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Title: COPPER TOLERANCE IN FOUR FUNGAL SPECIES

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This study was initiated to determine the tolerance to copper of Aspergillus niger, Stemphylium sarcinaeforme, Monilinia fructicola and Fusarium oxysporum f. sp. lycopersici. Included were strains of the latter two fungi that were induced to tolerate relatively high levels of copper by continual culture on sublethal levels of the toxicant. Some of the effects of copper on the physiological activities of the copper-tolerant and parent strains were studied.

The tolerances of the mycelia of Fusarium and Monilinia were increased 12- and 8-fold, respectively. The spores of the tolerant strains of Fusarium and Monilinia could withstand concentrations of copper 60 and 17 times higher, respectively, than the parent strains when measured by the inhibition of spore germination. Measured by the ability of spores to form colonies after a copper treatment, the tolerant strains of Fusarium and Monilinia were 50 and 5 times more

tolerant, respectively, than their parent strains.

Polymodal dosage-response curves were obtained for the inhibition of spore germination of Aspergillus, Stemphylium and both strains of Monilinia, following a two-hour treatment with copper sulfate. Nearly linear dosage-response curves were observed for the parent and tolerant strains of Fusarium, but complete inhibition of germination was not reached even at a concentration of 1M $CuSO_4$. The polymodal curves were not a characteristic of the particular treatment method nor were they influenced by pH or caused by differential uptake of copper as the concentration increased.

The ED_{50} concentration of copper for inhibition of spore germination increased when filtrates from spore suspensions of parent and tolerant strains of Monilinia and Fusarium growing on solid media were included in the spore treatment solutions. When spores were treated with copper sulfate in their own exudates, the ED_{50} concentrations were increased 22-, 56-, and 375-fold for the tolerant and parent strains of Fusarium and the parent strain of Monilinia, respectively. However, exudates from the tolerant Fusarium strain were 5-fold more effective in protecting spores of the parent Fusarium strain from inhibition of spore germination by copper than the parent strain exudates. Exudates from the tolerant Fusarium strain protected spores of the parent Monilinia strain, but the protection was less than with exudates from either Monilinia strain.

Nine different amino acids were present in exudates from the tolerant strain of Fusarium but only two were present in the parent strain exudates. The exudates from the tolerant and parent Monilinia strains contained 15 and 14 amino acids, respectively. The concentration of individual amino acids in the exudates varied between the tolerant and parent strains of both fungi, but generally, concentrations were higher in exudates from the tolerant strains.

The Fusarium strains were grown in liquid culture and the amino acid content of the culture filtrates and the free and bound amino acids in the spores and mycelium were determined. As with the exudates, more kinds and higher concentrations of amino acids were present in culture filtrates, spores and mycelium of the tolerant strain than the parent strain. Addition of copper to the growth medium increased the concentration of amino acids with both strains, except that the concentration of free amino acids in the tolerant strain was decreased.

The amounts of copper remaining in culture filtrates from the lmM copper treatment of the two Fusarium strains were nearly equal, but there was almost twice as much copper with the free amino acids from the spores and mycelium of the tolerant strain as the parent strain. On the other hand, higher copper concentrations were found with the bound amino acids of the spores and mycelium of the parent strain than the tolerant strain. This suggests that copper is bound

at different sites in the two strains. The amino acids in the culture filtrates and the free amino acids in the spores and mycelium can combine with copper and reduce its toxicity. Since more amino acids are produced by the tolerant strain than by the parent strain, this could explain the acquired tolerance.

Copper Tolerance in Four Fungal Species

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COPPER TOLERANCE IN FOUR FUNGAL SPECIES

INTRODUCTION

The fact that bacteria and insects develop resistance to antibiotics and insecticides, respectively, suggests that fungi may become resistant to fungicides. Copper compounds have been used extensively as fungicides for many years but there is relatively little evidence that fungi develop tolerance to them under field conditions. Some fungal species are highly tolerant of copper and other species have been induced to tolerate copper in synthetic culture media (2, 40).

Taylor (46) showed that spores of Physalospora obtusa collected from orchards regularly sprayed with Bordeaux mixture were more resistant to copper than those from unsprayed orchards. Horsfall (12) suggested that Phytophthora infestans may have developed resistance to copper as demonstrated by reduced effectiveness of Bordeaux mixture for control of potato blight over the years.

Attempts to develop resistance to copper in fungi have been more successful. Ashida (2) cited 14 cases of acquired resistance to copper by filamentous fungi in his review of the adaptation of fungi to metal toxicants.

