Fischer (6) in detail. In general, it resembles T. caries in most morphological aspects but it is considered distinct from the common wheat smut,

T. caries by a combination of the following characteristics (18):

(1) T. contraversa cause greater dwarfing and tillering in the host;

(2) the spores of T. contraversa are usually encased in a hyaline sheath;

(3) the reticulations are deeper and the areolae wider in T. contraversa than in T. caries;

(4) the spore balls of T. contraversa are generally hard and compact;

(5) seed treatment with chemicals is ineffective as a control method of T. contraversa;

(6) teliospore germination is more difficult for T. contraversa than that for T. caries.

The nuclear phenomena in T. contraversa also resemble that of T. caries (18). The teliospores germinate by sending forth a germ tube (promycelium) in which reduction division of the nucleus occurs. Approximately 8 to 16 haploid nuclei are formed from nuclear divisions in the promycelium, and each haploid nucleus in the promycelium migrates into a separate primary sporidium. As soon as a primary sporidium is mature, it may germinate directly and continue to grow on artificial media as mononucleate hyphae. However, the germ tube of primary sporidium usually fuses with an adjucent sporidium and thus

initiates the binucleate parasitic stage of the fungus. The binucleate cell will then germinate and form binucleate hyphae. The binucleate hyphae are capable of infecting young wheat seedlings. As the wheat plant begins to form seeds the bunt fungus produces teliospores very rapidly within the seed coat to produce the "smut" or "bunt" ball. During teliospore formation the nuclear condition changes from the haploid binucleate form of the hyphae to the diploid mononucleate form of the mature teliospore.

C. Growth of smut fungi on artificial media

The first investigator who studied the development of smut fungi in artificial media was probably Brefeld in 1833 (7, p.221). Since then, increasing attention has been given to the cultivation of the smuts.

In 1924, working with Tilletia tritici and T. foetans, Sartoris (33) found that the mycelium of these two fungi developed best on a heavy oatmeal agar. He also observed that malt extract in solution was the best liquid medium, and dextrose was the best sugar solution for this purpose. The addition of calcium nitrate and potassium nitrate to the solution favored the formation of mycelium, while magnesium sulfate and potassium dihydrogen phosphate inhibited its formation. However, while acting negatively on Tilletia, potassium dihydrogen phosphate and magnesium

sulfate promoted the formation of mycelium and caused the secondary spores of Ustilago hordei and U. henfleri to conjugate and form resting spores. Sartoris also found that slightly acid medium was suitable for the smuts studied, and sugars were not fermented by the four species studied.

For the wheat bunt fungi T. caries and T. foetida, Kienholz and Heald (22) found that 4% sucrose was the best source of carbon. They also found that plain agar or soil extract agar were the most suitable media for T. levis and T. tritici for germinating the smut spores. Media containing added sugars usually caused heaped type of growth to form, while in the medium containing no added sugars the culture remained flat.

On studies of cultural conditions of T. tritici, Halbsguth (11) noted that sucrose, glucose, and levulose were the most suitable carbon sources, while alanine and asparagine were the best sources of nitrogen. The optimum pH range was found to be 5.5 to 6.5, and temperature had a pronounced influence on the growth. The dry weight of cultures was proportional to thiamine concentration, but the proportionality was dependent upon the composition of the medium.

In studies of the nutritional requirements of T. caries, Zscheile (43) visually compared the surface

growth of the fungus. He found that a certain concentration of total solutes permitted the most growth of T. caries. At higher concentrations of total solutes no growth occurred, and at low concentrations growth was scanty. The optimum pH for both germination of teliospores and mycelial growth was between 6.0 and 8.6. As to the minor mineral elements he found that a distinct stimulation occurred when traces of iodide, zinc, and manganese were added. Molybdenium was stimulatory only when nitrate was substituted for organic nitrogen. Thiamine was the only essential vitamin for T. caries and maximum effect was obtained at a concentration of 0.01 mg. per liter. L-asparagine was the most effective source of nitrogen.

More recently, Siang (34) studied the physiology and nutritional requirement of Tilletia caries and T. contraversa. He also used visual observation to detect differences in fungus growth but several of his observations were recorded as dry weight of the surface mycelium. He found that the pH optimum for growth of both fungi was from 6.1 to 7.6, but T. caries grew slightly better in alkaline medium than did T. contraversa. This pH range was narrower than that found by Zscheile (43). In both species, Siang found that dextrose, levulose, and sucrose gave the best growth among the six sugars tested.

Raffinose and maltose were slightly effective, but mannose did not support fungus growth. The optimum sugar concentration for growth of T. contraversa was 2% while that of T. caries was 4%.

Siang further demonstrated that the organic nitrogen compounds were much more readily utilized by both organisms than inorganic forms of nitrogen. Asparagine was the best source of nitrogen for T. contraversa while casein hydrolysate was the most effective for T. caries. Among the 14 amino acids tested, all except DL-methionine and DL-threonine served as nitrogen sources for both species.

In a study of vitamin requirements, Siang observed that T. caries and T. contraversa require an exogenous source of thiamine. The thiamine requirement could be replaced only by the thiazole moiety of thiamine.

It has long been recognized that only the binucleate stage of the bunt fungi cause infection on host. This suggests that the binucleate stage of these fungi differs physiologically and pathologically from the mononucleate stage. The binucleate stages of the bunt fungi had long been considered as obligate parasites which were difficult or impossible to culture on sterile media. Trione and Metzger (37) were not able to isolate the parasitic stage of T. caries from infected tissue, but they successfully

cultured the binucleate stage of T. caries through its life cycle beginning with germinating fused primary sporidia. Kendrick (21) observed the production of teliospores of T. caries in a culture which originated from a single primary sporidium.

MATERIALS AND METHODS

Organism: The organism used in this study was Tilletia contraversa Kühn (Conners, 1954), race D-3, monosporidial isolate No. 459-3 which was obtained by the courtesy of Dr. E. L. Kendrick, U. S. Department of Agriculture, Regional Smut Research Laboratory, Washington State University, Pullman, Washington. Dr. Kendrick isolated this organism as a single primary sporidium, The fungus colony which resulted from the primary sporidium, thus, consisted of haploid, monocaryotic, non-parasitic hyphal cells.

Basal medium: The basal medium, called T-19 was designed by Dr. E. J. Trione, Science Research Institute, Oregon State University. The composition of medium T-19 is shown in Table 1. A comparison of T-19 with Zscheile's MT-1 medium (43) is given in Table 2. Media T-19 and MT-1 are similar but differ quantitatively as well as qualitatively. Medium T-19 was developed to support good growth of T. contraversa while medium MT-1 was developed for T. caries.

The chemicals used were of C. P. grade except chelated iron (sodium ferric diethylene triaminepenta-acetate, 10% Fe, from Geigy Chemical Company) and no further purification was attempted. Since minor elements

were not studied, regular distilled water was of sufficient purity and was used throughout all experiments. Except for heat labile compounds, all media and glassware were sterilized in the autoclave at 15 pounds pressure for 15 minutes. Heat labile compounds were sterilized by filtration through bacterial-retaining millipore filters (Millipore Filter Corporation, Bedford, Mass.).

The pH of the media was adjusted to 6.0 with 1N NaOH before autoclaving and checked after autoclaving. Growth inhibiting substance may be formed if the sugar were in contact with phosphate (5) or with amino acids (16, 27) during autoclaving. Therefore, the minerals, sugar and thiamine, and amino acids were placed in separate flasks prior to autoclaving. On cooling, these three components were added together, and the pH determined with a Beckman Zeromatic pH meter.

Inoculum: Stock cultures were maintained on T-19 agar and incubated at 12°C. To start inoculum for the nutrition experiments, the mycelium from the stock cultures was transferred to liquid T-19 medium and incubated as described below. The mycelium of the rapidly growing shake cultures was collected by centrifugation from the liquid T-19 medium. The cells were washed twice with sterilized water, suspended in distilled water, and 5 ml. of the suspension was used to inoculate each flask

in an experiment.

Growth of cultures: In each experiment the cultures were grown in 500 ml. Erlenmeyer flasks, each containing 150 ml. of the liquid medium. Since growth in shaking cultures was found to be much better than that in stationary cultures, a reciprocating shaker with 9 cm. amplitude, running at 105 cycles per minute was used for all experiments. The fungus cultures were found to reach maximum dry weight when incubated for 7 days on the shaker in a constant temperature room at 70°F. Thus in most experiments the cultures were harvested after 7 days of incubation.

Measurement of growth: The dry weight of the mycelium produced provided an accurate measure of growth. A Buchner funnel under vacuum was used to filter the hyphae from the culture suspension. The hyphae were washed with distilled water to remove excess medium and then dried to constant weight at 50° to 60°C in an oven. The dry mycelium was weighed on an analytical balance.

Statistical analysis of the results: The results were analyzed by standard statistical procedures and compared at the 1% and 5% levels for least significant differences. The data presented in all tables and figures are the average of 3 or 4 replications. The

calculated least significant differences are presented with all data.

EXPERIMENTAL RESULTS

1. The Effect of Initial pH on Growth

Under given conditions, a fungus will grow maximally only in a certain range of initial pH values of the medium. The pH range which allows optimum growth, and the pH ranges which inhibit growth vary with the species. In order to determine these values for T. contraversa, the initial pH of the medium was adjusted to various levels ranging from 3.0 to 8.5 before autoclaving, and checked after autoclaving. On the seventh day of growth, the fungus was harvested, dried, and weighed. The final pH of the medium was also measured. The results are presented in Table 3, and the average rate of growth of the fungus was plotted against pH in Figure 1. It was found that the optimum initial pH for this fungus lies between 6.0 and 8.0. Growth was not obtained above pH 8.0 or below 4.0.

The medium changed somewhat during growth and the final pH tended to approach neutrality. With acid medium, the final pH increased to 7.5-8.0 and with alkaline medium, it decreased to the same range.

