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Title: THE INFLUENCE OF SULFUR AND CARBOHYDRATE NUTRITION OF FUSARIUM OXYSPORUM F. SP. LYCOPERSICI ON RESISTANCE TO METHYLISOTHIOCYANATE

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Sulfur nutrition markedly affected the susceptibility of F. oxysporum f. sp. lycopersici to the toxic action of methylisothiocyanate (MIT). Fungal spores from sulfur-deficient cultures were resistant to, and in some cases were even stimulated by MIT. High levels of sulfur in the growth medium either as organic or inorganic compounds increased the cellular sulfur content, susceptibility to MIT, and generally increased uptake of MIT. However, the amount taken up was not necessarily correlated with susceptibility of the fungus to the toxicant. Similarly cellular sulfur content affected the susceptibility of F. oxysporum f. sp. lycopersici to other sulfur-containing fungicides.

When a solution of MIT was mixed with cysteine in solution, a reaction occurred causing a change in the characteristic U. V.

absorption spectrum of MIT, confirming previous work; however, no evidence of a similar reaction between MIT and methionine was noted. No spectral shift of MIT was detected when microconidia of F. oxysporum f. sp. lycopersici were incubated in MIT solutions for varying lengths of time.

Various experimental designs were employed to test the hypothesis that MIT's mode of action may involve a loss of cell membrane integrity. Cells treated with MIT showed little evidence of changes in membrane permeability.

Fusarium oxysporum f. sp. lycopersici grown in culture medium containing methionine was much less affected by treatment with MIT than when grown on high levels of cysteine or inorganic sulfur. Methionine-grown cultures were further characterized by the production of an offensive, pungent odor.

Quite striking morphological differences were noted between microconidia treated with MIT and untreated cells. The treated cells were incapable of somatic cell division, whereas untreated cells divided normally.

Inhibition of colony formation by germinated spores, morphological characteristics, and the reactivity of MIT with sulfhydryl compounds suggest that the mode of action of MIT may be related to mitotic disruption. Analogous modes of action have been proposed for a number of other isothiocyanates and thiocyanates on plant, animal and fungal cells.

By varying the carbohydrate supplied to the fungal spores after treatment with MIT, significant changes in susceptibility to MIT were observed. Fructose and gluconate in the medium reduced susceptibility to MIT relative to toxicity on a glucose-containing medium. Fructose and sucrose significantly stimulated growth of untreated cultures. The relatively high production of furans from fructose during autoclaving of the culture medium was not responsible for the stimulated growth.

The Influence of Sulfur and Carbohydrate Nutrition of *Fusarium oxysporum f. sp. lycopersici* on Resistance to Methylisothiocyanate

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INTRODUCTION

Fungicides are generally non-specific cell poisons (29) whereas other pesticides often have a highly specific action (e. g. organophosphate insecticides inhibit cholinesterase activity and triazine herbicides selectively inhibit the Hill reaction in higher plants). Nevertheless, it is important to study the mode of action of fungicides because an understanding of their action promotes intelligent use in plant disease control and aids in design of more selective toxicants for the future.

Methyliosothiocyanate (MIT) is a highly volatile compound with a broad spectrum of activity against soil fungi, insects, nematodes, and weed seeds (30, 57). MIT is a constituent of several commercial soil fumigants including Trapex and Vorlex and is the principle biocidal agent arising from the decomposition of Vapam (sodium N-methyldithiocarbamate) and Mylone (3, 5-dimethyltetrahydro-1, 3, 5-thiadiazine-2-thione) in soil (13, 58).

Studies (14, 32, 57) have suggested that MIT's reactivity with cellular thiols may account for its fungitoxicity. However, there is disagreement on the site within fungal cells where these reactions occur and on the consequences of the reactions (57, 60). Wedding

and Kendrick (60) suggested that the reaction between MIT and cellular thiols occurs on the spore surface and that this directly or indirectly, through disruption of membrane permeability, causes fungitoxicity. Goksøyr (13) demonstrated that the reaction between MIT and SH-compounds was pH dependent, and apparently occurred on the spore surface. Other reports have related toxicity of MIT to binding with sulfhydryl, amine, and alcohol groups on respiratory enzymes (30, 35, 57).

If MIT toxicity is associated with reactions between MIT and sulfhydryl compounds, then alterations in fungal sulfur nutrition should influence toxicity and uptake of MIT. An understanding of the influence of sulfur nutrition on fungal sulfur content and resistance to MIT was one of the major objectives of this study.

Preliminary studies on the action of MIT on spores of F. oxysporum f. sp. lycopersici suggested that fructose, gluconate and acetate may significantly reduce toxicity by circumventing biosynthetic reactions inhibited by MIT. Thus, a second phase of the present study was to investigate further the influence of carbon nutrition on the fungitoxicity of MIT to this fungus.

An understanding of the influence of sulfur and carbon nutrition on MIT toxicity would provide the necessary background information for the design of meaningful experiments to determine the exact

modes of action of MIT and may aid in predicting the conditions under which MIT would be most effective in plant disease control.

LITERATURE REVIEW

Sulfur Nutrition

Fungi require sulfur for growth and reproduction (26), and most can use inorganic forms (e.g. sulfate, sulfite and sulfide) (24). Fusarium lini, Baker's yeast, and a number of other fungi can reduce elemental sulfur and utilize it (59), while other fungi that are unable to use inorganic sulfur can supply their requirement from organic sulfur compounds (24).

Although many fungi utilize S-containing amino acids as a sulfur and nitrogen source, these compounds are not utilized as a sole carbon source, apparently because many fungi are unable to break the C-S bond without some other energy source (15, 61).

The metabolically important sulfur-containing compounds in fungal cells include enzymes and other proteins; the free amino acids cysteine, cystine and methionine; the tripeptide glutathione; the vitamins thiamine and biotin; and coenzyme A (24). Much of the sulfur in fungal cells is in a reduced organic form, usually R-SH, but it is also found in R-S-R' compounds such as methionine and thiamine, and as R-S-S-R' compounds (e.g. cystine) (5).

The sulfur content of fungal cells varies with the amount of sulfur in the growth medium and may be influenced by the nitrogen: sulfur ratio (24). Fungal species vary greatly in their sulfur

content from a low of 0.11% in Rhodotorula gracilis (24) to a high of 19.6% in Trametes suaveolens (26).

The sulfur requirement of Fusarium spp. in a chemically defined medium is usually satisfied by including an inorganic sulfate (19, 28, 34). Growth of F. oxysporum f. cubense was stimulated ten-fold over sulfur-deficient controls when $MgSO_4 \cdot 7H_2O$ was included in the medium at concentrations of 0.03, 1.0 and 3.0 g/l (2). The sulfur requirement of F. oxysporum f. sp. lycopersici in defined culture media is usually satisfied by $MgSO_4 \cdot 7H_2O$ (15, 19).

The Mode of Action of Fungitoxic Sulfur Compounds

Many fungicides contain sulfur in one form or another ranging from simple compounds (e.g. elemental sulfur and CS_2) to compounds with relatively complex structures such as captan (N-trichlormethyl-thio-4-cyclohexene-1,2-dicarboxyamide) and the dithiocarbamates. Elemental sulfur was probably the first fungicide used by man and it is still used to some degree but its fungicidal mode of action is still not fully understood (59).

Toxicity of several sulfur-containing fungicides is thought to be due to their reaction with essential sulfur metabolites in fungal cells (27, 36). Captan, for example, reacts through its $-SCCl_3$ group with cellular thiols such as coenzyme A, cysteine, and glutathione This reaction is probably responsible for the inhibition of a wide

range of metabolic processes in fungal cells treated with captan, including inhibition of citrate synthesis by oxidation of the thiol of coenzyme A, and the inactivation of enzymes with functional sulfhydryl groups (36, 37). Furthermore, the uptake of captan by fungal spores appears related to the cellular thiol contents. An increase in cellular thiols is correlated with increased captan uptake (49). Electron photomicrographs of captan-treated spores suggest that captan binds with the sulfhydryl rich proteins in the nucleus (47).

Folpet (N-trichloromethylthio phthalimide) an analog of captan, binds with several enzymes of the glycolytic pathway in Saccharomyces pastorianus (54) probably by combining with the thiol, amino, or hydroxyl groups of the enzymes. In some cases the addition of excess glutathione to spore suspensions in Folpet solutions resulted in the liberation of Folpet from the spore (54). The phthalimide moiety of Folpet was rapidly decomposed by S. pastorianus and thus could not have been the toxiphore of Folpet.

The chelation of metal ions by dialkydithiocarbamate fungicides involves the sulfur atoms in the dithiocarbamate molecule as a portion of the ligand (57). Chelation may be involved in fungitoxicity in one of several ways. Chelation can rob the fungus cell of needed metal ions, however the addition of excess metal ions to inhibited cells seldom reverses the toxic effects of chelating agents (17). The metal chelate may be toxic itself, as appears to be the case in

bacteria treated with 8-hydroxyquinoline (17). Chelating agents may combine with metals inside fungal cells and there disrupt cellular function by inhibiting metal containing enzymes (57). Smale (56) concluded that fungitoxicity of sodium dimethyldithiocarbamate (Na-DMDT) is influenced by prior treatment of fungal spores with solutions of soluble metal salts. At low Na-DMDT concentrations a toxic 1:1 complex forms between metal ions and Na-DMDT. As the concentration of fungicide increases a 1:2 complex forms which is less toxic and results in an inversion zone of decreasing fungitoxicity. As the concentration of Na-DMDT is increased even further, complexes form between Na-DMDT and other metal ions which are toxic and result in a second region of inhibition in the dosage response curve.

The decomposition of various dithiocarbamates to their corresponding isothiocyanates is well documented (57), and the importance of this conversion for the fungitoxicity of the ethylenebisdithiocarbamates was suggested by Klŏpping and van der Kerk (21). The reactivity of the diisothiocyanates with sulfhydryl groups of essential enzymes is probably the primary mode of fungitoxic action of these compounds (57). Owens (35) and Rich and Horsfall (46) doubt that the action of dithiocarbamates can be attributed to their conversion "in situ" to isothiocyanates. In some instances the corresponding isothiocyanate is less toxic to test fungi than is the parent

dithiocarbamate (46). Thorn and Ludwig (57) suggest that additional work with cell-free systems is needed to resolve this conflict, but inhibition of isolated enzyme systems may not be indicative of the action of fungicides within fungal cells. Studies with isolated enzyme systems for example, do not account for factors involved in uptake and detoxification within the fungal cells.