The mechanism of resistance to copper in fungi is still not well understood. Intensive studies of the growth and metabolism of fungi with naturally occurring and induced tolerance should provide

additional information about the resistance mechanism. Several Japanese workers (1, 4, 14, 30, 32) have studied copper-tolerant and parent strains of yeasts and proposed mechanisms to account for their resistance to copper. However, there have been relatively few studies of copper resistance mechanisms with the filamentous plant pathogenic fungi.

Strains of Fusarium oxysporum f. sp. lycopersici (Sacc.) Snyd. and Hans. and Monilinia fructicola (Wint.) Honey became tolerant of high levels of copper when cultured on media containing sublethal concentrations of copper sulfate. This study was designed to determine the influence of copper on mycelial growth, spore germination and colony formation by spores of these two fungal strains, their parent strains and two other fungi, Stemphylium sarcinaeforme (Car.) Wilt., a copper-susceptible fungus, and Aspergillus niger v. Tiegh., a resistant one. A second phase of this study was to determine some of the effects of copper compounds on the physiological activities of the copper-tolerant and parent strains of Fusarium and Monilinia to gain information on the mechanism of resistance to copper in fungi.

The mode of fungitoxic action of copper is still not well understood and information gained in studies of resistance should provide additional insight into how copper compounds disrupt the metabolism of fungi.

LITERATURE REVIEW

The mode of action of metals as fungicides generally is believed to be their reaction with essential enzymes of the cell, thereby preventing the normal functioning of the enzymes (16, 23). Owens (36) studied the action of copper on several enzyme systems in vitro. The experiments were conducted using pancreatic amylase, malt amylase, catalase, and polyphenol oxidase, all of which are dependent for activity on amino groups, sulfhydryl groups, iron, and copper, respectively. Pancreatic amylase activity was inhibited 74% by copper chloride at 10^{-3}M. Under these same conditions, malt amylase was inhibited 69%, there was no inhibition of catalase, and a slight increase in polyphenol oxidase activity was observed. When a copper-zinc-chromium complex was used, the two amylases were inhibited 69% and 68%, respectively, by a 10^{-3}M concentration. The complex failed to inhibit catalase, and increased polyphenol oxidase activity by 32%, as compared with a 12% increase with copper chloride. These results suggest that enzymes relying on amino and sulfhydryl groups for activity may be similarly susceptible to inactivation by copper compounds.

Byrde and co-workers (6) surveyed the action of copper on various enzymes of Sclerotinia laxa. They found a marked depression in fumarase, aldolase and cytochrome c oxidase activity, a slight

depression of polyphenol oxidase, glutamic dehydrogenase, isocitric dehydrogenase, cytochrome c reductase and catalase activity, and no effect on peroxidase, succinic dehydrogenase or alkaline phosphatase activity. There was stimulation of hexokinase and DPN oxidase activity. There is some discrepancy between the results obtained by Owens and Byrde and his co-workers. Owens found that catalase was unaffected by copper and polyphenol oxidase was stimulated, but Byrde's group found a slight decrease in activity of both enzymes. A general conclusion from these collective investigations is that copper affects the activity of most enzymes although not all are affected equally or in the same manner. Copper depressed the activity of cyrochrome c oxidase but exerted a compensating effect by stimulating the alternative DPN oxidase system. Since the studies were done with isolated enzymes it would be difficult to speculate if the same inhibitions might occur in intact fungal cells. The work by Byrde's group may be more representative of the effect of copper on fungi since the enzymes were from a fungal source.

Many factors could govern the inhibition of enzymes in cells by copper. One of these factors and perhaps the most important is penetration of copper into the cell and to a particular intracellular site of enzymatic activity. There is much controversy over the site of action of copper. Somers (42, 43) suggested that the fungistatic action of cations is related to the strength of their covalent bonding

at the cell surface with imidazole, carboxyl, phosphate or sulfhydryl groups, but that fungicidal action is within the cell following disruption of membrane permeability and subsequent diffusion of metal ions into the cell and is caused by the secondary reactions that follow.

Miller (21, 22, 25) disagreed with this concept and expressed the opinion that the action of copper is entirely within the cell. He suggested that metal ions enter the cell more easily than indicated by Somers. Miller's suggestion is based on studies of spore exudates in which as much as 30% of the cell constituents, based on radioactive phosphorus, were released when spores were collected in water. The loss of compounds had no effect on germination. An additional release of cell constituents occurred with treatment of the spores with metals, including copper (24). McBrien and Hassall (18) found that when Chlorella vulgaris was treated with copper, cell potassium was released. The release of potassium was not an exchange reaction. They suggested that the primary toxic effect of copper was the increase of cell permeability, but they did not consider this as the most important effect of copper since cells with disrupted permeability were still capable of growth.