In a medium which is not strongly buffered the pH changes during the period of rapid growth. The changes in pH of the medium during growth was studied in 2 media with initial pH values of 6.0 and 8.3. The results are

presented in Figure 2. It is noteworthy that an extracellular pigment(s) produced by this organism exhibited a color change with pH change. The pigment(s) was dark reddish brown at pH 4.0 but it became lighter in color as the pH was lowered to 3.0 or raised to 7.0. At pH values below 3.0 or above 7.0 no pigmentation was observed.

2. Carbon Nutrition

Carbon occupies a unique position among the essential elements required by living organisms. The composition, structure, and configuration of organic compounds are the key factors which must be considered in relation to utilization of organic compounds by fungi. Since more is known about carbohydrates and related compounds as carbon sources, and about the manner in which they are assimilated and dissimilated than any other classes of organic compounds, all of the carbon compounds employed in this experiment were carbohydrates.

Carbon sources were added to the basal medium in a concentration of 20 gm. per liter. Since carbohydrates contain about the same percentage of carbon in the molecules, it is possible to compare their effectiveness as carbon sources. The growth of T. contraversa on 7 different carbon sources is presented in Figure 3. In general, glucose is utilized more effectively by fungi than any other sugar and is often considered a universal

carbon source. The dwarf bunt fungus, however, grew much better on sucrose and fructose than on glucose. Galactose, maltose, and soluble starch supported poor growth and lactose was not utilized as a carbon source.

In another experiment, sucrose was shown to be superior to its moieties, glucose and fructose, as a carbon source. As shown in Table 4, the combination of glucose and fructose together in the medium produced more growth than either alone but not as much growth as sucrose alone. Similar results have been reported by Haskins and Weston (13) working with Karlingia rosea, and by Herrick (15) with Stereum gausapatum Fries.

For many fungi, an increase in carbohydrates beyond an optimum concentration results in a decrease in growth. This phenomenon may be ascribed to the fact that other constituents of the medium, especially nitrogen, become limiting at the higher carbohydrate concentrations. That is, within limits, growth is increased at higher carbohydrate levels provided that adequate nitrogen is supplied. The data of Figure 4 exemplify this type of response. At constant composition of the remainder of the medium, growth increased as the concentration of sucrose increased, but above 25 gm. sucrose per liter the dry weight decreased.

3. Nitrogen Nutrition

Nitrogen is used by fungi for functional as well as structural purposes. In general, nitrogen sources can be classified into inorganic and organic compounds. The former includes ammonium salts, nitrates, and nitrites, while amino acids, amides, proteins, and peptides belong to the latter. In the present investigation, both organic and inorganic compounds were tested as sources of the essential element nitrogen.

The nitrogen compounds were added to the basal medium in amounts calculated to give a nitrogen concentration equivalent to the nitrogen in 3 grams of L-asparagine per liter. All of the nitrogen atoms in the molecules were assumed to be utilized equally. Urea was sterilized by filtration through bacterial-retaining millipore filters due to its ease of destruction by autoclaving. A comparison of eight organic and inorganic compounds as sources of nitrogen was given in Figure 5.

Of the eight compounds studied, L-asparagine supported the best growth. The addition of L-glutamic acid to L-asparagine did not increase the dry weight significantly even though the nitrogen concentration in the medium was increased to twice the value in the experiment with L-asparagine alone. The growth on L-asparagine alone and L-glutamic acid alone were 1240 mg. and 440 mg.,

respectively. When these two amino acids were combined the yield was 1270 mg.

Moderate growth was supported by casein hydrolysate, potassium nitrate, L-glutamic acid, urea, ammonium tertrate, and glycine. Ammonium sulfate was a poor source of nitrogen probably because of the lowering of pH of the medium which accompanied the utilization of ammonium nitrogen.

It is difficult to establish an optimum amount of nitrogen for a culture (1, p.241). In principle, any factor may change the apparent optimum concentration of the nitrogen source, but the nitrogen demand is directly correlated with the carbon supply. The dependence upon carbon is illustrated in Figure 6. The nitrogen requirement increased as the sugar concentration increased. At 5, 10, and 40 grams of sucrose per liter of medium, the optimum concentration of L-asparagine were found to be 1, 3, and 7 grams per liter, respectively.

4. Vitamin Requirements

It is known that in addition to the essential elements which yield energy and are used for structural purposes, cells also require certain vitamins for normal growth and development. Similarly, fungi require minute amount of specific organic compounds for growth, reproduction, and other vital functions. Some fungi can synthesize these

compounds, while others must obtain them exogenously.

It is generally assumed that a fungus which is independent of an externally supplied vitamin either is capable of synthesizing the compound or does not need the vitamin in its metabolism. Some fungi do not grow on synthetic media because they are unable to synthesize certain vitamins.

Vitamin deficiencies among the fungi have been detected only for certain members of the water soluble B-complex group. Since the most common vitamins involved are thiamine, biotin, inositol, pyridoxine, nicotinic acid, and pantothenic acid, those six compounds were studied in this experiment in addition to p-aminobenzoic acid and riboflavin. Pantothenic acid was supplied as calcium salt.

In studying the vitamin requirements of this organism, great care was taken to use glassware and chemicals which were free of vitamins. The flasks were washed both with detergent and with dichromate-sulfuric acid solution, rinsed thoroughly with tap water, followed by distilled water. Since vitamin contamination may occur from dust and cotton plugs, stainless steel caps were used instead of cotton plugs. The medium was boiled with acid washed activated carbon (Darco Department, Atlas Powder Company, New York) for 5 minutes to remove any

vitamins present in sucrose and amino acids (25, p.421). Vitamin solution were sterilized by filtration and were added to the cooled sterilized basal medium separately. Each vitamin was added to give a final concentration in the basal medium of 10 mg. per liter.

For the vitamin studies, the inoculum was prepared as follows: The hyphae which were growing in T-19 liquid medium were collected by centrifugation, washed three times with sterile distilled water, and then transferred into the vitamin-free culture medium. After four to five days the fungus started to grow actively. The hyphae were collected, and transferred again to a newly prepared vitamin-free medium of the same composition. The hyphae which grew after the second transfer were harvested, resuspended into sterile distilled water, and used as the inoculum.

As shown in Table 5, the only vitamin required by T. contraversa was thiamine. The other vitamins tested were not essential to this organism. The pH decreased in all media except that containing thiamine. This change in pH may have influenced the growth and development of the mycelium.

The effect of the concentration of thiamine on growth was also investigated. As shown in Figure 7, the optimum thiamine concentration was found to be 40 µg. per liter

in T-19 medium, and half of the maximum growth was obtained at about 7 µg. per liter.

Thiamine can be synthesized chemically by coupling the two moieties, 2-methyl-4-amino-5-methylpyrimidine and 4-methyl-5,β-hydroxyethylthiazole, commonly referred to as pyrimidine and thiazole, respectively. Thiamine deficient fungi differ in their ability to utilize or synthesize these two moieties. Most thiamine-deficient fungi can synthesize thiazole and couple the two moieties to make the complete thiamine molecule. It may be assumed that only the intact molecule has the activity as a growth factor.

To determine whether T. contraversa requires (1) the intact molecule of thiamine; (2) both moieties; (3) thiazole alone; or (4) pyrimidine alone, the fungus was grown in media containing the respective components at approximately equimolar concentrations. The concentration of thiamine hydrochloride was 5 mg. per liter. The results (Table 6) demonstrated that thiazole was required for growth. No significant difference in total dry weight of the hyphae was detected in the medium containing thiamine compared to a medium containing the two moieties of thiamine. Likewise, the total dry weight of hyphae produced in the medium containing the thiazole moiety alone equaled the hyphal production in the thiamine

containing medium. This indicated that T. contraversa was able to synthesize the pyrimidine moiety and complete the synthesis of thiamine when furnished with the thiazole moiety.

5. Inorganic Nutrition

In the inorganic nutrition studies, only the response of T. contraversa to concentration gradients of four macroelements: phosphorus, potassium, sulfur, and magnesium; and one microelement: calcium, was investigated. No attempt was made to find the most suitable source for these elements except for sulfur. Also no special purification of the media was made in these experiments. Since in some cases, precipitates appeared after autoclaving, the pH was adjusted after autoclaving to approximately 6.0 with sterilized 1N HCl and 1N NaOH.

Phosphorus: Dibasic and monobasic potassium phosphate were omitted from the basal medium. To replace phosphorus and potassium they were supplied as sodium dihydrogen phosphate and potassium chloride, respectively. Potassium chloride was added at the concentration of 5 millimolar in the culture medium. As shown in Table 7, the dry weight increased with increasing phosphorus in the medium. However, the phosphorus concentration in T-19 was 0.5 millimolar, but the dry weight obtained was much higher than that from 0.5 millimolar phosphorus in

the test medium. This probably is due to the different sources for phosphorus and potassium in these two media.

Magnesium: Magnesium sulfate was omitted from the basal medium, and to replace magnesium and sulfur they were supplied in the form of magnesium chloride and sodium thiosulfate, respectively. The latter compound was added at the concentration of 0.5 millimolar. Maximum yield was obtained at 1.0 millimolar magnesium, which was the same concentration in T-19 medium. The results were presented in Table 8.

Potassium: Monobasic and dibasic potassium phosphate in the basal medium were replaced by potassium chloride and sodium dihydrogen phosphate. The results were presented in Table 9. The dry weight of hyphae did not increase significantly at concentrations of potassium greater than 10 millimolar. The potassium concentration in T-19 medium, however, was 0.55 millimolar, but the yield was much better in this medium than that obtained in the test medium at 10 millimolar potassium. This, again, may be ascribed to the different composition of the medium.

Sulfur: Since sulfur plays an important role in enzyme systems, special attention was given to survey the various sources of this element.

Most fungi can attain all their needs for sulfur from inorganic sulfate. They are capable of reducing the

sulfate radical and incorporating it into organic molecules. Some fungi, however, require a specific organic source of sulfur.

In the present experiment, several organic and inorganic sulfur compounds were tested for their effects on hyphal growth. They were added to the basal medium at the concentration of 1.0 millimolar sulfur, and sterilized by filtration except magnesium sulfate and sodium sulfate which were autoclaved. The source of magnesium was magnesium chloride.