The decomposition of Vapam and Mylone to MIT and a number of other related compounds in soil has been demonstrated (13, 58). The antifungal potential of MIT was recognized in the early 1950's when Klöpping and Van der Kerk (21), using a roll culture technique, determined the antifungal spectra of a number of dithiocarbamates and isothiocyanates. The assay employed two plant parasitic fungi, Botrytis cinerea and Penicillium italicum and two saprophytic fungi, Aspergillus niger and Rhizopus nigricans. Classification of the compounds by their antifungal spectrum was based on the relative resistance of the four fungi to the various compounds. MIT was originally classified in group II-III (20) based on its effectiveness against B. cinerea and P. italicum and low toxicity to A niger and R. nigricans. Later MIT was reclassified without comment and placed in group III-IV indicating nearly equal sensitivity of all four test fungi (20). Kotter (22) determined the effect of MIT on 36 fungal species and concluded that in general saprophytic fungi were more resistant to MIT than were parasitic species.

MIT inhibits keto acid metabolism of Neuospora sitophilia without affecting citrate synthesis or its metabolism (35). The dithiocarbamate fungicides tested either inhibited citrate synthesis (ferbam and thiram) or caused citrate to accumulate in the treated cells (ziram, nabam and maneb).

The action of MIT is often contrasted with its related dithiocarbamate, sodium N-methyldithiocarbamate. With Rhizoctonia solani, both compounds inhibit glucose metabolism in the intact mycelium, probably by different modes of action (60). Wedding and Kendrick (60) suggested that MIT did not inhibit an active enzyme as Goksøyr (14) suggested, but they rather believed that MIT reacted with some sulfhydryl constituent on the surface of the spore, producing a physical disruption of the plasma membrane. They felt that this disruption of the membrane caused either a loss of essential cell compounds or allowed the entrance of other toxic materials. The lack of evidence to support these speculations was pointed out by Munnecke (32).

In a later study, Goksøyr (13) again suggests that MIT toxicity is due primarily to its reaction with cellular thiols. The reaction of glutathione and cysteine with MIT was demonstrated by changes in the U.V. absorption spectrum of MIT, and an apparent reaction of MIT with intact cells of Saccharomyces cervisiae was noted.

Many aspects of the fungicidal mode of action of MIT are still

unclear and in many cases published results are conflicting. The mode of action of MIT has been ascribed to a number of factors including disrupting cell permeability, altering or inhibiting glucose metabolism, and binding with various cell components.

Carbon Nutrition

Fungi are heterotrophic and hence require an exogenous source of preformed carbon and nitrogen compounds for growth and reproduction, but there is an immense diversity in the type of organic compounds required. Among the sugars, glucose is readily utilized by most fungi, but the few species that cannot grow on glucose can use other sugars, e.g. Ustilago spp. and Leptomitus lacteus (26). Most fungi use fructose and mannose equally as well as glucose (7), and optimum growth is not always obtained with glucose as the carbon source, for example, Stereum gausapatum grows better on fructose (24).

Pentoses, sugar alcohols, oliogosaccharides and polysaccharides can serve as carbon sources for many fungi (24, 26, 40). In some cases carbon compounds other than carbohydrates can satisfy the carbon requirements for fungal growth, e.g. amino acids, organic acids and alkaloids (40).

Studies on carbon nutrition of Fusarium species are numerous (12, 34, 61). The growth response of F. avenaceum, F. culmorum,

F. equiseti, F. graminearum and F. nivale to different carbon sources has been determined (23) and only F. nivale grew better on fructose than on glucose. Growth on sucrose by F. avenaceum surpassed growth on glucose.

The growth response of F. oxysporum var. nicotianae was studied on 24 different carbon sources (61). Growth on xylose, fructose, mannose, maltose, cellobiose, sucrose, trehalose, raffinose and melitriose surpassed growth on an equivalent amount of glucose. F. oxysporum f. cubense grows equally well on glucose, fructose, L-arabinose, xylose, mannose, sorbitol, sucrose and maltose (2). Carbohydrate polymers such as xylan and pectin similarly support good growth of F. oxysporum f. cubense, but many Fusarium oxysporum formae utilize inulin poorly (39). The biochemical mechanisms for carbohydrate utilization in Fusarium spp. have been reviewed (34).

Fusarium lycopersici converts xylose readily into fatty acids but grows better on fructose than on glucose or xylose (12). Fusarium oxysporum f. sp. lycopersici grows on a number of pectic substances as well as glucose and galacturonic acids (38).

Fusarium spp. may utilize many other compounds as a sole carbon source. Fusarium oxysporum f. cubense, for example, grows on many amino acids, but not cysteine and methionine (2). Fusarium lycopersici can utilize acid-hydrolized casein as a carbon source (25).

The utilization of amino acids as the sole carbon source by F. oxysporum var. lycopersici was studied by Gottlieb (15), and growth was obtained on 20 of the 26 amino acids tested. None of the sulfur-containing amino acids supported growth, nor did tyrosine or creatinine. Fusarium spp. are also capable of utilizing cyanide as a carbon and nitrogen source (34).

Sterilization of culture media by autoclaving may cause the breakdown of sugars in the media to furan compounds (7, 33). Ketose sugars form furans, primarily 5-hydroxymethylfurfural (5-HMF), more readily than do aldose sugars (33). Furan type compounds can be either inhibitory or stimulatory to fungal growth (7).

The carbon nutrition of most fungi is poorly understood. The bulk of the studies were carried out before 1940 when sophisticated instrumentation was lacking (34). Lilly (24) comments that much of the work done on carbon nutrition is not of a critical nature and encourages more detailed studies on carbon nutrition. Cochrane (7) suggests that additional studies are warranted with species of the same genus but from different habitats.

Carbon nutrition influences the susceptibility of Colletotrichun phomoides to the sulfur-containing fungicide sodium-pyridinethione (50). The fungus was most susceptible to pyridinethione when grown on a medium containing sucrose or lactose but was most resistant when grown on xylose or glucose-containing media Preliminary

studies in our laboratory indicated that the sensitivity of F. oxysporum f. sp. lycopersici to MIT can be influenced by the sugar used in the medium.

GENERAL METHODS AND MATERIALS

Culture Media

The standard chemically defined liquid medium used throughout this study contained the following ingredients: 25 g glucose, 1.25 g KNO_3, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.31 g K_2HPO_4, 0.94 g KH_2PO_4, 10 mg $FeCl_3 \cdot 6H_2O$, 4 mg $ZnSO_4 \cdot 7H_2O$, 2.5 mg H_3BO_3, 1.0 mg $CaCl_2$, 0.9 mg $MnCl_2$, 0.275 mg $CuCl_2$, 0.01 mg MoO_3, and distilled water to make one liter. The media were prepared in two parts. One part contained the bulk of the mineral salts, glucose and half of the distilled water, while the second part contained the phosphates and the remaining water. The two portions of the medium were autoclaved separately and combined prior to use. If other materials such as vitamins or amino acids were added, they were filter sterilized (Millipore HA filter) and added to the medium at the same time as the phosphate. Difco "Bacto" agar was added to the medium at 2% (w/v) to solidify it. All media were adjusted to pH 6.0.

Potato dextrose agar (PDA) was prepared from 200 g potatoes, 20 g dextrose, 20 g agar and tap water to make one liter.

Maintenance of *Fusarium oxysporum* f. sp. *lycopersici* in culture

Cultures of *F. oxysporum f. sp. lycopersici* (Sacc.) Snyd. and

Hans. Race 5-6 were maintained on PDA slants and transferred every 30 days. The inoculum for liquid shake cultures was obtained by first transferring a small portion of a fungal colony from a slant culture to the center of PDA in a petri plate. Four to six days later 7 mm plugs were cut from the periphery of the growing colony and the plugs were placed in either 200 ml of medium in 500 ml Kjeldahl flasks on a wrist action shaker, or in 100 ml of medium in 250 ml Erlenmeyer flasks on a rotary shaker. Liquid shake cultures were grown at 18-23 C under approximately 16 hours of subdued light and 8 hours of darkness.

Fungitoxicity of MIT

Toxicity tests were conducted with spores (microconidia) collected from fungal cultures grown four days in liquid medium on a wrist action shaker. The cultures were filtered through four layers of cheesecloth, and then through Whatman #4 filter paper. The uniform microconidial suspension in the filtrate was adjusted to the desired concentration by dilution with sterile distilled water. The spores in 50 ml aliquots of the suspension were collected on 1.2μ Millipore filters, then resuspended in 50 ml of sterile distilled water or solutions of MIT ($CH_3N=C=S$, furnished by Nor Am Agricultural Products, Inc.), and finally incubated on a wrist action shaker for two hours. Following the incubation period the spores were

collected on 1.2μ Millipore filters then suspended in 50 ml of sterile distilled water, and diluted with sterile distilled water until a concentration of 40,000 spores/ml was reached. One ml of this spore suspension was plated on PDA in petri plates, and the number of germinated spores in 100 was determined after 16 hrs. incubation. Further dilutions of the spore suspension were made until a concentration of about 100 spores/ml was achieved. This spore suspension (0.5 ml) was then plated on PDA in petri plates, the plates were incubated for 3-4 days, and then the Fusarium colonies were counted. Results were expressed as percent inhibition based on distilled water controls.

Estimation of Fungal Growth

The mycelium and spores from shake cultures were collected on tared 0.8μ Millipore filters, dried at 100°C for 24 hours, and reweighed to determine dry weight.

Linear growth was estimated in Ryan growth tubes (26). Chemically defined culture medium was added to the sterile tubes to a thickness of 5-7 mm. After the medium had solidified, a 7 mm disk of inoculum was placed at one end of the medium and linear growth was measured daily. Results were expressed as the mean growth rate in mm/day.