An increase in permeability of spores following copper treatment may aid penetration of the spore by copper, but the increase in exudates may more than compensate for this. Miller (23) found that compounds in filtrates from suspensions of conidia of various species

of fungi retarded the uptake of silver by conidia. The spore exudates may also be able to retard uptake of copper.

Owens and Miller (38) in experiments to determine the site of action of copper, disintegrated conidia of Neurospora sitophila and Aspergillus niger that had been treated with sublethal amounts of metal ions and separated the components into a cell wall fraction, two particulate fractions and a water-soluble fraction. The particulate fractions were obtained by methods for isolation of mitochondria and microsomes. Their results demonstrated that the cytoplasmic contents had more affinity for metal ions than the spore wall fraction. Metal toxicants accumulated in the particulate fractions in quantities much greater than those required for inhibition of isolated enzymes. Since spore germination was not inhibited at these metal concentrations, it was suggested that the enzymes were protected from the toxicant by interaction of the metals with substances other than enzymes in a non-specific manner. In the case of copper, transfer of the spores to a toxicant-free medium results in leaching of the toxicant in sufficient quantities to allow germination. Thus, the fungistatic toxicity is concentration dependent and reversible when the metal concentration falls below a critical level.

Some fungi are naturally more resistant to copper than others. For example, Starkey and Waksman (44) isolated two fungi, Acontium velatum and a fungus belonging to the Dematiaceae, from a saturated

solution of copper sulfate. Still other fungi can adapt in culture to tolerate high copper concentrations. Horsfall (12) suggested that the ability of fungi to release acid into the media in which they are grown is associated with resistance to copper, possibly because sorption of copper is reduced at lower pH.

In some fungi, hydrogen sulfide production may be responsible for resistance to copper. For example, Yamasaki and Suwa (47) found that copper-resistant strains of Piricularia oryzae that produced large amounts of hydrogen sulfide accumulated more copper in their mycelium than strains that produced little hydrogen sulfide. A similar relationship between hydrogen sulfide production and copper resistance was found in yeast (13, 14, 33, 34). Ashida, Higashi and Kikuchi (3) using electron microscopy, demonstrated that hydrogen sulfide producing yeasts accumulated large quantities of copper sulfide in the cell walls, but they could not determine the location of the copper in the protoplasm. Ashida and Nakamura (4) found no difference in hydrogen sulfide production between tolerant and susceptible strains in their early growth stages, and some resistant strains produce no hydrogen sulfide. There was also no indication that resistant cells were less permeable to copper and thus, another method of resistance must exist.

Generally, copper-resistant fungal cells contain more copper than cells of susceptible strains (14, 32, 35). This was thought due

to binding with sulfur-containing compounds other than hydrogen sulfide, but sulfur-starved cells retained their copper resistance, so resistance is apparently not entirely dependent upon sulfur-containing compounds (4).

Another possible mechanism of resistance to copper is the activation of a metabolic pathway bypassing the copper-inhibited reaction. Murayama (28, 29, 30) showed that of the enzymes in the tricarboxylic acid cycle of yeast, activity of citrate and glutamate dehydrogenases was greater in the resistant than the parent strain. Arakatsu (1) induced a yeast to tolerate copper and found it was able to synthesize glutamic and aspartic acids in the presence of copper while the parent strain was unable to make these acids with copper present in the medium.

An effect of copper toxicity on fungi appears to be a general reduction in the amount of free amino acids in the cells. Tandon and Chandra (45) found that sublethal amounts of copper affected the metabolism of Curvularia penniseti, by decreasing free amino acids and completely suppressing tyrosine production. Siegel and Crossan (41) in a similar study showed that the number of free amino acids was generally reduced in Colletotrichum capsici, but specific amino acids were either missing, reduced, normal or increased in amount. Most amino acids were reduced in concentration but tyrosine was increased and cystine and glycine were missing. In the bound amino

acids there was an increased concentration of valine and tyrosine. Murayama and co-workers (27, 31) found no difference in the free amino acids between resistant and susceptible yeast strains when grown on copper-free medium, but resistant cells produced less glycine and serine and more peptide-like substances in a copper-containing medium.

It is difficult to assess the significance of these findings on copper toxicity, but there appear to be more differences in the metabolism of amino acids than in the other metabolic systems. This may indicate that the site of action of copper as well as the site of the resistance mechanism may be found in the pathways concerned with amino acid metabolism.

MATERIALS AND METHODS

Media

Potato dextrose agar (PDA) was the main growth medium for culturing fungi in this study. The PDA was prepared from the infusion of 200 g of potatoes, 20 g of dextrose and 20 g of agar in one liter of water.