As shown in Table 10, most sulfur compounds tested were utilized quite well. Sodium hydrosulfite, however, was toxic to the fungus for less growth was obtained in the medium containing this compound than was obtained in the medium with no sulfur added. The apparent increase in dry weight in the latter medium was possibly due to the sulfates of microelements added to the basal medium. The inorganic salt, sodium thiosulfate, was the most effective sulfur compound tested in this experiment for stimulating hyphal growth. Cystine was somewhat inferior to cysteine. Sulfamic acid supported good growth, while thiourea was less efficient.

Since sodium thiosulfate was found to be the best source for sulfur in the above experiment, it was used as the source of sulfur to investigate the effect of sulfur

concentration on the growth of T. contraversa. Magnesium was supplied as chloride as before. A sulfur concentration in the test medium of 6.4 millimolar resulted in maximum growth of hyphae as shown in Table 11. The dry weight was decreased at 12.8 millimolar sulfur. However, a point to be mentioned here is that sodium thiosulfate in this experiment was autoclaved instead of sterilized by filtration. As a result the maximum growth obtained was lower than the growth in T-19 basal medium which used magnesium sulfate as a sulfur source. This indicated that autoclaved sodium thiosulfate was not utilized as effectively as filter sterilized sodium thiosulfate.

Calcium: The requirement of calcium on the growth of fungi has been recognized recently. Some representatives of all the major groups of fungi have been reported to require calcium for stimulating growth (1, p.311), yet some fungi do not respond to added calcium by increasing dry weight (35). Therefore, this experiment was designed to determine whether T. contraversa required calcium for growth. Although calcium was reported as a microelement in fungi by Steinberg (35), no special purification of the medium was made in the present experiment. Calcium was supplied as calcium chloride at various concentrations. The pH of the medium was checked and adjusted with sterilized 1N HCl after autoclaving. In contrast to the

results obtained with the above four elements, the growth of T. contraversa decreased as the concentration of calcium in the medium increased, as shown in Figure 8.

Table 1

Composition of T-19 Basal Medium

Compound	Stock Solution		Growth Medium	
	amt. per liter	molar conc.	amt. per liter	molar conc.
KH_2PO_4	61.30 gm.	0.45 M	10 ml.	4.50 mM
K_2HPO_4	11.42 gm.	0.05 M	10 ml.	0.50 mM
$MgSO_4.7H_2O$	24.65 gm.	0.10 M	10 ml.	1.00 mM
$CaCl_2$	5.55 gm.	0.05 M	10 ml.	0.50 mM
Na-ferric diethylene triaminepentaacetate	2.00 gm.	0.20 %	10 ml.	20.00 mg./l
$ZnSO_4.7H_2O$	352.00 mg.	1.224 mM	10 ml.	12.24 µM
$CuSO_4.5H_2O$	38.10 mg.	0.153 mM	10 ml.	1.53 µM
$MnSO_4.H_2O$	3.10 mg.	0.018 mM	10 ml.	0.18 µM
$Na_2MoO_4.2H_2O$	2.54 mg.	0.011 mM	10 ml.	0.11 µM
L-Asparagine			3 gm.	22.70 mM
L-Glutamic acid			3 gm.	20.40 mM
Sucrose			20 gm.	58.50 mM
Thiamine hydrochloride			5 mg.	14.80 µM
Distilled water make to 1000 ml. at pH 6.0				

Table 2

Comparison of T-19 Medium with MT-1 Medium

Compound	Molar Concentration	
	T-19	MT-1
KH_2PO_4	4.50 mM	0.9 mM
K_2HPO_4	0.50 mM	1.5 mM
$MgSO_4$	1.00 mM	0.5 mM
$CaCl_2$	0.50 mM	0.75 mM
$MnSO_4$	0.18 μM	3.0 μM
$ZnSO_4$	12.24 μM	18.0 μM
$CuSO_4$	1.53 μM	
Na_2MoO_4	0.11 μM	
Na-ferric diethylene triaminepentaacetate	20.00 mg./l (Fe 2.00 mg./l)	
KI		10.0 μM
Ferric tartrate		2.5 mg./l (Fe 0.24 mg./l)
DL-Asparagine(L- in T-19)	22.70 mM	2.0 mM
L-Glutamic acid	20.40 mM	2.0 mM
Thiamine hydrochloride	14.80 μM	3.0 μM
Sucrose	58.50 mM	47.0 mM

Table 3

Effect of Initial pH on the Growth of *Tilletia contraversa*

pH				Final
Expected	Actual	Final	mg. Dry Weight	Color of Medium
3.0	3.25	3.25	38	yellow
3.5	3.75	3.82	50	red
4.0	4.20	5.07	270	deep brown
4.5	4.70	6.81	740	brown
5.0	5.25	7.00	960	brown
5.5	5.75	7.53	1080	brown
6.0	6.30	7.82	1150	brown
6.5	6.65	7.75	1140	light brown
7.0	7.15	7.88	1170	yellow
7.5	7.60	7.84	1220	yellow
8.0	8.00	7.51	1130	yellow
8.5	8.45	7.85	200	yellow

LSD 5% = 63 mg. LSD 1% = 87 mg.

Table 4

Effect of Mixed Carbon Source on the Growth of *Tilletia contraversa*

Carbon Source	Amount added, gm./liter	mg. Dry Weight
No sugar	--	5.8
Sucrose	20	740
Glucose	20	260
Fructose	20	450
Glucose + Fructose	10 each	530

LSD 5% = 54 mg. LSD 1% = 77 mg.

Table 5

Effect of Vitamins on the Growth of *Tilletia contraversa*

Vitamin	Dry Weight mg.	Initial pH	Final pH
No vitamin	23	5.85	3.70
Nicotinic acid	27	5.80	3.60
p-Aminobenzoic acid	27	5.80	4.00
Biotin	28	5.85	3.70
Calcium pantothenate	43	5.80	3.60
Riboflavin	32	5.80	3.45
Inositol	26	5.80	3.50
Pyridoxine	23	5.80	3.60
Thiamine hydrochloride	980	5.85	6.90

LSD 5% = 28 mg. LSD 1% = 39 mg.

Table 6

Response of *Tilletia contraversa* to Thiamine and Its Moieties

Compound	mg. Dry Weight
Pyrimidine	45
Thiazole	700
Pyrimidine + Thiazole	660
Thiamine	660
No vitamin	23

LSD 5% = 89 mg. LSD 1% = 127 mg.

Table 7

Responce of *Tilletia contraversa* to Phosphorus

Phosphorus concentration	Dry Weight		Final pH
millimolar	mg.	% control	
0.00	180	21.4	5.40
0.05	250	29.8	5.40
0.10	300	35.8	5.50
0.20	360	42.9	5.50
0.30	400	47.7	5.70
0.40	460	54.7	5.90
0.50	580	69.0	7.15
1.00	660	78.5	7.65
2.00	760	90.5	7.70
Control (T-19)	840	100.0	7.85

LSD 5% = 27 mg. LSD 1% = 36 mg.

Table 8

Response of *Tilletia contraversa* to Magnesium

Magnesium concentration	Dry Weight		Final pH
millimolar	mg.	% control	
0.00	290	22.8	6.05
0.05	890	70.0	6.85
0.10	940	74.0	7.55
0.30	1100	86.7	7.15
0.60	1210	95.4	7.10
1.00	1270	100.0	7.30
5.00	1250	98.5	7.40
10.00	1280	101.0	7.15
Control (T-19)	1270	100.0	7.80

LSD 5% = 26 mg. LSD 1% = 36 mg.

Table 9

Response of *Tilletia contraversa* to Potassium

Potassium concentration	Dry Weight		Final pH
millimolar	mg.	% control	
0.00	200	15.8	5.45
0.10	500	39.4	5.75
0.50	710	56.0	6.75
1.00	760	59.8	7.45
3.00	790	62.2	7.35
6.00	790	62.2	7.20
10.00	930	73.2	7.60
30.00	960	75.6	7.35
Control (T-19)	1270	100.0	7.80

LSD 5% = 34 mg. LSD 1% = 47 mg.

Table 10

Effect of Different Sulfur Compounds on the Growth of *Tilletia contraversa*

Compound	Formula	mg. Dry Weight
No sulfur		145
Sodium hydrosulfite	$Na_2S_2O_4$	7
Thiourea	$CS(NH_2)_2$	440
L-Cystine	$[-SCH_2CH(NH_2)COOH]_2$	520
Magnesium sulfate	$MgSO_4$	620
Cysteine hydrochloride	$HSCH_2CH(NH_2.HCl)COOH$	640
DL-Methionine	$CH_3SCH_2CH_2CH(NH_2)COOH$	650
Sodium sulfate	Na_2SO_4	670
Sulfamic acid	NH_2SO_3H	710
Sodium thiosulfate	$Na_2S_2O_3$	760

LSD 5% = 26 mg. LSD 1% = 35 mg.