Experiments designed to test the efficiency of various carbon

sources in promoting colony formation were conducted in an identical manner to the procedure described in the previous section except a two hour distilled water wash was substituted for chemical treatment and the spores were placed on chemically defined medium with various carbon sources rather than PDA. Carbohydrate determinations were made using a phenol-H_2SO_4 reagent (10). Appropriate blanks and controls were prepared for each determination.

RESULTS

The Effect of Sulfur Nutrition on the Fungitoxicity of MIT

The high reactivity of MIT with thiols (35) suggests that fungitoxicity of MIT may vary with the sulfur content of fungal cells. To test this hypothesis, F. oxysporum f. sp. lycopersici was grown on the defined shake culture medium, containing various levels of sulfur. A low sulfur medium was prepared by substituting $MgCl_2$ and $ZnCl_2$ for $MgSO_4$ and $ZnSO_4$ respectively in the basic medium, thus reducing the sulfur content to less than 1.0 gm S/liter. A completely sulfur-free medium is impractical because traces of sulfur are necessary to maintain fungal growth (19).

Media containing sulfur at concentrations higher than those normally used were prepared by adding either Na_2SO_4, cysteine, or methionine to the medium. A medium with an amount of sodium (NaCl) equivalent to that supplied in Na_2SO_4 was included to discount any interference of sodium ions on the fungitoxicity of MIT.

Spores from cultures grown on different levels of inorganic sulfur were exposed to MIT two hours and then they were plated on nutrient medium. Spore germination and colony formation were subsequently recorded.

As the inorganic sulfur in the medium was increased, F. oxysporum f. sp. lycopersici generally became more susceptible to MIT

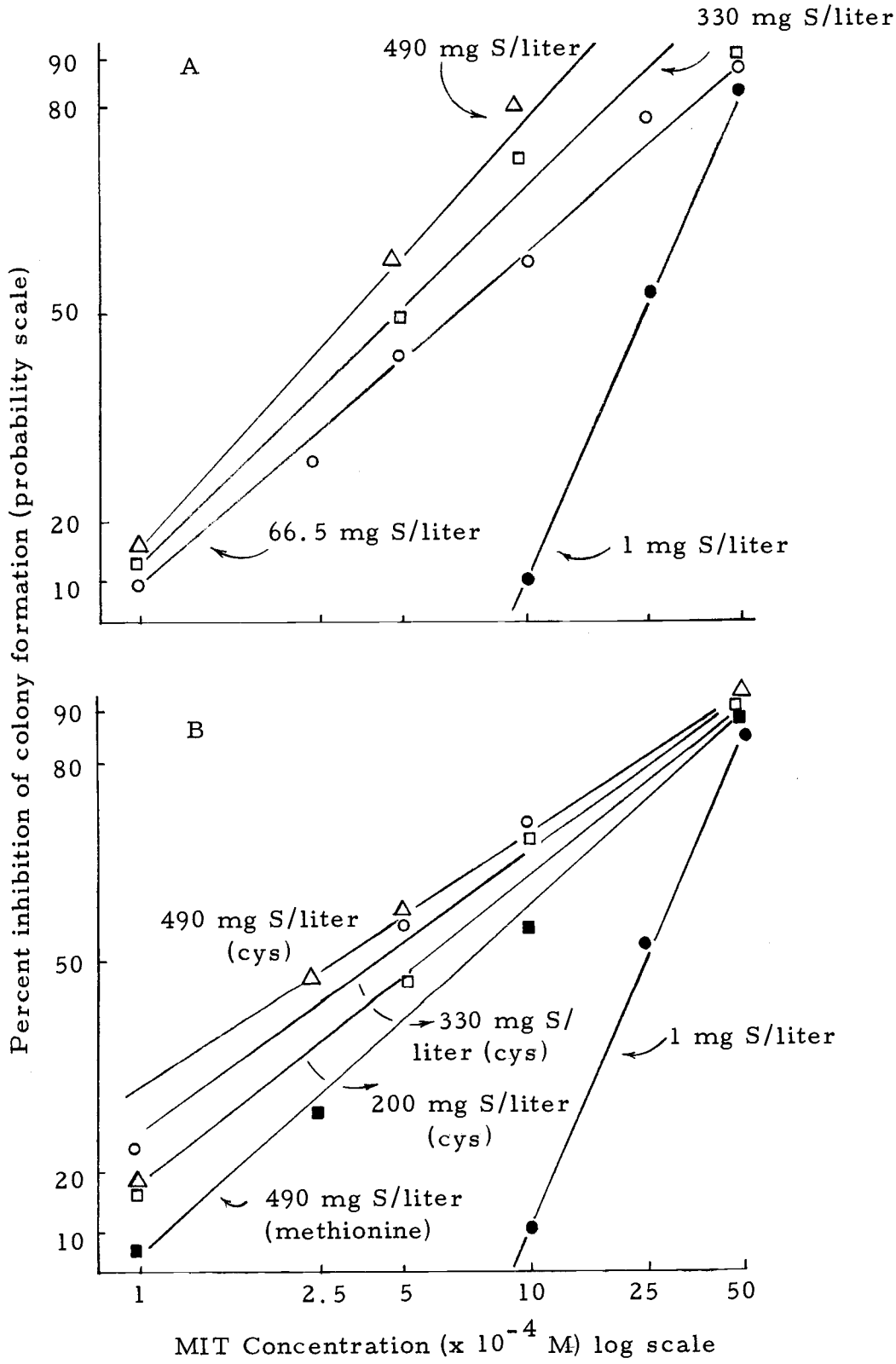
(Figure 1A). The slope of the dosage response lines indicates less variation in susceptibility to MIT within the spore population at the lowest sulfur level.

Microconidia from cultures supplied with cysteine or methionine in addition to 66.5 mg/liter sulfur as sulfate were similarly assayed with MIT.

The dosage response lines of microconidia supplied with excess sulfur as cysteine (Figure 1B) were similar to those obtained with spores from cultures with excess sulfur as sulfate (Figure 1A), but they were slightly flatter than the one obtained with spores supplied with sulfur as methionine. Microconidia from sulfur-deficient cultures are relatively resistant to MIT, and generally as the sulfur level in the medium is increased, susceptibility to MIT increases. However, microconidia from methionine-supplied cultures are more resistant to MIT than microconidia from cultures supplied with equivalent sulfur as cysteine or sulfate. At higher MIT concentrations, colony formation is uniformly inhibited regardless of the sulfur content of the media.

At all MIT concentrations, spore germination was less affected by the toxicant than was colony formation. The dosage response lines for inhibition of spore germination had a similar slope over a range of sulfur levels (Figure 2), and were similar to those for colony formation except for the line of inhibition of colony formation by

Figure 1. The influence of (A) inorganic and (B) organic sulfur nutrition on the subsequent inhibition of colony formation by microconidia of F. oxysporum f. sp. lycopersici treated with MIT.


A
B
330 mg S/liter
490 mg S/liter
66.5 mg S/liter
1 mg S/liter
490 mg S/liter (cys)
330 mg S/ liter (cys)
200 mg S/liter (cys)
490 mg S/liter (methionine)
90
80
50
20
10
1
2.5
5
10
25
50
Percent inhibition of colony formation (probability scale)
MIT Concentration (x 10^{-4} M) log scale

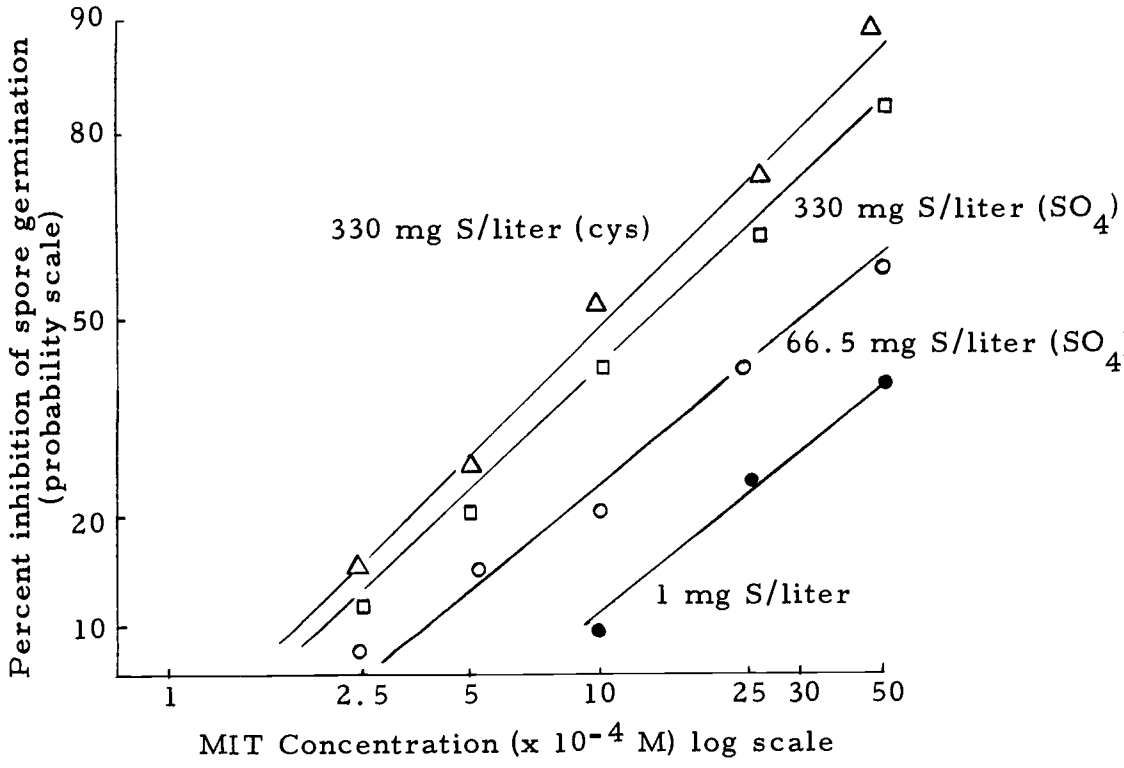


Figure 2. The influence of inorganic and organic sulfur on inhibition of spore germination of F. oxysporum f. sp. lycopersici treated with MIT.

spores from the sulfur-deficient medium.