A more defined medium than PDA was used in some experiments. One liter of this medium contained: 25 g glucose, 20 g agar, 1 g peptone, 1 g yeast extract, 2.5 g KNO_3, 500 mg $MgSO_4 \cdot 7HOH$, 310 mg K_2HPO_4, 940 mg KH_2PO_4, 10 mg $ZnSO_4 \cdot 7HOH$, 10 mg $FeCl_3 \cdot 6HOH$, 1.25 mg H_2BO_3, 1.0 mg $CaCl_2 \cdot 2HOH$, 0.9 mg $MnCl_2 \cdot 4HOH$, 0.275 mg $CuCl_2 \cdot 2HOH$ and 0.01 mg MoO_3. In some experiments, strains of Fusarium were grown in a liquid medium with the same ingredients but without agar, peptone and yeast extract. To prevent precipitation of the metals in liquid media, the phosphates were autoclaved separately and then mixed with the other ingredients when cool. All media were adjusted to pH 6.2.

Fungi

Copper-tolerant strains of Fusarium and Monilinia were produced by continual culture on PDA containing sublethal levels of copper sulfate. To obtain the desired copper concentration,

$CuSO_4$. 5HOH was dissolved in sterile water and one ml was added to 20 ml of liquid PDA at 44°C in test tubes. The PDA-copper medium was thoroughly mixed, poured into petri plates and inoculated after it had solidified with a 7mm disc cut from the growing edge of an established fungus colony on PDA. Transfers were made to fresh media monthly and the copper concentration of the medium was increased whenever the fungus began growing well. Fungal isolates maintained on PDA without copper are referred to as the parent strains in this study.

Although copper-tolerant strains of Fusarium and Monilinia grew on relatively high levels of copper, sporulation was almost completely inhibited. Thus, for experiments requiring spores of these strains, the fungi were transferred to PDA without copper before they were needed (7 days with Monilinia and 12 days with Fusarium). Although significantly fewer spores were produced by the copper-tolerant Monilinia strain, they appeared normal and the percentage and rate of germination were similar to that of the parent strain. Similarly, fewer spores were produced by the tolerant strain of Fusarium but although the spores appeared normal, their germination rate was slow and the germ tubes were more compact with less branching than those from spores of the parent strain. Spores from the parent strain of Fusarium usually germinated 100% but those from lots of the copper-tolerant strain varied from 85 to 90%.

Aspergillus niger was included in this study because it is highly resistant to copper, possibly through the release of acid into the medium on which it is grown (12). Stemphylium sarcinaeforme does not excrete acid into the growth medium and was included as a copper-sensitive fungus (12).

Copper Toxicity to Mycelial Growth

Inhibition of fungal mycelial growth was determined on PDA containing copper. Three plates of each copper concentration were prepared and inoculated with 7mm plugs cut from the periphery of fungal colonies on PDA. The highest copper concentration on which each fungus grew was recorded four weeks after inoculation.

Copper Toxicity to Spore Germination

Two methods were used to determine the influence of copper on spore germination. Spores were collected from colonies of each fungus growing on PDA when sporulation was at the maximum. Ten ml of a sterile 1% glucose solution were pipetted onto each plate to be harvested and the plate was rubbed with a sterile rubber policeman. The plates were rinsed twice with five-ml aliquots of the glucose solution which were added to the spore suspension. Fusarium and Aspergillus spore suspensions were filtered through Whatman no. 4 filter paper and Monilinia and Stemphylium spore suspensions were

filtered through four thicknesses of Kimwipes (disposable wipers, type 900-S, Kimberly-Clark Corp., Neenah, Wisconsin).

The spore concentration was determined in a Leavy-Hausser corpuscle counting chamber and the suspension was then diluted to twice the desired final concentration: 20,000 spores/ml for Stemphylium and 100,000 spores/ml for the other fungi. Solutions of copper sulfate were prepared in a 1% glucose solution at twice the desired final concentration.

The spore suspension and copper sulfate solution were thoroughly mixed in a 1:1 ratio and a few drops were placed in depressions on glass slides. The depression slides were placed on glass rods in petri plates containing moistened filter paper discs to maintain a humid atmosphere. Spore germination was determined in four replicate samples by counting the number of germinated spores per hundred. Counts were made after 24 hours of incubation, except for the tolerant strain of Fusarium which required 48 hours for germination. The percentage germination of the untreated Aspergillus spores was always low, about 30%, probably because of nutrient deficiencies. It was felt, however, that nutrients added to stimulate germination might interact with copper in the medium and confound the experiments. Spores were considered to be germinated when the germ tubes were as long as the width of the spores.