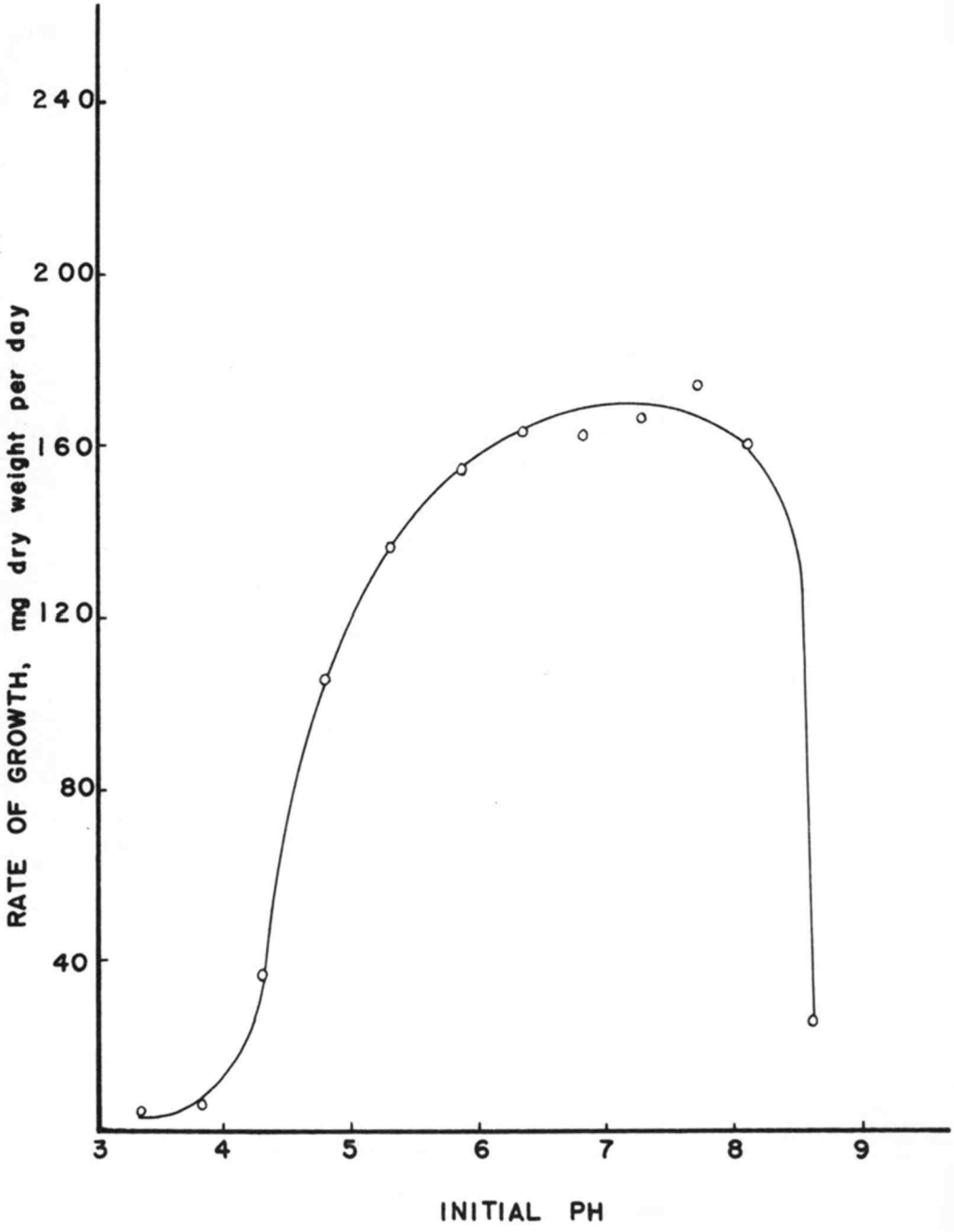
Table 11

Response of *Tilletia contraversa* to Sulfur

Sulfur concentration	Dry Weight		Final pH
millimolar	mg.	% control	
0.00	180	14.0	6.35
0.05	520	40.3	6.45
0.10	550	42.6	6.25
0.20	540	41.8	6.35
0.40	670	52.0	6.25
0.80	660	51.1	6.25
1.60	720	55.8	7.05
3.20	810	62.8	7.05
6.40	830	64.4	7.15
12.80	740	57.4	7.20
Control (T-19)	1290	100.0	7.65

LSD 5% = 48 mg. LSD 1% = 66 mg.