Fungal Cell Sulfur Content and MIT Toxicity

Microconidia from cultures supplied with different levels and types of sulfur compounds were assayed for sulfur content to determine if a correlation existed between sulfur nutrition, sulfur content of spores, and susceptibility to MIT. Microconidia from cultures of F. oxysporum f. sp. lycopersici growing on different levels and types of sulfur compounds were collected, washed and dried at 100°C for 24 hours. The sulfur content of the spores was determined in a Leco Sulfur Analyzer following oxygen combustion of the sample in an induction furnace and titration of the SO_2 in an acidic starch-KI solution. The amount of titrant (KIO_3) required to maintain the endpoint in the starch-KI indicator solution for the samples was compared to that for cysteine and cystine standards.

As the sulfur in the culture medium was increased, the sulfur content of the spores generally increased (Table 1). Microconidia from cultures supplied with sulfate and cysteine had a significantly higher sulfur content than microconidia from cultures supplied with equivalent amounts of sulfur as sulfate only. Sulfur nutrition directly influences the level of sulfur content of the spores, and since MIT susceptibility is correlated with sulfur nutrition, MIT toxicity may be influenced by the sulfur content of the spores.

Table 1. The influence of sulfur nutrition on the sulfur content and amount of titrateable surface sulfhydryl groups on microconidia of F. oxysporum f. sp. lycopersici.

Sulfur source	Total sulfur content of medium (mg/liter)	Sulfur content of microconidia (% dry weight)	Surface sulfhydryl content in μ moles cysteine equivalents
Sulfate	1.0	0.5	20
Sulfate	66.5	0.7	62
Sulfate	330	0.8	65
Sulfate	490	0.9	75
Sulfate 66.5 mg/liter cysteine (0.05% w/v)	200	0.8	55
Sulfate 66.5 mg/liter cysteine (0.10% w/v)	330	1.4	69
Sulfate 66.5 mg/liter cysteine (0.15% w/v)	490	2.2	84

Determination of Titrateable Surface Thiols

A significant amount of the sulfur content of fungal spores is in the sulfhydryl groups of compounds on the exterior surface of the spores and it has been suggested that these compounds play a role in the uptake and toxicity of fungicides (32). To determine the surface sulfhydryl content of spores, a modification of the method of Hess and Sullivan was used (16). Spores from F. oxysporum f. sp. lycopersici cultures grown on various levels of sulfur were collected, washed three times by centrifugation, and finally suspended in distilled water. The spore suspension was adjusted to 5.0×10^6 spores/ml and concentrated HCl was added to bring the HCl concentration to 2% (w/v). Ten ml aliquots were withdrawn and 10 ml of HCl (4% w/v) and 10 ml of aqueous KI (5% w/v) were added to each sample. The samples were then titrated to a permanent yellow color with a 0.06 M KIO_3 solution. The amount of KIO_3 required to obtain a permanent yellow color in each of the samples was compared to a standard curve obtained with cysteine and the results expressed as cysteine equivalents in μ moles.

As was the case with total sulfur content, surface thiols increased as the sulfur levels in the growth medium increased (Table 1). Uptake and toxicity of MIT may be dependent on a reaction of MIT with exposed thiols at the spore surface (13, 57, 60). Decreased toxicity of MIT at the higher sulfur levels where there would be increased

surface thiols, however, is not consistant with this suggestion.

MIT Uptake as a Function of Spore Sulfur Content

The uptake of MIT by spores from cultures of different sulfur content was determined by spectrophotometric measurements of the MIT content of solutions in which known quantities of spores had been incubated. After a two hour incubation period, the spores were removed by filtration through a Millipore filter (1.2μ) and a sample of the filtrate was assayed for MIT. A fresh suspension of spores was added to the original MIT solution and this procedure was repeated every 2 hours during the course of the experiment. MIT solutions without spores were assayed every two hours to correct for the loss of MIT by volatilization. The spores collected from the treatment solutions were plated on PDA to determine their ability to form colonies.

Microconidia from sulfur-deficient and high sulfur media took up significantly more MIT than spores produced on an intermediate level of sulfur (Table 2). MIT uptake is probably not linked to metabolic activity because heat-killed spores took up as much or more MIT than the viable cells. The general assumption that increased uptake of MIT should lead to increased toxicity (e.g. increased inhibition of colony formation) appears unwarranted in that sulfur-deficient spores take up relatively large amounts of MIT and are not

Table 2. The influence of sulfur nutrition on MIT uptake and fungitoxicity to microconidia of *F. oxysporum f. sp. lycopersici*.

Culture Type	Spore condition[a]	Accumulative MIT uptake (μg)	Percent inhibition of colony formation by MIT (5×10^{-4} M)
Low Sulfur (1.0)	viable	49	10% (stimulation)
	heat killed	45	---
Intermediate Sulfur (66.5)	viable	19	38
	heat killed	22	---
High Sulfur (330)	viable	51	66
	heat killed	65	---

[a] Both heat-killed and viable spores were used to determine if metabolic activity was a requirement for toxicant uptake.

inhibited. This experiment indicates that the relationship between uptake and fungitoxicity of MIT can be greatly influenced by the previous sulfur metabolism of the spores. The loss of membrane integrity and the subsequent increased uptake and toxicity of MIT suggested by Wedding and Kendrick (60) as the main action of MIT is not therefore a valid hypothesis for MIT action at least with sulfur-deficient cells. Surface sulfhydryl groups probably are not required for MIT uptake, because sulfur-deficient spores have a low surface sulfhydryl content, high MIT uptake, but low susceptibility to MIT.

MIT Stimulation of Sulfur-Deficient Cultures

An experiment was designed to determine if sulfur-deficient cultures were generally stimulated by toxic concentrations of sulfur-containing fungicides. Fusarium oxysporum f. sp. lycopersici was grown on a sulfur-deficient medium in shake culture for four days to allow utilization of the sulfur contaminants in the medium and the sulfur introduced with the inoculum plug. On the fourth day, MIT, carbon disulfide, captan, and $MgSO_4$ as a control were added to the cultures under sterile conditions and growth was measured daily by determining the increase in dry weight of the cultures.

In a companion experiment the influence of MIT and captan on growth of the fungus on normal sulfur media was determined. The fungicides were added 36 hr after initiation of the experiment which

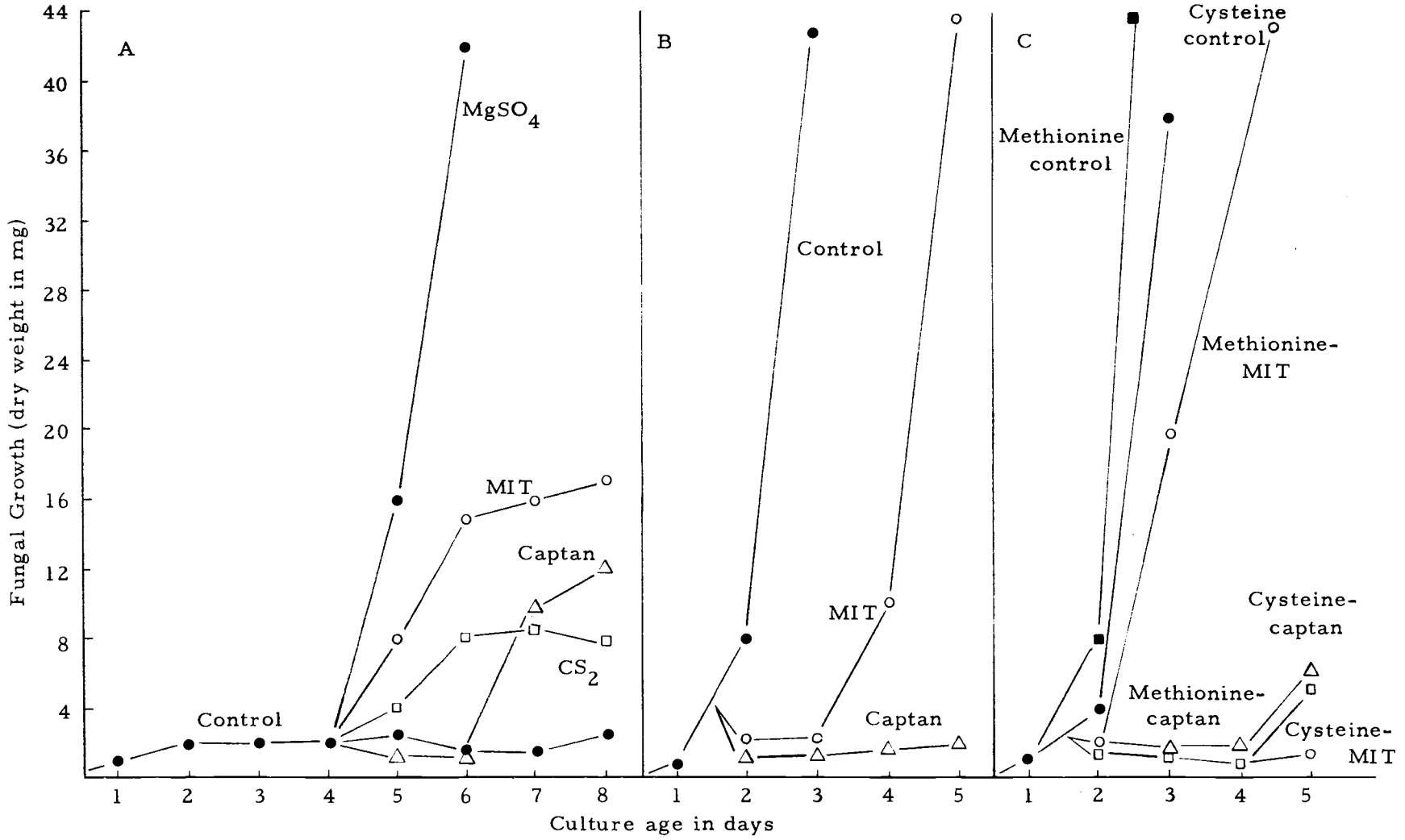
gave about the same fungus to toxicant ratio as in the experiments with sulfur-deficient cultures. The viability of the cultures was monitored by periodically removing small samples which were streaked on the surface of PDA in petri plates.