In a second method for measuring inhibition of spore germination, five ml each of the spore suspension and copper solution were

pipetted into glass vials. The vials were plugged with serum caps and then rotated on a motor-driven wheel for two hours. The spores were removed from the treatment solution by filtration through an 0.8μ Millipore filter and then resuspended in 10 ml of sterile 1% glucose. Three large drops of this suspension were placed in separate locations on the defined agar medium in petri plates and 24 hours later the percentage spore germination was determined. Monilinia spores would not germinate well on the defined medium, so PDA was used for this fungus.

The ability of treated spores to form colonies on nutrient agar was determined by spreading about 75 spores over the surface of the defined agar medium or PDA and counting the colonies which subsequently formed. At least three plates were made for each treatment.

Physiological Studies

Copper Uptake by Spores

The amount of copper removed from solution by fungal spores was estimated after two hours. Large amounts of spores were required for the copper determination so the spores were treated in 400 ml quantities. The treated spores were collected on a Millipore filter (0.8μ) and then resuspended in 10 ml of glass distilled water

containing 1% glucose. Copper was determined by a modification of the method of Giorgio, Cartwright and Wintrobe (8). Five ml of concentrated nitric acid and 2.5 ml of concentrated sulfuric acid were added to the spore suspension and the mixture was boiled until the solution became yellow. After cooling, five ml of concentrated nitric acid and one ml of concentrated sulfuric acid were added and the solution was boiled again until 5-10 ml of liquid remained. The solution was cooled, 10 ml of glass distilled water was added, then 1N ammonium hydroxide was added until the solution gave a pink color with phenolphthalein. The copper was extracted from this aqueous solution with 10 ml of a 0.1% (wt/v) solution of zinc dibenzyldithiocarbamate in carbon tetrachloride. The extraction was carried out by shaking the hydrolysate with the carbon tetrachloride solution for two minutes in a separatory funnel. The carbon tetrachloride layer was removed and filtered through Whatman no. 1 filter paper. The absorption of the solution at 435mμ was compared with a standard curve for the absorption of copper dibenzyldithiocarbamate obtained from known quantities of copper sulfate which had undergone the same hydrolysis and extraction procedure.

Amino Acid Determinations

Both strains of Fusarium were grown in liquid shake cultures with various levels of copper. The dry weight of mycelium and spores, the number of spores produced, and the pH were determined at each copper concentration. The mycelium and spores were collected on previously weighed 0.8μ Millipore filters and then dried to constant weight at 55°C.

The free amino acids in the dried mycelium and spores were extracted by boiling in three 50-ml quantities of 80% ethanol. The extracts were filtered through 0.22μ Millipore filters and evaporated to dryness. The extracted mycelium and spores were hydrolyzed in 50 ml of boiling 6N HCl for eight hours. More 6N acid was added (as required) to maintain a nearly constant volume of the mixture during hydrolysis. The hydrolysates were evaporated to dryness under vacuum, redissolved in water, filtered through Whatman no. 1 filter paper, evaporated again and dissolved in exactly 10 ml of glass distilled water.

The total amino acid content of the culture filtrates and the ethanol extracts and acid hydrolysates of the mycelium and spores was determined by the method of Moore and Stein (26). The absorption of the unknowns at 570 mμ was compared with a standard curve of the absorption of leucine at the same wavelength.

Determination of Copper

The copper content of culture filtrates and the ethanol extracts and acid hydrolysates of the mycelium and spores was determined in the same manner as the copper determination previously described for copper uptake by spores, except that culture filtrates and ethanol extracts were not hydrolyzed and no further hydrolysis was made of the acid hydrolysates.

Determination of Simple Sugars

The amount of simple sugars in the culture filtrates was determined by the phenol-sulfuric acid method of Hodge and Hofreiter (11). Glucose was used as the standard.

Chromatographic Determinations

Paper chromatography was used for determining individual amino acids, simple sugars and organic acids in the culture filtrates, and the amino acids in the acid hydrolysates and ethanol extracts of mycelium and spores of the Fusarium strains.

Chromatography of the amino acids in the hydrolysates required that the samples be desalted first. An aliquot of the hydrolysate was passed through a 2 x 4cm column of Dowex 50w x 8 cation exchange resin in the hydrogen form. The amino acids were eluted from the

column with 1N ammonium hydroxide. The solution was evaporated to dryness under vacuum and then brought up to the original volume with glass distilled water.

The amino acids were separated by two-dimensional descending chromatography according to the method of Grable, Presley and Templeton (10) using reagent grade n-butanol, glacial acetic acid, and glass distilled water (4:1:1) in the first direction and absolute methanol, 95% ethanol and glass distilled water (45:45:10) with 0.5 g urea added, in the second direction. The chromatograms were sprayed with 0.2% ninhydrin in ethanol, then heated for 15 minutes at 90°C, and finally fixed with acidified copper nitrate.