FIGURE 1. EFFECT OF INITIAL PH ON THE GROWTH OF *T. CONTRAVERSA*



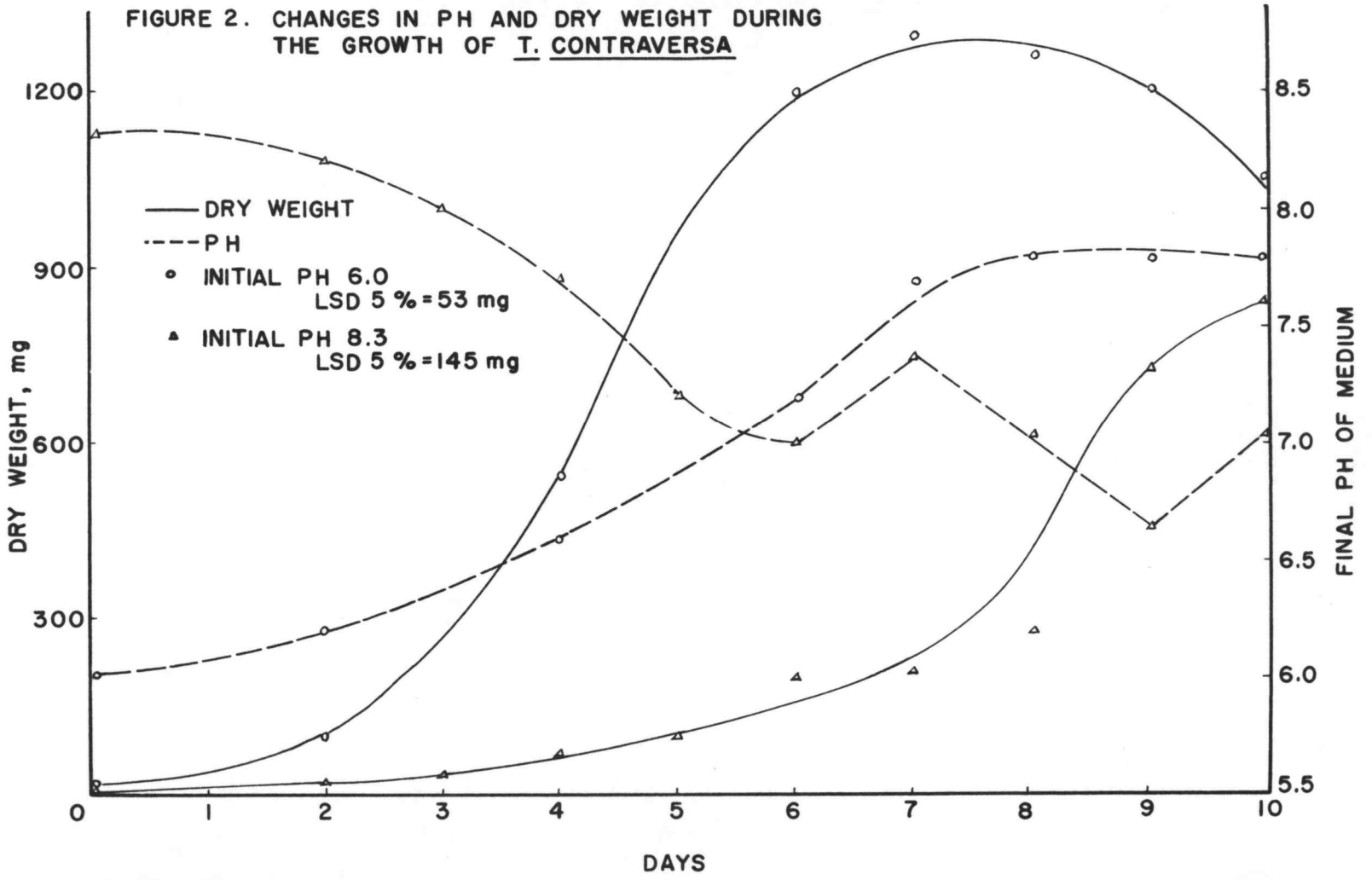


FIGURE 2. CHANGES IN PH AND DRY WEIGHT DURING THE GROWTH OF T. CONTRAVERSA

FIGURE 3. EFFECT OF DIFFERENT CARBON SOURCES ON THE GROWTH OF *T. CONTRAVERSA*

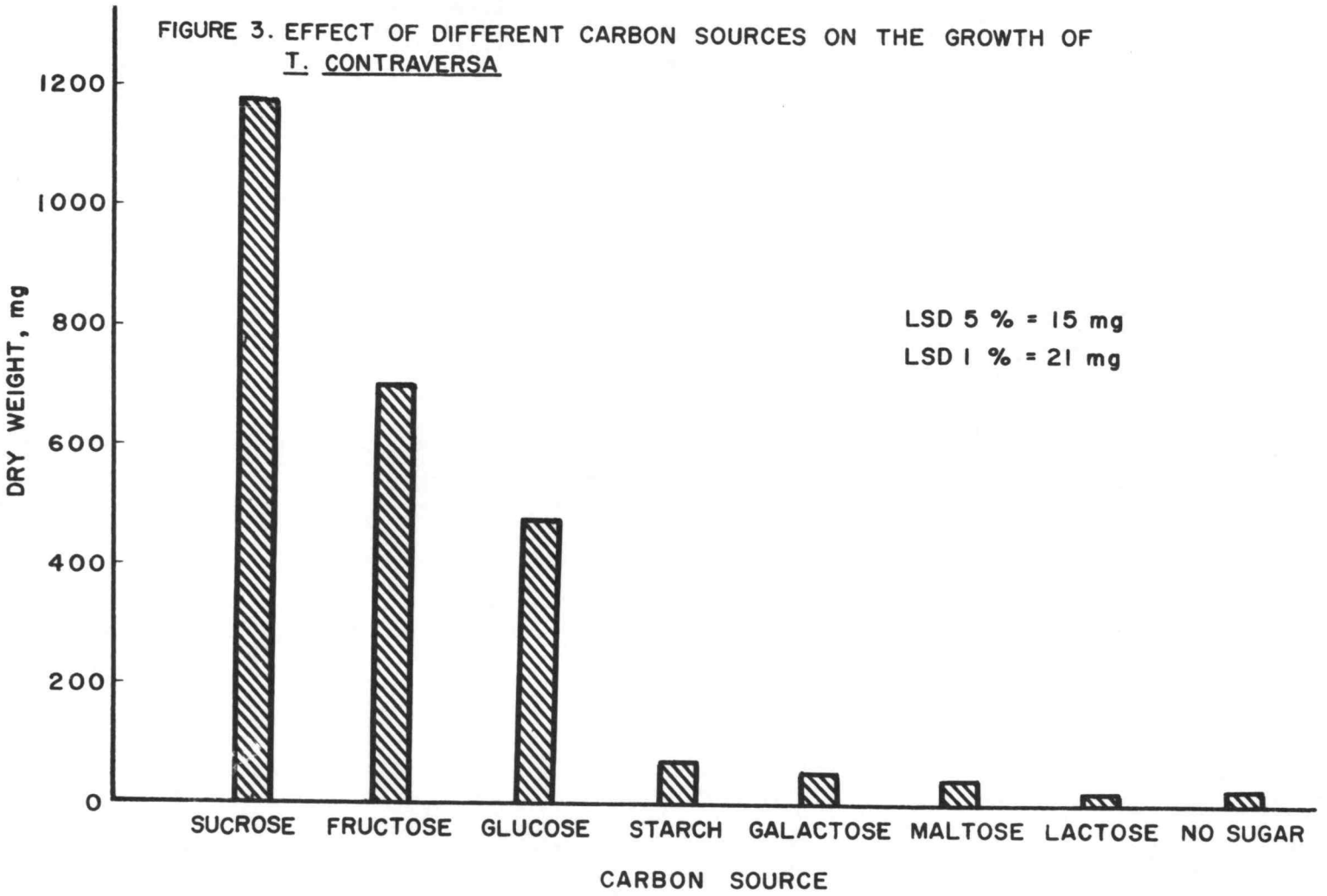


FIGURE 4. EFFECT OF SUCROSE CONCENTRATION ON THE GROWTH OF *T. CONTRAVERSA*

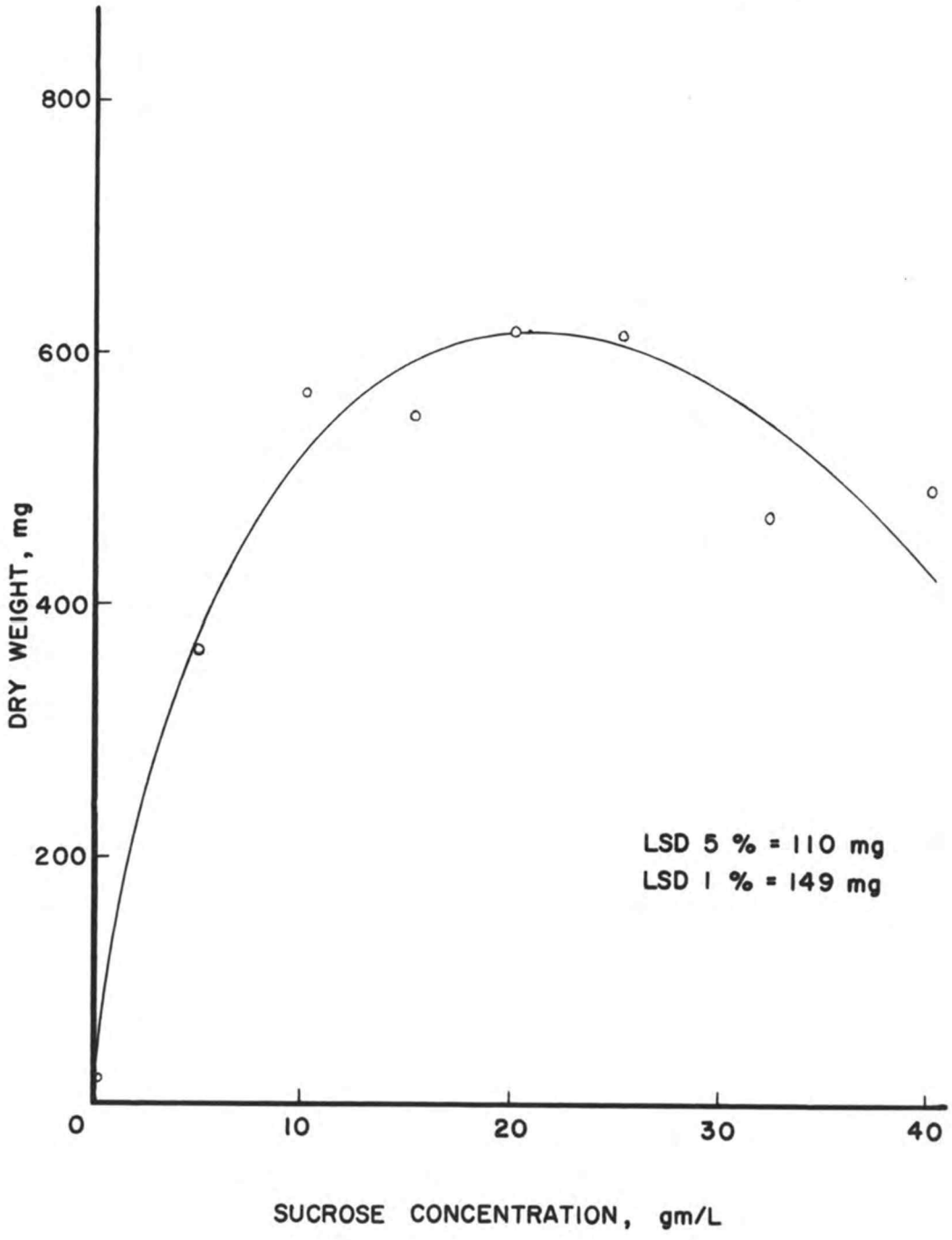


FIGURE 5. EFFECT OF DIFFERENT NITROGEN SOURCES ON THE GROWTH OF T. CONTRAVERSA
(ASPARAGINE AND GLUTAMIC ACID ARE IN L-FORM)