Stimulation of the sulfur-deficient cultures by normally toxic concentrations of sulfur-containing fungicides (Figure 3A) indicates that the sulfur content of fungal cells may generally influence the susceptibility of the spores to sulfur-containing fungicides and that the phenomenon is not unique to MIT. When deficient in sulfur, the spores may be able to detoxify the fungicides to supply their sulfur requirement.

Fungal growth in cultures supplied with normal sulfur was completely inhibited by captan (Figure 3B), but the cultures were capable of growth when removed from the captan-containing medium. MIT surpressed growth for about two days but after that growth was similar to the control. Resumption of growth in this case was probably due to loss of MIT from the culture by volatilization.

Cultures grown on excess sulfur as cysteine or methionine were assayed for inhibition by captan and MIT in a similar manner. Growth in cysteine-supplied cultures was almost completely inhibited by both MIT and captan as was growth in methionine cultures treated with captan (Figure 3C). Fungal cells removed from these cultures grew in fresh medium. MIT only briefly supressed fungal growth in

Figure 3. Growth of F. oxysporum f. sp. lycopersici in shake media with various sulfur levels and fungicides added at 10^{-4} M. A) Sulfur-deficient medium with $MgSO_4$ and the fungicides added on the 4th day following initiation of the experiment. B) Normal sulfur (66.5 mg SO_4/liter) with the fungicides added 36 hr after initiation of the experiment. C) Excess sulfur with the fungicides added 36 hr after experiment initiation.


A
B
C
Fungal Growth (dry weight in mg)
Culture age in days
Control
MgSO4
MIT
Captan
CS2
Control
MIT
Captan
Methionine control
Cysteine control
Methionine-MIT
Cysteine-captan
Methionine-captan
Cysteine-MIT

cultures supplied with excess sulfur as methionine as it did in cultures that had received normal sulfur nutrition. The failure of cysteine-supplied cultures to show this phenomenon may be due in part to the reaction of MIT with cysteine (13) to form a relatively nonvolatile product.

The Reaction of MIT with Cellular Sulfur Compounds

The reaction of MIT with the sulfhydryl group of cysteine and cellular thiols of Saccharomyces cerevisiae has been established (13), and thus, experiments were designed to determine if similar reactions could be obtained with F. oxysporum f. sp. lycopersici. A solution of MIT (7.5×10^{-4} M) and cysteine-HCl (7.5×10^{-3} M) was adjusted to pH 6.5 with 0.1 NaOH. The reaction between MIT and cysteine was indicated by a shift in the ultraviolet absorption spectrum (Figure 4). When microconidia from cultures deficient in sulfur, with normal sulfur, or with excess sulfur were incubated for two hours with the reaction products of MIT and cysteine no fungitoxicity could be detected (Figure 5).

Methionine was tested for reaction with MIT by a method identical to that used for cysteine. No detectable reaction product was formed with methionine over the pH range of 4.5 to 7.5. The failure of methionine to react with MIT may explain the loss of toxicity in MIT-methionine cultures from which the MIT may have escaped by volatilization.

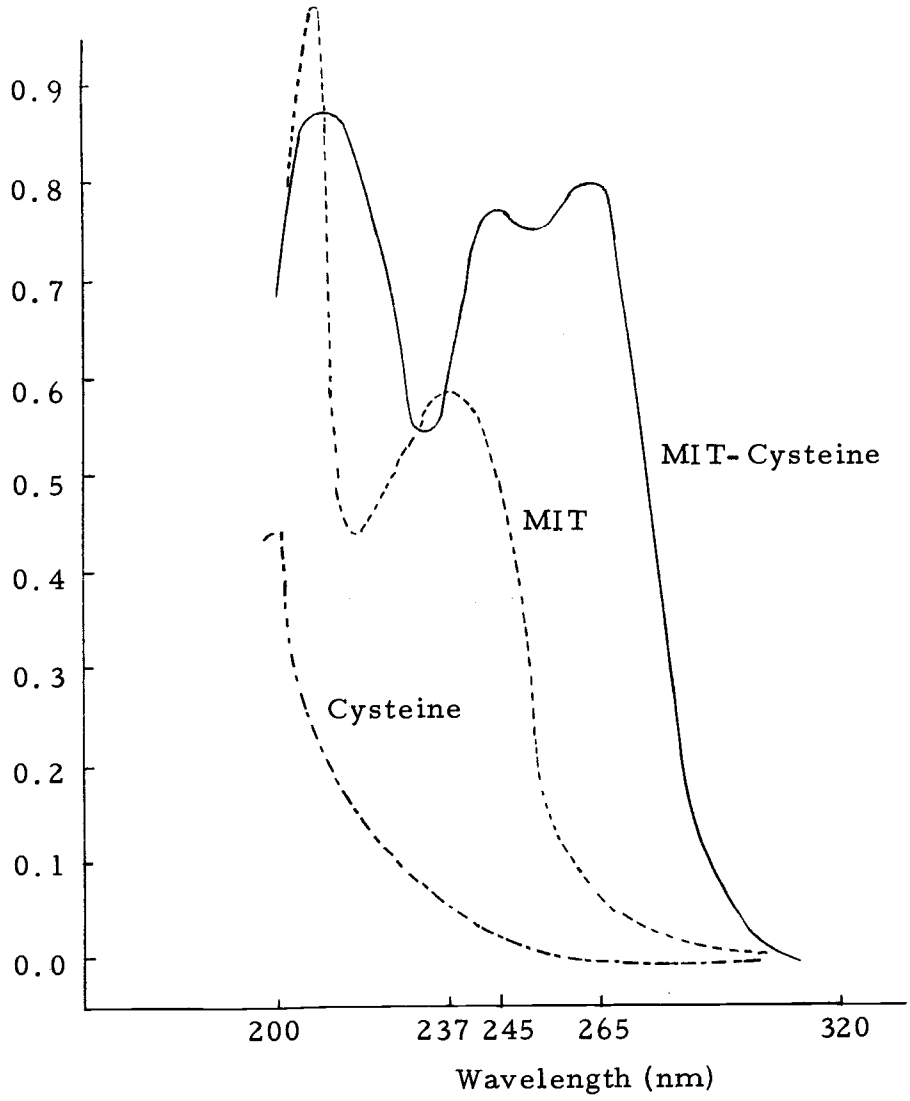


Figure 4. Ultraviolet spectra of MIT, cysteine-HCl, and MIT-cysteine reaction mixture at pH 6.5.

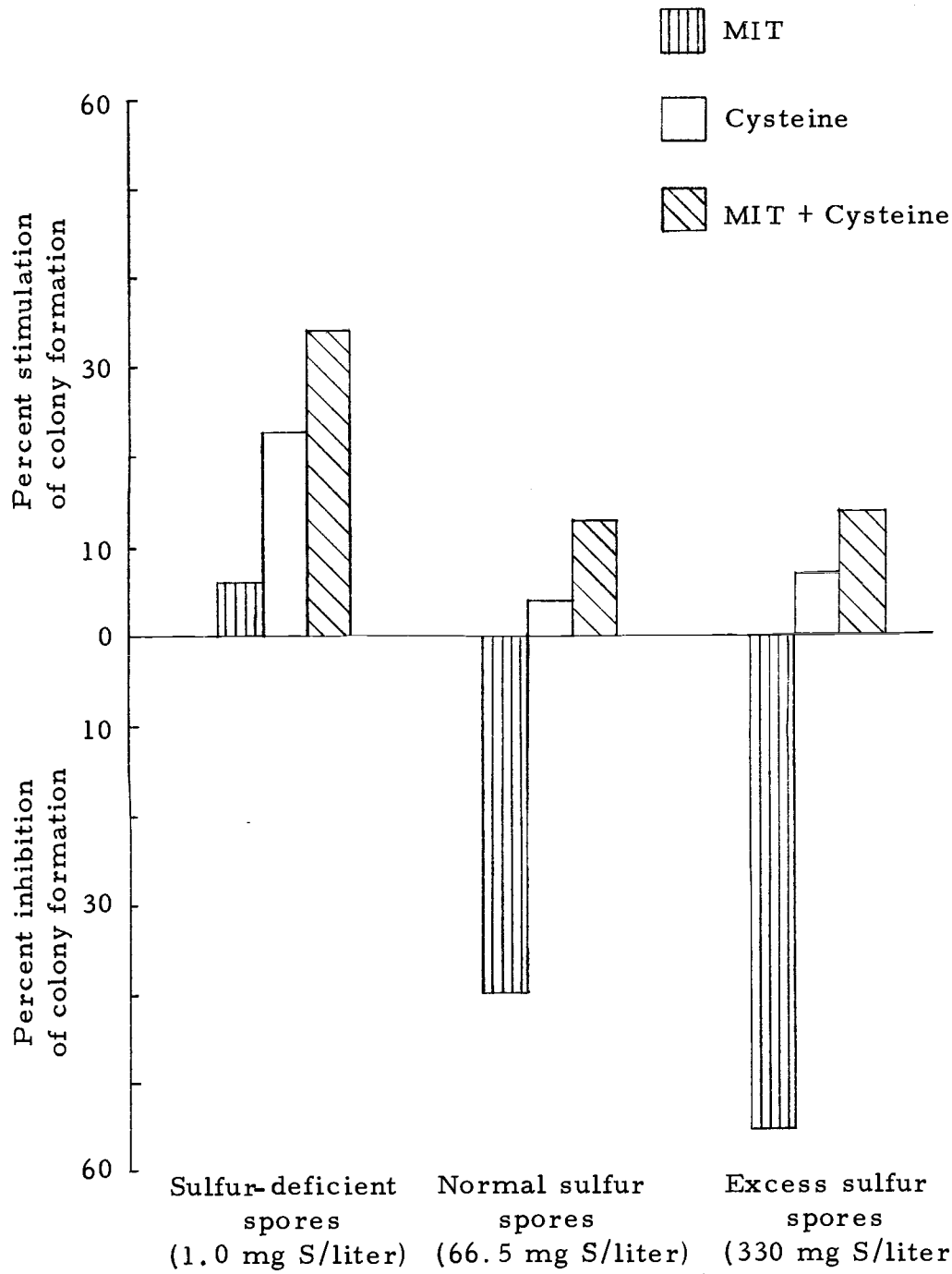


Figure 5. The influence of MIT, cysteine and an MIT-cysteine mixture on colony formation by microconidia of F. oxysporum f. sp. lycopersici grown on media with deficient, normal and excess sulfur.