Simple sugars were separated by one-dimensional descending chromatography with butanol:acetic acid:water (4:1:1) and developed by spraying with a solution prepared with 1.69 g of 2-amino biphenol, 0.9 g of oxalic acid, 84 ml of acetone, 5 ml of glycerol and 10 ml of distilled water (9). The spots containing the sugars became visible after heating the sprayed chromatograms for five minutes at 110°C.

Organic acids were separated by one-dimensional ascending chromatography in a solvent-indicator solution containing 85 ml of 77% ethanol, 15 ml of 88% formic acid, 60 mg of sodium formate and 50 mg bromphenol blue. When the chromatograms were dry, the organic acids appeared as yellow spots on a dark blue background.

RESULTS

Inhibition of Mycelial Growth

The maximum concentration of copper in the growth medium at which a fungus will grow provides an approximation of its tolerance to copper. The strains of Fusarium and Monilinia were designated as copper-tolerant strains because of their ability to grow on media containing high levels of copper. To determine the degree of tolerance of all of the fungi, they were transferred to media with a range of copper concentrations and the maximum concentration at which growth occurred was recorded after four weeks.

The copper-tolerant strains of Monilinia and Fusarium had increased in tolerance about 8- and 12-fold, respectively, over their parent strains (Table 1). The normally resistant Aspergillus was about four times as tolerant as Stemphylium and the parent strains of Monilinia and Fusarium.

Table 1. The influence of copper on mycelial growth of various fungi.

Fungus	Highest concentration of copper in PDA allowing growth (ppm)
parent Fusarium strain	250
tolerant Fusarium strain	3,000
parent Monilinia strain	250
tolerant Monilinia strain	2,000
Stemphylium	250
Aspergillus	1,000

The tolerance developed by culturing fungi on media containing a fungicide often is rapidly lost when the fungi are returned to a fungicide-free medium. Genetic adaptation is generally considered more stable than physiological adaptation (2). Although this was not a study on the genetics of copper resistance, it was considered worthwhile to determine the stability of tolerance of the copper-tolerant strains. Cultures of the tolerant strains were maintained for one year on PDA without copper with monthly transfers to new media. Following this period, these cultures were able to grow at the same copper concentrations as the fungi transferred directly from media containing copper. Thus, copper tolerance was extremely stable and may have been due to genetic mutation.

Inhibition of Spore Germination and Colony Formation

The ability of a fungus to grow on media containing a fungicide indicates the tolerance of its mycelium to the fungicide, but not necessarily the tolerance of its spores. The tolerance of the spores of the various fungi to copper was determined by the two methods described earlier. Copper concentrations ranged from $1x10^{-7}$M to 1M. A probit regression analysis of the data was made (5).

Tolerance of the spores from the copper-tolerant Fusarium strain increased over those from its parent strain about 60-fold and spores from the tolerant Monilinia strain increased about 17-fold

over those from its parent strain when in continual contact with copper (Figure 1). The slopes of the dosage-response curves for the inhibition of spore germination of the various fungi were relatively similar with the exception of the slope for Aspergillus which was much flatter, indicating more variability in copper tolerance in this spore population.

Fungal spores that germinate in the presence of a fungicide may not form colonies even when removed from the toxicant, and in other cases spores that fail to germinate in the presence of a fungicide may germinate and form colonies when removed from the toxicant (12). An experiment was designed to differentiate between spores that were dead or inhibited from forming colonies by copper from those that were inhibited from germinating but still capable of forming colonies after 24 hours of treatment. Spores were removed from their treatment solutions by filtration, an aliquot was placed on solid media and the subsequent number of colonies that formed were counted. The ED_{95} concentration of copper required to inhibit germination of the spores in the treatment solutions was much less than that required to kill or inhibit colony formation of the spores when removed from the toxicant, especially with Stemphylium and Aspergillus (Table 2). Thus, Aspergillus and Stemphylium have more tolerance to the fungicidal action of copper than to its fungistatic action. Increased tolerance to the fungicidal activity by Fusarium, (50-fold), was greater