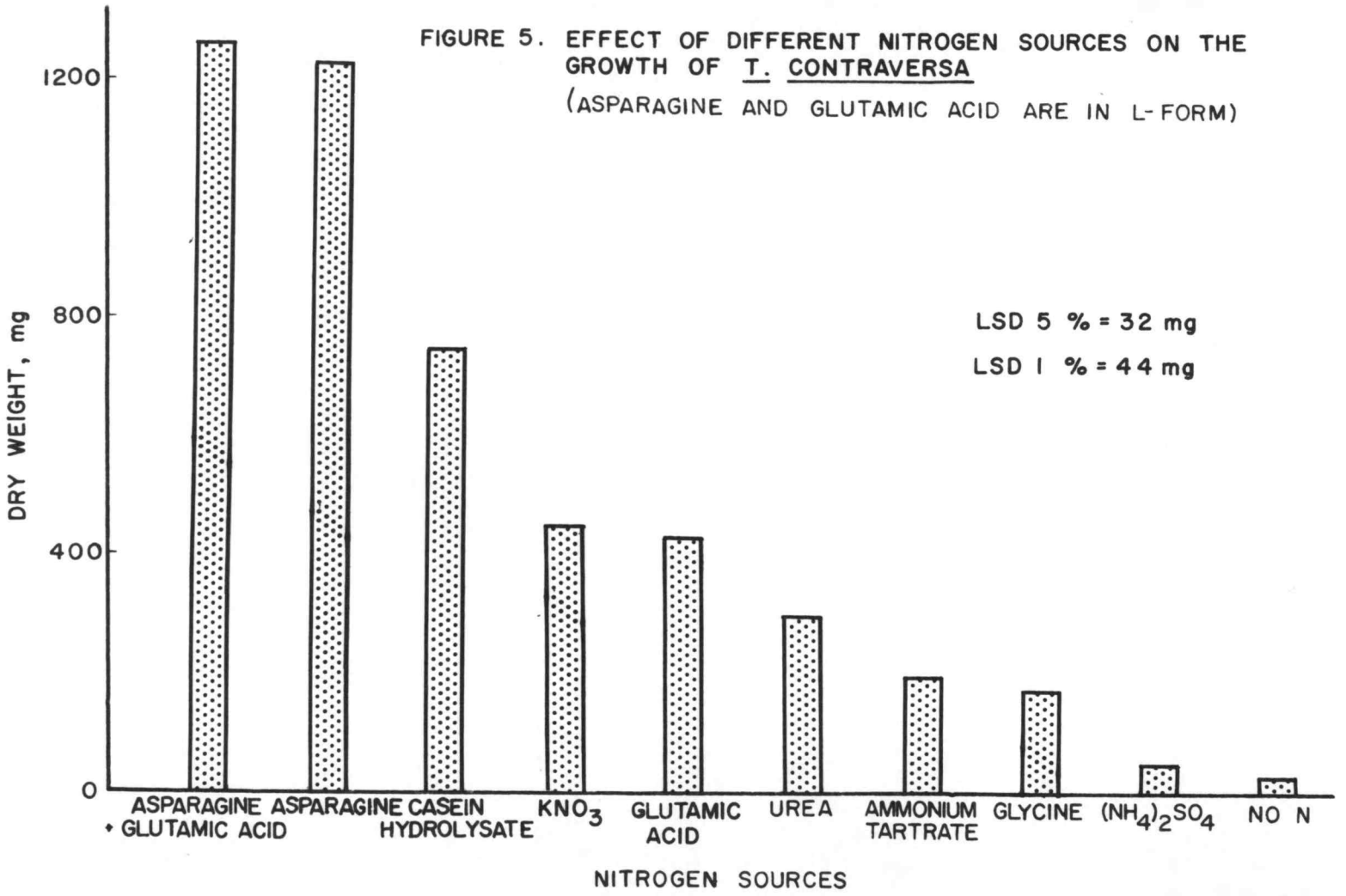


FIGURE 6. RESPONSE OF T. CONTRAVERSA TO L-ASPARAGINE IN DIFFERENT CONCENTRATIONS OF SUCROSE

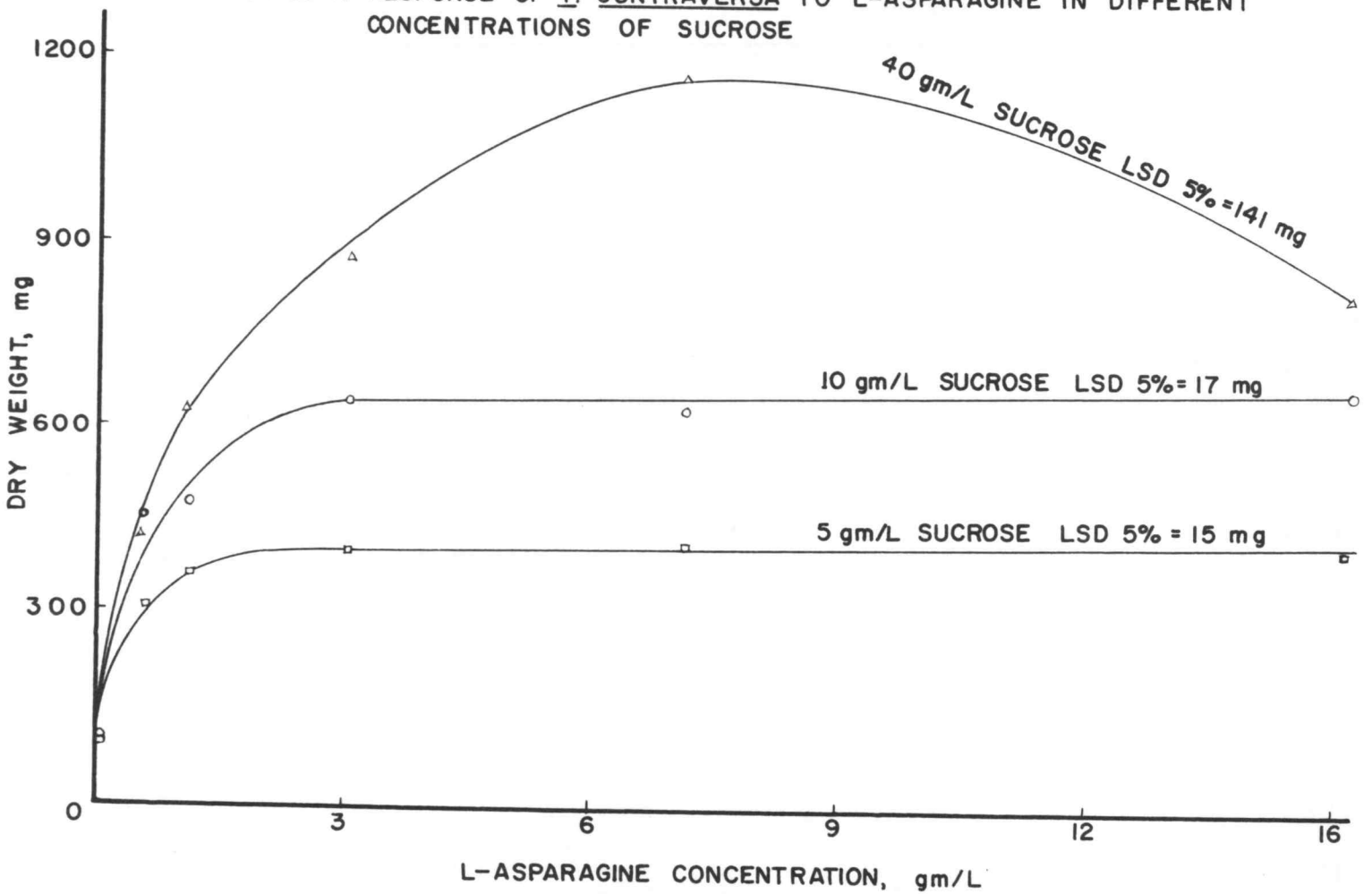


FIGURE 7. EFFECT OF THIAMINE CONCENTRATION ON THE GROWTH OF *T. CONTRAVERSA*

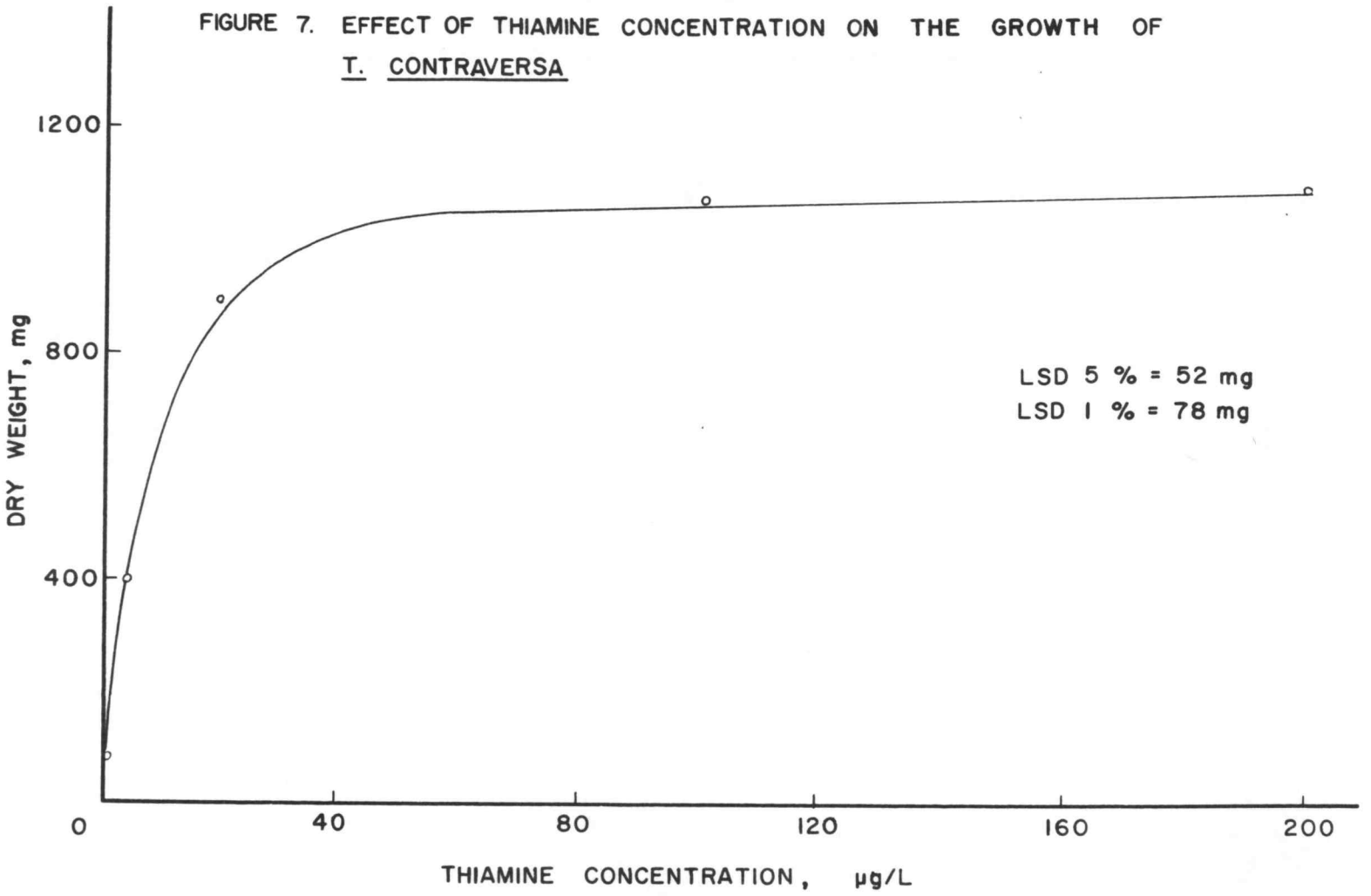
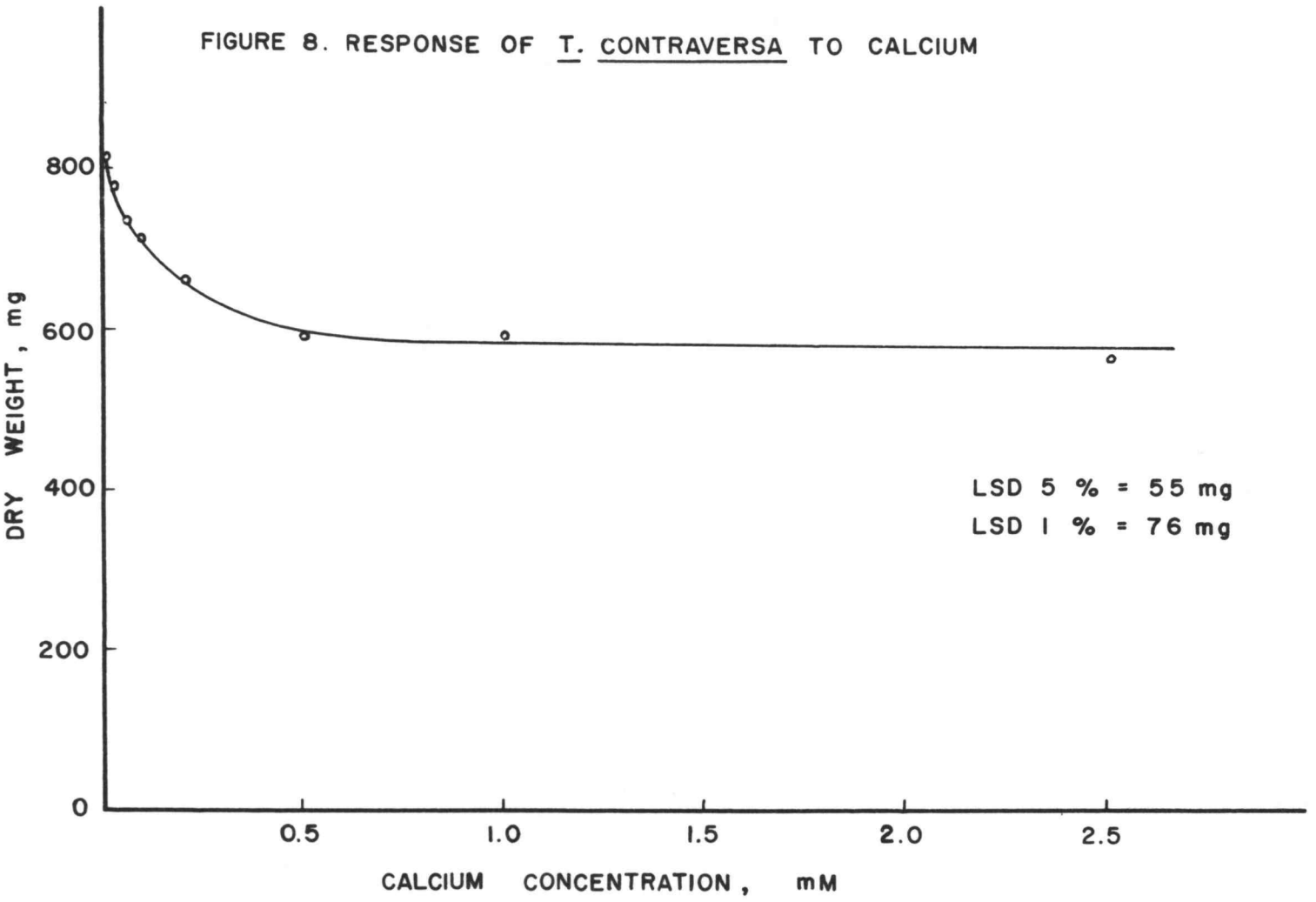