Goksøyr (13) demonstrated a spectral shift in MIT solutions when incubated with cell suspensions of S. cervisiae at pH 6.5. Microconidia of F. oxysporum f. sp. lycopersici were similarly incubated with unbuffered MIT solutions (pH 5.0 - pH 5.7) for 2 min, 1, 18, and 22 hr but no spectral shifts were detected, and thus, the Fusarium spores probably did not react with MIT as the yeast cells did.

Spore Membrane Permeability and MIT Toxicity

The disruption of spore membranes by MIT has been suggested as a mode of toxic action (60). Membrane disruption could facilitate uptake of toxic quantities of MIT, as well as allow loss of vital metabolites from the damaged spores. To test this possibility, the ambient solutions surrounding microconidia treated with MIT were analyzed. Ion leakage was monitored by measuring the conductivity of the ambient solution (51), and analyses were made for carbohydrates by the phenol-H_2SO_4 test (10) and for amino acids by the ninhydrin method. Results of the phenol-H_2SO_4 test were compared to glucose standards while those of the ninhydrin assay were compared to alanine standards.

Ion leakage was similar whether the microconidia were incubated in MIT or in distilled water (Table 3).

MIT failed to significantly influence carbohydrate leakage from treated microconidia (Table 4), but did cause a significant increase

Table 3. Electrolyte loss from MIT-treated microconidia of F. oxysporum f. sp. lycopersici.

Treatment of microconidia	Resistance in the ambient solution expressed as x 10^{-4} ohms one and two hours after treatment	
	1 hr.	2 hr.
Distilled water	12.5	27.5
MIT (1 x 10^{-3} Molar)	13.3	20.0

Table 4. The loss of metabolites from MIT-treated microconidia of F. oxysporum f. sp. lycopersici.

Treatment	Relative amounts of metabolites lost from one million spores in 4 hrs.		Percent colony inhibition
	Carbohydrate (mg)	Ninhydrin positive materials (mg)	
Distilled water control	7	2.0	0
1 x 10^{-4} M MIT	6	2.5	3
7.5 x 10^{-4} M MIT	7	3.1	23
1.0 x 10^{-3} M MIT	7	3.2	42
5.0 x 10^{-3} M MIT	7	3.0	65

in the ninhydrin positive materials lost. However, this loss was independent of increasing fungitoxicity of MIT between 7.5×10^{-4} and 5.0×10^{-3} M, and thus, disruption of fungal spore membranes as other workers have proposed (20), is probably not the major toxic action of MIT.

The Atypical Response of Methionine-Grown Cultures to MIT

Throughout this study, F. oxysporum f. sp. lycopersici cultures supplied with methionine were less susceptible to MIT than those grown on corresponding levels of other sulfur compounds, and the sulfur content of the mycelium plus spores was significantly higher when methionine was present in the growth medium (Table 5). However, when spores only were analyzed, the sulfur content of those from cysteine cultures was highest. Thus, methionine appears to concentrate in the mycelium while cysteine accumulates more readily in the spores.

Methionine-grown cultures were further characterized by the production of an offensive, pungent odor. This odor was not noticeable from cultures supplied with other forms of sulfur. When a stream of air was passed through the methionine cultures and bubbled through 3% mercuric chloride the volatile compounds formed a precipitate characteristic of the way methanethiol, dimethyl disulfide, or dimethylsulfide form precipitates from mercuric chloride (53).

Table 5. The sulfur content of F. oxysporum f. sp. lycopersici grown on various sulfur sources at 490 mg sulfur/liter of media.

Sulfur Source	Sulfur content in percent dry wt.		ED_{50} conc.
	Mycelium and spores[1]	Spores[2]	MIT
Methionine	0.78	1.67	9.5×10^{-4} M
Cysteine	0.56	2.20	4.0×10^{-4} M
Na_2SO_4	0.28	.85	4.0×10^{-4} M

[1] Total sulfur determinations by Paar Bomb Oxygen Ignition, gravimetric sulfate. Charlton Laboratories, Portland, Ore.

[2] Induction furnace combustion and KI-KIO_3 titration.

Fusarium culmorum has been reported to release similar volatile sulfur compounds when supplied with methionine (53).

Morphological Changes of Microconidia Treated with MIT

The greater influence of MIT on colony formation than on spore germination suggests that one aspect of the action of MIT might involve disruption of cell division possibly through mitotic poisoning. This is based on the assumption that spore germination does not require mitosis while cell division and thus colony formation does.

Microconidia collected from a 4-day-old shake culture of F. oxysporum f. sp. lycopersici were treated for two hours with MIT (2.5×10^{-3} M) and plated on PDA. Microscopic observations were made periodically to detect the influence of MIT on germination and colony formation.

Treated spores germinated as rapidly as the untreated ones but were characterized by the formation of long, narrow germ tubes that were highly vacuolated, lacked septa and branches and subsequently failed to produce sporophores with conidia. The untreated spores formed germ tubes that were about equal in diameter to the spore, lacked significant vacuoles, rapidly formed numerous septa at regular intervals, became highly branched and soon began sporulating.

The nuclei of the microconidia of F. oxysporum f. sp.

lycopersici are at the limit of resolution of light microscopes (1), and thus, direct observations of nuclear division were not feasible in this study. Nevertheless, the observations of treated spores are consistent with the hypothesis that mitosis is inhibited by MIT. Mitosis as observed in Fusarium spp. by electronmicroscopy is similar to that in higher plant cells (1), and thus, MIT-induced mitotic aberations could limit somatic cell division.

The Effect of Carbohydrate Nutrition on the Fungitoxicity of MIT

Preliminary experiments with microconidia of F. oxysporum f. sp. lycopersici indicated that MIT was significantly less toxic to spores when they were incubated on fructose following treatment than when incubated on glucose as the sole carbon source.

Additional experiments were designed to determine if selected sugars and acetate uniquely affect the ability of treated spores to germinate and form colonies. F. oxysporum f. sp. lycopersici was grown on the defined medium, the microconidia were collected and treated for one hour with MIT (10^{-3} M). The spores were then plated on the defined solid media at pH 5.5 containing various carbon sources. The experiment was repeated at 0.5 pH unit intervals up to 7.5.

The microconidia were particularly susceptible to MIT when

incubated following treatment on the acetate medium below pH 7.0 (Figure 6A). On the carbohydrate substrates, optimum toxicity occurred at pH 6.0, and the microconidia were somewhat more resistant to MIT when incubated on the fructose and gluconate media than on the glucose medium. Resistance to MIT on fructose and gluconate was even more evident, however, in inhibition of colony formation at pH 6.0 and below (Figure 6B). Treated spores responded similarly on acetate and glucose media.

Untreated microconidia consistantly produced 20-50% more colonies on fructose medium than when germinated on media containing the other sugars or acetate even though the percentage germination was similar on all three substrates (95-98%). Additional experiments were designed to study the stimulatory action of fructose as a carbon source for colony formation, because of its possible association with the toxic action of MIT.

Glucose or fructose at concentrations ranging from 0.01 to 0.35 M were added to the defined solid medium in petri plates and 0.5 ml of spore suspension containing 100 spores/ml were spread over the surface of the medium. After incubation for 16 hr, the spore germination percentage was determined and 5-7 days later the number of colonies produced was counted. Sucrose and mannose at 0.05 M were tested in a similar manner.

Colony formation on the fructose-containing medium was

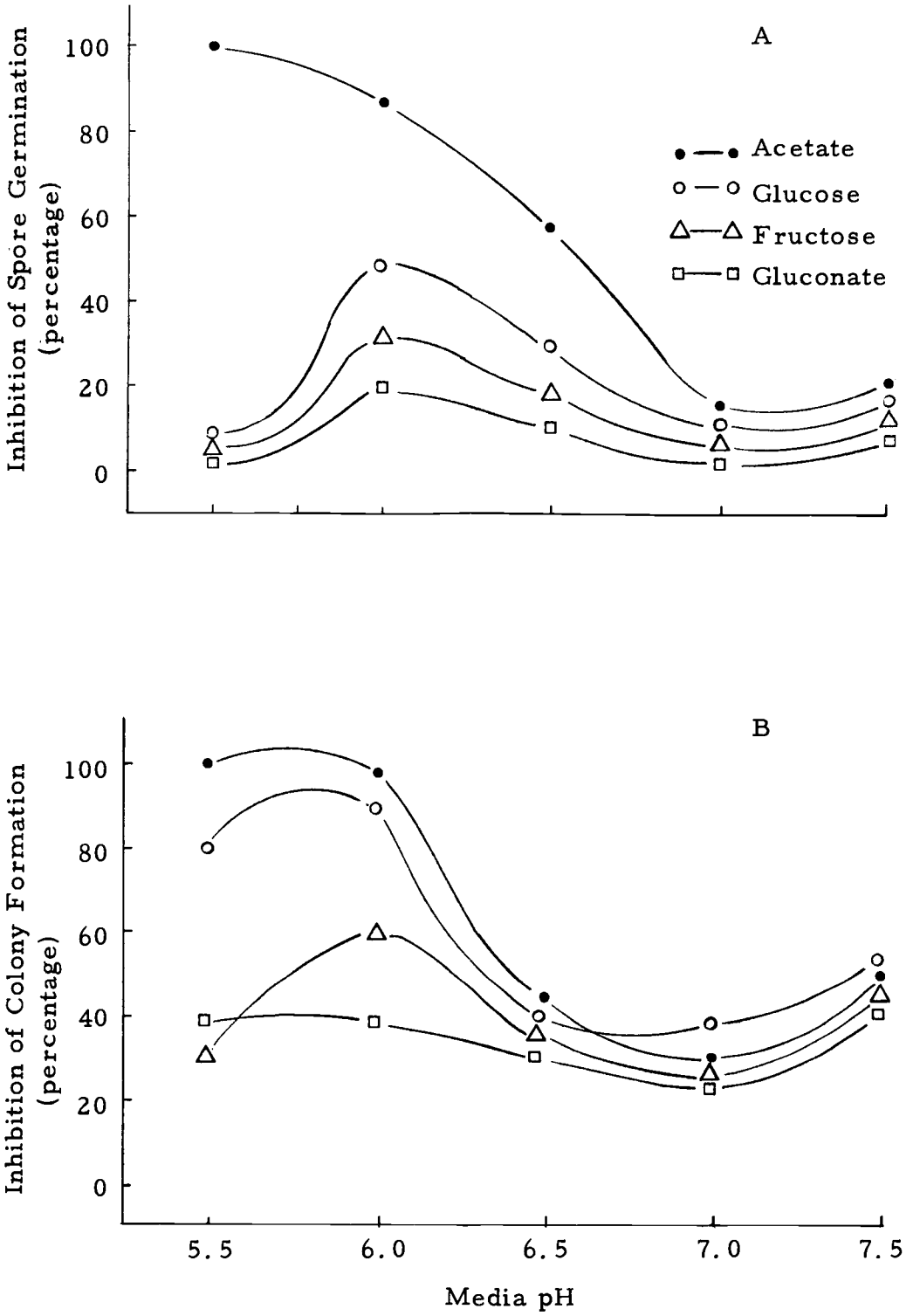


Figure 6. Inhibition of (A) spore germination and (B) colony formation by F. oxysporum f. sp. lycopersici spores treated with 1 x 10^{-3} M MIT and subsequently incubated on media at various pH's.