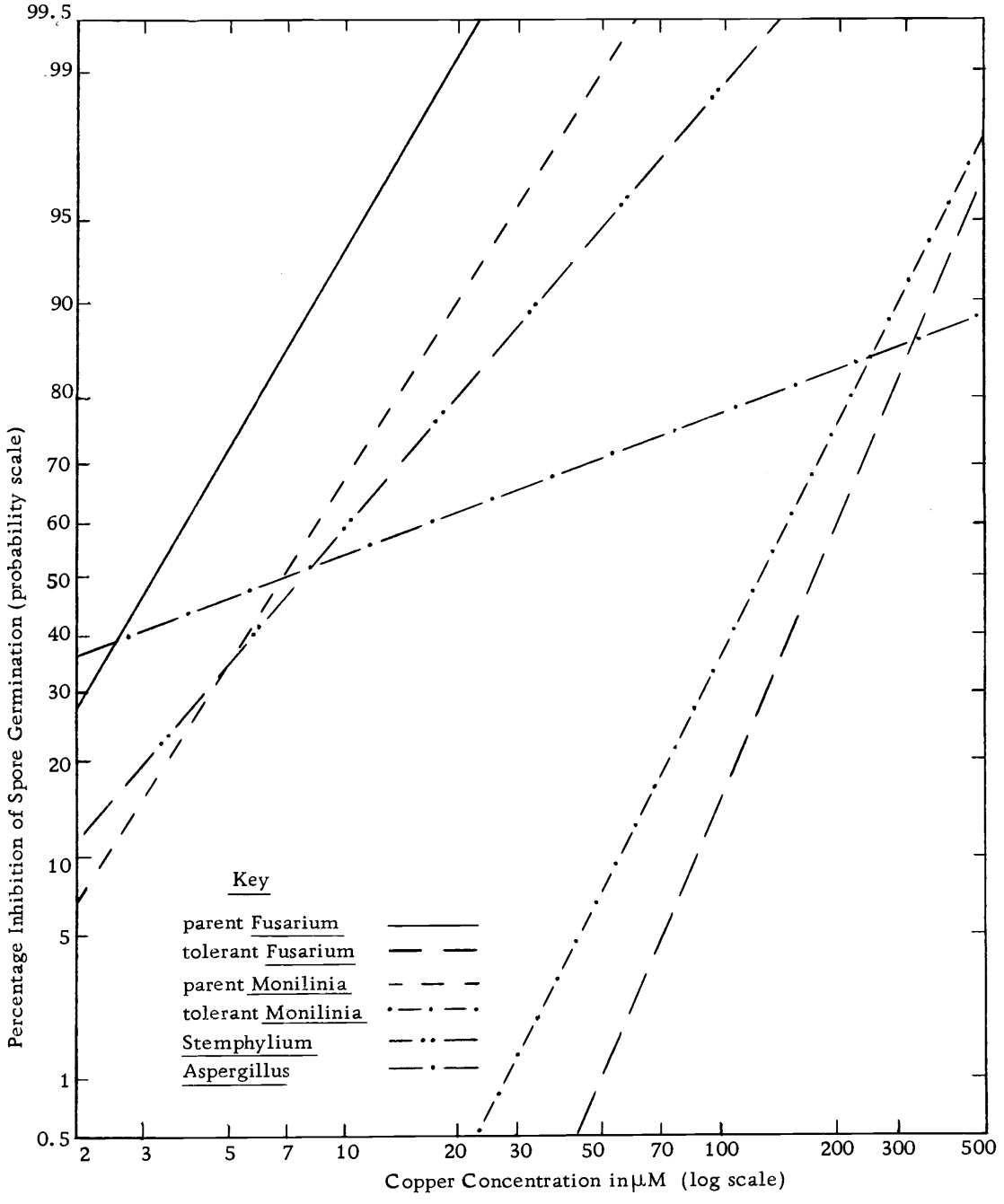


Figure 1. The influence of copper on germination of spores of various fungi in continual contact with copper sulfate.

than the increase by Monilinia (5-fold). Similarly, Fusarium had a greater increase in tolerance to fungistatic activity than did Monilinia (44-fold versus 13-fold).

Table 2. The influence of copper on germination and colony formation by various fungi.

Fungus	Inhibition of spore germination, ED_{95} concentration (μM)	Inhibition of colony formation, ED_{95} concentration (μM)
parent Fusarium	10	10
tolerant Fusarium	440	500
parent Monilinia	29	100
tolerant Monilinia	380	500
Stemphylium	52	1,000
Aspergillus	1,240	500,000

Spores were treated with copper sulfate for two hours to determine if short treatments were as effective as the longer exposure periods. Spores were collected on Millipore filters, (0.8μ), after two hours in the treatment solutions. The spores were then resuspended in 1% glucose and this suspension was placed directly onto solid media for determination of germination and inhibition of colony formation.