FIGURE 8. RESPONSE OF *T. CONTRAVERSA* TO CALCIUM



DISCUSSION

In the earlier nutritional studies of Tilletia contraversa the fungus was grown in liquid in still culture, and Siang (34) reported that no growth was obtained when the inoculum was submerged. In the present investigation, however, excellent growth was obtained by submerged culture on a shaker. Both results indicated that T. contraversa was obligatory aerobic. In fact, it was found that when the submerged liquid cultures from the shaker were permitted to stand for few days without shaking, the cells were killed in the poorly aerated solution.

The measurement of the slow growth on agar media was not used in this study because it did not represent the full expression of the growth potential of this organism. In the present study the amount of growth of the fungus was determined by measuring the dry weight of the mycelium produced in liquid medium. With the assistance of standard statistical analysis, it was possible to compare the effectiveness of various culture media. In most experiments, the differences between replications were very small.

The maximum growth attained by T-19 basal medium was somewhat variable in different experiments. This may be attributed to small changes in temperature, humidity, and

other conditions. Thiamine in T-19 basal medium was autoclaved together with sucrose. The destruction of thiamine during autoclave is probably one of the factors affecting the variation in dry weight.

The pH limits for a given fungus as determined in different laboratories are often not in agreement with one another. This may be due to the fact that the composition of the medium and the conditions employed are different. In the present studies only the influence of initial pH on growth was studied and no buffer was employed. Siang (34), in his investigation, used various concentrations of H_3PO_4, KH_2PO_4, K_2HPO_4, and K_3PO_4 as buffers. However, as shown in the later part of this thesis, the amounts of both potassium and phosphorus affect growth. Therefore, the results obtained in Siang's pH experiment reflect the effect of pH as well as the different compositions of the media. For this reason, pH studies in fungus nutrition are difficult to analyze.

Furthermore, almost any factor in the environment may change the pH optimum. Those factors include temperature (32, 41); source of nitrogen (20); concentration of calcium and magnesium (38); time of harvest (23, 30); and growth factor supply (24). In a medium without buffer, the effect of time of harvest on the optimum initial pH is especially pronounced. This effect was

shown in Figure 2. On the sixth day, the dry weight in the pH 6.0 medium was 5 times as much as that in the pH 8.3 medium, but on the tenth day of incubation the dry weight in the pH 6.0 medium was only 1.3 times as much as that in the pH 8.3 medium. This result indicated that the time for maximum growth at a given initial pH was different from that at other initial hydrogen ion concentrations. Lehman (22), when growing Diaporthe Sojae in a medium containing inorganic salts and glucose, found that growth starting at pH 3.1 was much slower than at pH 4.0, but dry weight of mycelium produced at the former pH value nearly equalled that attained in the latter. Neal (30) also found a similar results with Fusarium vasinfectum Atk. However, the pH change during growth may be an important factor to be considered at this point. As shown in Figure 2, the hydrogen ion concentration in the pH 6.0 medium decreased gradually while that in the pH 8.3 medium increased gradually during growth. From these results, it seems that T. contraversa had the tendancy to change the unfavorable pH to the pH suitable for its growth by adjusting the metabolic processes. Lilly and Barnett (25, p.168) attributed the pH change of a medium during the growth of a fungus to be the combined result of four metabolic processes: (1) utilization of anions; (2) utilization of cations; (3) formation of acids; and (4)

production of bases.

For the source of carbon, sucrose was found to be the best among the seven carbohydrates studied. This result was in agreement with that of Kienholz and Heald (22) for T. caries and T. foetida, and that of Siang (34) for T. caries and T. contraversa. The optimum concentration of sucrose was found to be the same as that found by the latter worker. However, Siang stated that glucose and fructose were as good as sucrose for the growth of T. contraversa. This is due to the fact that he did not measure the growth quantitatively but determined the relative growth by visual observation. In the present studies, exact measurements of dry weight were recorded. It was found that sucrose, the disaccharide, was superior to the other two monosaccharides, and fructose was better than glucose (Figure 3). Starch supported poor growth in this experiment. Nevertheless, the dwarf bunt fungus grows well on seeds which contain starch. This probably is due to the ability of the parasitic stage of the fungus to secrete amylases which function in the hydrolysis of starch.

The fact that T. contraversa utilized sucrose better than it does glucose and fructose, and the combination of glucose and fructose did not support as much growth as sucrose alone indicated that sucrose was a better precurser of the preferred in vivo carbon source than was

either glucose or fructose. A possible participating enzyme is sucrose phosphorylase. This enzyme has no detectable action on starch, maltose, lactose, and arabinose (9, p.446). The reaction which sucrose phosphorylase catalyzes is as follows:

Sucrose + H_3PO_4 $\rightleftharpoons$ Glucose-1-phosphate + Fructose

Glucose-1-phosphate may then be converted to glucose-6-phosphate through the catalytic action of phosphoglucomutase, or react with hexoses to form other disaccharides. Although sucrose phosphorylase has only been reported in bacteria, it is quite possible that this enzyme functioned in the utilization of sucrose by T. contraversa.

The optimum concentration of sucrose in T-19 basal medium was found to be 20-25 grams per liter. Beyond this concentration, the dry weight of mycelium decreased significantly. Treschow (36) and Ward et al. (39) obtained similar results for other fungi. Although the exact explanation for this phenomenon has not yet been established, Cochrane (1, p.86) interpreted the decrease in growth at high concentration of sugar as a result of the formation of toxic metabolic products.

However, a consideration to be mentioned here is that all tests of assimilability of the carbon sources were based on experiments with the pure compounds. Under natural conditions, fungi grow upon a mixture of carbon

compounds and the results of tests with individual compounds cannot serve as a final test of assimilability. Horr (19) found that galactose was a poor source of carbon for Aspergillus niger. The dry weight per culture produced with 10 gm. per liter of galactose was only 45 mg. while the same amount of glucose in the culture medium produced 410 mg. dry weight per culture. However, when these two sugars were added together to the culture medium, the dry weight per culture increased to 1150 mg. This result indicated that the utilization of galactose was favored by the presence of glucose, or vice versa.

Another problem related to assimilation is adaptive growth. Some fungi can grow in a poor source of carbon after a long period of incubation. Therefore, to investigate the growth potential of an organism more thoroughly it would be better to measure the growth curve for each source of carbon.

In the study of nitrogen requirements, it was found that the organism utilized both organic and inorganic forms of nitrogen. In the medium containing ammonium sulfate, the hydrogen ion concentration increased as a result of utilization of the ammonium ion. This development of acidity probably influenced the growth of the fungus. In the medium containing potassium nitrate, however, the pH remained near neutral, and better growth

was obtained in this medium than in the medium containing ammonium sulfate. Therefore, the relative assimilability of ammonium and nitrate salts might be attributed to the acidity of the nutrient solution. Urea, which was a better source of nitrogen than potassium nitrate in Siang's (34) study, was not as effective as potassium nitrate in the present study.

One of the objects for the present investigation was to develop a synthetic medium which supported good growth of T. contraversa. For this reason, L-asparagine, L-glutamic acid, glycine, and casein hydrolysate were chosen in the study of organic nitrogen sources because most investigations have shown that these compounds support good growth of many fungi (1, p.256). L-Asparagine was also found to be an excellent source of nitrogen for Tilletia species by Halbsguth (11), Zscheile (43), and Siang (34). The growth response of T. contraversa to the above individual amino acids and mixture of amino acids were very diverse. Foster (8, p.495-504) reported that the response of fungi to amino acids may vary with the molecular structure of amino acids and the incubation period of fungi. The first effect was proved by the results shown in Figure 5. Among the three amino acids tested, L-asparagine was the most effectve as a source of nitrogen. This result was in agreement with that of Halbsguth (11)

for T. tritici, Zscheile (43) for T. caries, and Siang (34) for T. caries and T. contraversa. Glycine, the simplest amino acid, was inferior to either L-asparagine or L-glutamic acid. This was probably due to the development of acidity during the growth of the fungus since the pH in this medium decreased to 4.5 at the time of harvest.

Besides molecular structure, molecular configulation of amino acids also play an important role when used as sources of nitrogen. As Zscheile (43) demonstrated, L-asparagine gave ten times as much growth of T. caries as the D-form. Siang (34) also ascribed the ineffectiveness of DL-alanine to the presence of D-form in the chemical used. Fungi usually grow better on natural or artificial mixture of different amino acids than with any single amino acid (1, p.257-258). In the present study, however, casein hydrolysate was inferior to L-asparagine as a nitrogen source when supplied at the same concentration of nitrogen. Siang (34) reported similar results for T. contraversa. The major constituents of casein hydrolysate are glutamic acid, leucine, lysine, and serine and very little or no asparagine is present in this natural mixture of amino acids (14, p.109). L-Glutamic acid was found to be inferior to L-asparagine in the present study (Figure 5) as well as in Siang's study (34); leucine and lysine

supported poor growth of T. caries and T. contraversa in Siang's experiment (34); and serine was a poor source of nitrogen for T. caries in Zscheile's investigation (43). From these results, it may be tentatively concluded that L-asparagine alone was a better source of nitrogen than any of the above individual amino acids or the mixture of amino acids. The addition of L-glutamic acid to L-asparagine did not increase the dry weight significantly. This result indicated that when these two nitrogen sources were present in the medium at the same time they did not exert a combined additive effect on the total growth of the culture.