significantly higher than on the glucose-containing medium at concentrations of 0.01 and 0.05, but at 0.35 M there was no significant difference (Table 6). Colony formation on mannose similarly exceeded colony formation on glucose and on the sucrose medium which was similar to glucose.

Table 6. Colony formation from microconidia of F. oxysporum f. sp. lycopersici on defined media with different carbon sources.

Carbon source	Total number of colonies formed in 25 petri plates at three carbon source concentrations		
	0.01 M	0.05 M	0.35 M
Glucose	1011	933	1337
Fructose	1555	1277	1391
Sucrose	--	957	--
Mannose	--	1213	--

The stimulation of colony formation on a fructose-containing medium, suggests that fructose could similarly influence the mycelial growth rate and economic efficiency of this fungus. The growth of F. oxysporum f. sp. lycopersici in rotary shake culture on carbon sources at 0.05 M was determined by dry weight measurements four days after inoculation. Because the carbon sources were added to the medium at the same molar concentration, the actual carbon content of the media varied. Thus, an additional experiment was run

in which glucose or fructose was added to the medium at concentrations that were equivalent on a carbon basis to the other carbon sources.

The fructose-containing media generally supported more growth than the media with the other carbon sources except for the sucrose-containing medium (Table 7). Growth on the glucose medium was generally poorer than on the other media.

Economic coefficients (i.e. the ratio of carbohydrate consumed to the amount of growth) were determined for shake cultures of F. oxysporum f. sp. lycopersici grown on glucose, fructose, and sucrose. Four days after initiation of the experiment, mycelium and spores were removed from the culture by filtration through a 1.2μ Millipore filter and the fungal material air dried and weighed. Carbohydrates remaining in the filtrate were determined by the previously described phenol-H_2SO_4 method (10).

Generally, growth efficiency on glucose and fructose media was similar (Table 8). Sucrose was a slightly more efficient carbon source except at the highest concentration (3.6%) where the fungus on fructose media was significantly more efficient than on the other sugars.

The mechanism by which fructose stimulates colony formation from germinated spores is not clear, especially since growth efficiency on fructose is not greatly different from that on glucose. The

Table 7. Growth of F. oxysporum f. sp. lycopersici in shake medium containing various carbon sources.

Growth (mg. dry wt.) on various carbon sources at 0.05 M		Growth (mg. dry wt.) on fructose or glucose at concentrations equivalent to the carbon content of the various carbon sources at 0.05 M.	
		Fructose	Glucose
Glucose	361	376	---
Fructose	376	---	361
Mannose	355	376	361
Sucrose	792	780	715
Acetate	79	170	152
Gluconate	330	421	408

Table 8. Growth efficiency of F. oxysporum f. sp. lycopersici on three sugars.

Sugar	Economic coefficients[a] at four sugar concentrations (percent w/v) in the media			
	0.2%	0.9%	1.7%	3.6%
Glucose	44.1	41.3	42.8	34.7
Fructose	45.5	42.5	43.1	42.2
Sucrose	47.1	44.8	45.6	35.8

[a] Economic coefficient $= \frac{\text{mycelial dry wt., gm}}{\text{sugar consumed, gm}} \times 100$

conversion of fructose to glucose and vice versa, is readily accomplished by most microorganisms but possibly spores of F. oxysporum f. sp. lycopersici fail to make this conversion particularly when treated with MIT. The reduced toxicity of MIT to microconidia plated on fructose medium is not explained by stimulation of the fungus on the fructose medium.

Furan Toxicity

Ketose sugars (e.g. fructose) form furans when heated in slightly acidic solutions more rapidly than do aldose sugars such as glucose (33), and thus, furans might account for stimulated growth on fructose-containing media. Stimulation of fungal growth by furans has been reported (7), but these compounds e.g. 5-hydroxymethylfurfural (5-HMF) usually inhibit growth on microorganisms (7, 33).

The ultraviolet absorption spectra of the unautoclaved complete growth medium and its component parts were determined and compared with the spectrum of autoclaved medium and a standard solution of 5-HMF (Aldrich Chemical Co.). Reagent grade fructose in solution has a small furan peak at 285 nm, which is not shown by a glucose solution. Autoclaving caused the formation of about three times the amount of furans in the fructose-containing medium than in the glucose medium (Figure 7).

Autoclaved media containing either fructose or glucose as the sole carbon source were inoculated with microconidia of F. oxysporum

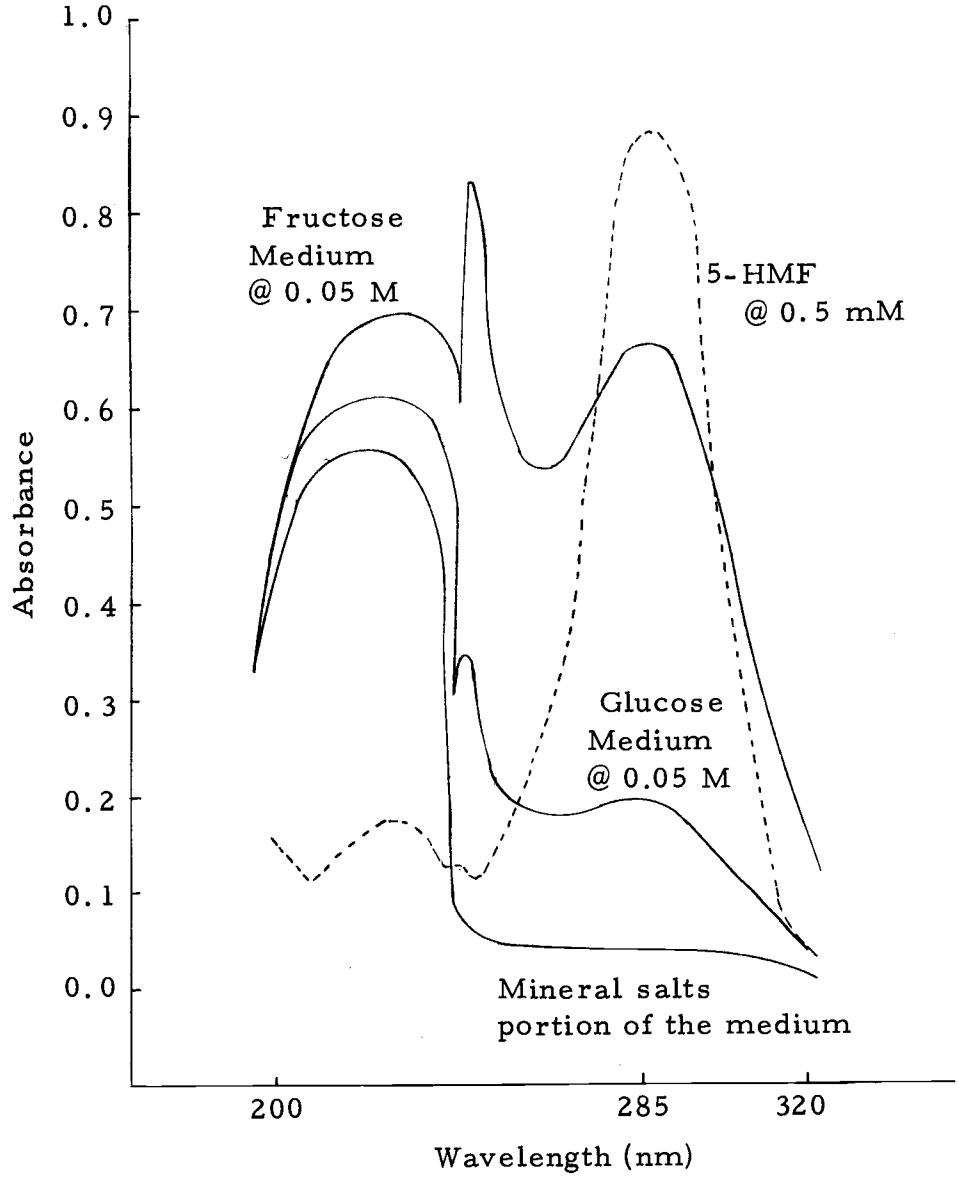


Figure 7. Ultraviolet absorption spectra of 5-hydroxy-methylfurfural, autoclaved fructose- and glucose-containing media, and the mineral salts portion of the culture medium.

f. sp. lycopersici and the furan concentration was estimated periodically by comparison of the culture filtrate absorption maxima at 285 nm with a standard curve prepared with 5-HMF. The furan compound was lost from the glucose- and fructose-containing media rapidly following inoculation, but was lost only slightly from the uninoculated media (Figure 8). The furan was either utilized along with the sugar or it was chemically altered by fungal action. Utilization of furans was demonstrated with Saccromyces spp. when they were present in the medium at concentrations as high as 6% (31).

To determine the toxicity of 5-HMF to F. oxysporum f. sp. lycopersici, increasing concentrations of reagent grade 5-HMF were added to the standard liquid medium containing either glucose or fructose as the carbon source. The medium was inoculated with a spore suspension of F. oxysporum f. sp. lycopersici and the cultures were incubated on a shaker. Growth was estimated by dry weight measurements four days later.

Cultures grown on the glucose medium were more susceptible to 5-HMF than those on the fructose medium (Table 9). The ED_{95} concentrations are high enough to suggest that 5-HMF has a relatively low toxicity to F. oxysporum f. sp. lycopersici, although it is toxic at low concentrations to many microorganisms (7, 33). Increased growth and colony formation by Fusarium on media containing fructose, thus, is probably not due to the presence or absence of furans in the culture media.

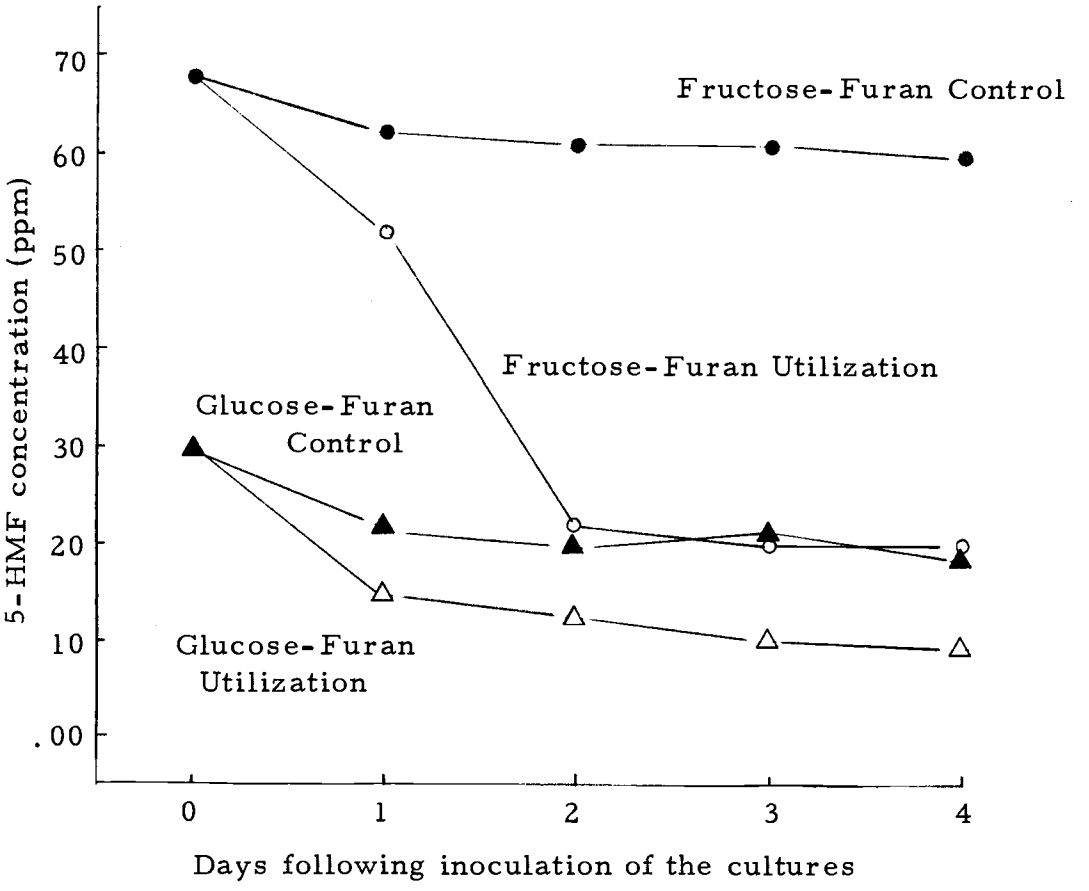


Figure 8. Disappearance of 5-HMF from culture media inoculated with Fusarium oxysporum f. sp. lycopersici.

Table 9. The fungitoxicity of 5-hydroxymethylfurfural to F. oxysporum f. sp. lycopersici growing in shake culture

5-hydroxymethylfurfural concentration in ppm	Percentage inhibition of growth (dry weight basis)	
	Glucose medium	Fructose medium
500	20	8
750	45	11
1000	68	15
5000	99	99

DISCUSSION

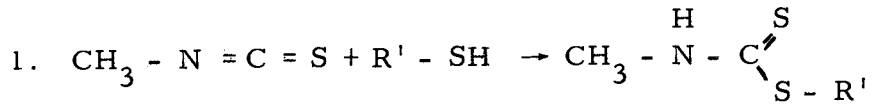
The sulfur content of the microconidia of F. oxysporum f. sp. lycopersici affects their susceptibility to MIT and other sulfur-containing fungicides. When the cellular sulfur level falls below about 0.5% on a dry weight basis, susceptibility to MIT is drastically reduced even though relatively large amounts of MIT are taken up by the spores. Cellular sulfur levels between 0.5% and about 0.85% increase susceptibility to MIT proportional to the sulfur level, but at sulfur levels greater than 0.85% susceptibility is not increased.

Uptake and fungitoxicity of MIT are not always directly related. Sulfur-deficient spores take up relatively large quantities of MIT but the fungus is stimulated rather than inhibited. Evidently, when sulfur is lacking, Fusarium can metabolize MIT to supply its needs. Cell surface thiol groups are probably not required for MIT uptake as the sulfur-deficient cultures took up relatively large amounts of MIT but had a low cell surface thiol content.

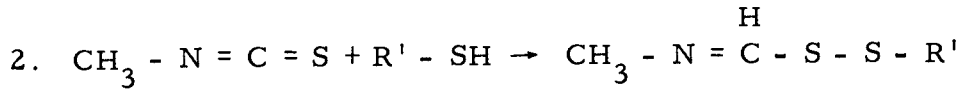
Fungitoxicity of MIT has been linked with its ability to react with cellular thiols (13, 35, 60), but the site of the reaction on or within the fungal cells, and the consequences of this reaction are poorly understood. Wedding and Kendrick (60) propose, with relatively little experimental evidence, that the reaction occurs on the surface of the spores. Goksøyr (13) agrees with this hypothesis

and presents evidence that the reaction at the spore surface is pH dependent; acid pH's are generally unfavorable for the reaction. However, unbuffered MIT solutions (pH 5.0 - 5.7) were highly toxic to F. oxysporum f. sp. lycopersici, and there was no evidence of a reaction between the spores and MIT similar to that described by Goksøyr (14). This is insufficient evidence to preclude a reaction between MIT and thiols at the spore surface, but it does suggest that the reaction need not occur on the surface of the spores to have toxic consequences. According to Wedding and Kendrick (60), the reaction of MIT with surface thiols damages membrane permeability and subsequently leads to toxicity. With Fusarium, however, there was relatively little damage to membrane permeability at toxic MIT concentrations. In addition, sulfur-deficient cells had a relatively low number of potential reaction sites (titratable sulfhydryl groups) on the spore surface but nevertheless took up relatively large amounts of MIT. It would appear that MIT is toxic by virtue of its reaction with cellular sulfur compounds within fungal spores and not at their surface.

The reaction between MIT and cellular sulfhydryl compounds theoretically results in the formation (Reaction 1) of dithiocarbamates (44), but it could also result in the formation (Reaction 2) of disulfide compounds (45) and substituted thioureas (30).



Dithiocarbamate Formation



Disulfide Formation

The energies required to form the dithiocarbamates and disulfides (6, 42) are about equal (70 and 73 Kcal/mole respectively). The disulfide would presumably be more easily degraded (detoxified) because biological systems commonly metabolize naturally occurring disulfides during many cellular processes (18). Since Fusarium spores were exposed to MIT for short time periods (e.g. two hours), toxicity would be favored by production of compounds like the dithiocarbamates that are less readily metabolized.

The sulfur molecule in methionine is relatively unreactive, which may partially explain why spores grown on methionine are less susceptible to MIT. Another consideration might well be that methionine as S-adenosyl methionine detoxifies MIT by methyl group transfer. Reid (43) has compiled an impressive list of toxic compounds to which methionine is antagonistic in mammalian systems. A similar system may be operative in fungal cells treated with MIT.

Failure of colonies to form from germinated Fusarium spores on nutrient media suggest inhibition of mycelial growth. MIT as the

active ingredient in Vorlex and Trapex completely inhibited mycelial growth of Diplodia zeae, Pyrenochaeta terrestria, Pythium debaryanum, Rhizoctonia solani and Sclerotium cepivorum at relatively low concentrations (21). Horsfall (17), while studying antimitotics, discovered that diphenyl inhibits mycelial growth at concentrations that fail to inhibit spore germination in a number of fungi. A number of thioureas and urethanes have a similar action (46), and the urethanes (oxygen analogs of dithiocarbamates) have been suggested to be mitotic poisons in a number of studies (3, 4, 9). Similarly, a dithiocarbamate, (Ferbam) caused chromosome aberations in Aspergillus niger and Allium cepa (41).

Thiocyanates (isomers of isothiocyanates) and thioureas, adversely affect mitosis in animal cells, and prevent sporulation by A. niger (45). Inhibition of sporulation is believed due to disruption of nuclear division. Thiothiazolidone, a cyclic relative of MIT, represses mitosis in plant root tips at levels as low as 7.8×10^{-6} M and is lethal to the cells at 6.2×10^{-5} M (8). Allyl isothiocyanate functioned as a mitotic poison by sticking chromosomes together after replication in A. cepa (3, 4).

The mitotic apparatus is rich in thiols and disulfide compounds whose interconversion is necessary for mitotic function (11, 52). These compounds present many potential reaction sites for MIT which could disrupt mitotic function and block cell division. MIT-treated

spores germinated readily, but the germ tubes failed to form septa and sporophores, and were unbranched, suggesting that nuclear division was in fact inhibited.

Captan is similarly reactive with cellular sulfur compounds and electron photomicrographs indicate that this fungicide becomes attached to the nuclear envelope of treated fungal cells (47, 48, 49). Similar studies with MIT are needed to establish its reaction with nuclear components.

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