Biotin, inositol, pyridoxine, nicotinic acid, calcium pantothenate, riboflavine, and p-aminobenzoic acid were demonstrated to be ineffective in stimulating the growth of T. contraversa. However, this fungus was found to require thiamine for growth. Zscheile (43) reported that thiamine was also required for the growth of T. caries.

Fungi differ in their ability to synthesize vitamins. Some fungi are able to synthesize the vitamin from exogenously supplied carbon, nitrogen, and metallic compounds; others are capable of making the vitamin if one or all of its intermediates are supplied; and still others are unable to synthesize any portion of the vitamin. T. contraversa apparently belongs to the second group; that

is, it can make the pyrimidine but not the thiazole part of the thiamine molecule. This result was in agreement with that of Zscheile (43) for T. caries and that of Siang (34) for T. caries and T. contraversa.

The function of vitamins is diverse. The metabolically active form of thiamine is thiamine pyrophosphate which is also called cocarboxylase. Thiamine pyrophosphate participates in decarboxylation of pyruvic acid and α-ketoglutaric acid, and in the transketolase reaction. Nagata et al. (29) described the decrease in oxalate accumulation as a function of thiamine. Since oxalic acid is quite a strong dicarboxylic acid, the decrease in pH in the thiamine-free medium in the present experiment may be explained by the reverse mechanism. Zscheile (43) also ascribed the increased acidity in the medium without thiamine to the accumulation of organic acids. When thiamine was present in the medium, the pH remained near 7.0 because thiamine promoted the complete respiration of carbon residues from the original carbohydrates. Grimm and Allen (10) suggested another function of thiamine in promoting cytochrome synthesis by Ustilago sphaerogena.

It has long been known that, for normal growth and function, inorganic salts must be supplied to all microorganisms. Of the inorganic elements identified in biological material, phosphorus, potassium, magnesium,

and sulfur are found to be present in high concentrations. They are termed the macroelements by microbiologists. In the present series of experiments, it was found that T. contraversa grew poorly without these elements. In Tables 7, 8, 9, and 11 it will be noticed that slight growth occurred in the media without phosphorus, magnesium, potassium, and sulfur, respectively. This might be due to the impurities of the medium and contamination from the glassware.

In general, the requirements of phosphorus, magnesium, potassium, and sulfur by fungi lie between 1-3 millimolar, 1 millimolar, 1-4 millimolar, and 0.1-0.6 millimolar, respectively (1, p.301-306). The optimum concentrations of the above elements for T. contraversa in T-19 medium were found to be 2 millimolar, 1 millimolar, 1 millimolar, and 0.4 millimolar, respectively. These results were in good agreement with the above generalization and demonstrated that the mineral requirements of T. contraversa were very similar to that of other fungi. However, these optimum concentrations may vary with the source of carbon, source of nitrogen, and other constituents of the culture medium (1, p.315-316).

Sartoris (33) found that when magnesium sulfate and potassium dihydrogen phosphate were added to malt extract or glucose solution, the growth of T. tritici and T.

foetans was decreased, but at concentrations lower than 25 millimolar these two compounds promoted the formation of mycelium. This indicated that high concentrations of magnesium sulfate and potassium dihydrogen phosphate inhibited the growth of the above two fungi. The inhibition of the growth of T. contraversa by high concentration of sulfur in the culture medium was also found in the present experiment (Table 11). Within certain limits of concentration the dry weight of the fungus increased as the sulfur content of the medium increased. The dry weight reached a maximum of 830 mg. at 6.4 millimolar sulfur. However, at 12.8 millimolar sulfur concentration, the dry weight decreased to 740 mg. Growth inhibition due to the other three macroelements, potassium, magnesium, and phosphorus were not observed within the concentrations studied in the present experiment.

The function of these inorganic elements in microbial nutrition are still under investigation. Phosphorus plays an important role in the chemical transformations and energy transfer. Thus, phosphate deficiency causes a number of metabolic disturbances. Potassium and magnesium ions are activators for many enzyme systems (25), while sulfur is directly incorporated into organic molecules and has a special role in the activity of some

enzymes. Calcium also functions in activation of enzymes, but inhibition of enzymes has also been reported (9, p.910).

The response of T. contraversa to calcium (Figure 8) is interesting because the result was entirely different from that obtained with phosphorus, potassium, magnesium, and sulfur. The dry weight of the fungus decreased when the concentration of calcium in the culture medium increased, and the inhibition of growth was maximum at 0.5 millimolar calcium. No further decrease in dry weight was observed as the calcium concentration was increased from 0.5 to 2.5 millimolar. Calcium has been reported to be essential for the growth of some fungi (1, p.311) and non-essential for others (35). Halbsguth (11) mentioned that the requirement of calcium by T. tritici was met by the impurities of the agar and other components of the media but he did not study the effect of calcium on the growth of the fungus. Zscheile (43), on the other hand, found that calcium was required for the growth of T. caries and the optimum concentration was 0.75 millimolar.

In contrast to the above observations, growth inhibition of fungi due to calcium has not been reported previously. The growth inhibition curve for the response of T. contraversa to calcium (Figure 8) may be explained in three ways. The first explanation is based on the

antagonism of ions. Calcium ion has been known to antagonize the effect of potassium, sodium, and copper ions (28) in some biological systems. A similar phenomenon was reported to occur in fungus nutrition by Omvik (31). Working with Geomyces vulgaris and other fungi, Omvik found that calcium antagonized the effect of manganese. Thus, the result of the present investigation might be due to the antagonistic effect of calcium ion with one of the metallic ions present in the culture medium. The fact that the inhibition of growth was maximum at 0.5 millimolar calcium suggested that calcium may have been competing with a microelement in low concentrations; for example, manganese, zinc, copper, etc.

The second explanation for the inhibition due to calcium may be based on enzyme inhibition. One or more of the enzymes which participate in the metabolic processes of the fungus may be inhibited by calcium ion. One of the reactions in which calcium ion acted as an inhibitor was the following, catalized by enolase (9, p.472):

2-Phosphoglyceric acid $\rightleftharpoons$ Phosphoenol pyruvic acid + H_2O

Both 2-phosphoglyceric acid and phosphoenol pyruvic acid are intermediate products in carbohydrate metabolism. If the reaction were blocked at this stage, the carbon residue could not undergo respiration completely and therefore the energy for growth would be decreased.

The third possible interpretation of calcium induced inhibition of growth could be based on organic acids. The metabolic intermediates of carbohydrates, the organic acids, may be released into the culture medium where the organic acids combine with calcium to form the corresponding calcium salts. The fungus may not be able to utilize the calcium salts of the organic acids for growth, thus, the amount of utilizable carbon source would be reduced. However, based on the fact that the inhibition of growth reached maximum at 0.5 millimolar calcium and no further decrease in dry weight occurred beyond this concentration, this third explanation seems to be unsatisfactory. If the decrease in dry weight were due to the precipitation of organic acids, further inhibition of growth would be expected as the concentration of calcium increased above 0.5 millimolar.

From the present investigation and that of others, it was revealed that the nutritional requirements of smut fungi vary with different species and to a lesser degree even among races within a given species.

SUMMARY

The organic and inorganic nutritional requirements of Tilletia contraversa Kühn, race D-3 were investigated. It was found that the rate and amount of growth of the fungus were dependent upon the composition of the medium in which the fungus was grown.

Among the seven carbohydrates tested, sucrose was the most effective as a source of carbon in promoting the development of mycelium. Glucose, fructose, or the combination of glucose and fructose could not equal sucrose as carbon sources in promoting maximum growth of the fungus. Soluble starch, galactose, and maltose supported poor growth, and lactose was not utilized as a carbon source. The optimum concentration of sucrose was found to be between 20-25 gm. per liter of the culture medium.

As a source of nitrogen, L-asparagine was found to induce better growth than the other seven organic and inorganic nitrogen compounds studied. The addition of L-glutamic acid to L-asparagine in the culture medium did not increase the dry weight significantly. The fungus was not able to utilize ammonium sulfate as a nitrogen source probably due to the development of acidity during growth. Casein hydrolysate was less effective than L-asparagine as a source of nitrogen. Potassium nitrate,

L-glutamic acid, urea, ammonium tartrate, and glycine supported moderate growth of the fungus. The optimum concentration of L-asparagine varied with sucrose content of the medium. It was found that as the concentration of sucrose in the medium increased, the amount of L-asparagine needed for maximum growth also increased.

Thiamine was the only vitamin required by Tilletia contraversa. Other vitamins studied in this experiment were not essential for the growth of the fungus. An experiment with the moieties of thiamine suggested that the fungus was able to synthesize the pyrimidine part of thiamine molecule and was capable of completing the synthesis of thiamine if furnished with thiazole. The optimum concentration of thiamine for maximum growth of the fungus was found to be 40 µg. per liter.

Phosphorus, potassium, magnesium, and sulfur were required for normal growth, and the optimum concentrations of these elements were found to be 2 millimolar, 1 millimolar, 1 millimolar, and 0.4 millimolar, respectively. Calcium was not needed by the fungus in the development of mycelium. The fungus utilized both organic and inorganic sources of sulfur, but sodium hydrosulfite was not utilized. Sodium thiosulfate was found to be the best among the nine sulfur compounds studied.

The pH of the medium, in all cases, was an important

factor for the growth of T. contraversa. The optimum initial pH range for this organism in T-19 basal medium was found to be 6.0-8.0. Either below 4.0 or above 8.0 no growth was obtained.

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