The results obtained when spores were in continual contact with copper were different from those obtained when spores were removed from the treatment solution after two hours (Figure 2). Polymodal dosage-response curves were obtained with some of the fungi following the two-hour treatment, whereas the dosage-response curves

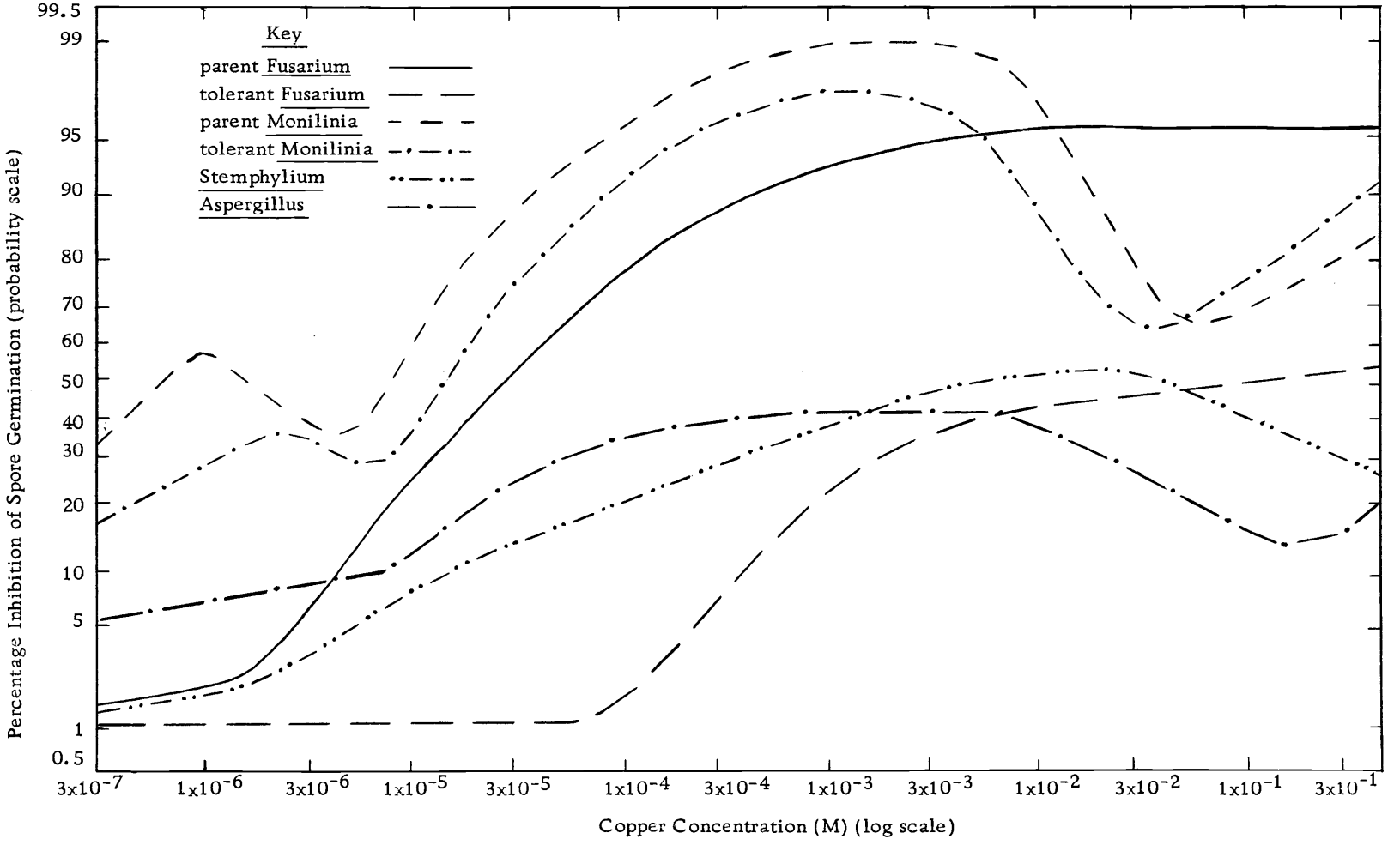


Figure 2. The effect of a two-hour treatment with copper sulfate on germination of spores of various fungi.

were linear when spores remained in the treatment solutions until they were germinated. The percentages of inhibition for both spore germination and colony formation were identical with the two-hour treatment. The most pronounced polymodal curves were obtained with the two Monilinia strains. The percentage inhibition of germination of the parent Monilinia strain is about the same at widely separated concentrations. For example, there was 80% inhibition at 2×10^{-5}, 3×10^{-2} and 3×10^{-1}M. The difference in inhibition between the two Monilinia strains is not great, relative to the difference in inhibition between the two Fusarium strains. Neither Fusarium strain has a polymodal dosage-response curve; however, complete inhibition was never obtained. Copper concentrations greater than 1M could not be used because this concentration was nearly a saturated solution of copper sulfate. The differences in response between Monilinia and Fusarium to copper treatment in this experiment may indicate that resistance in these fungi is by a different mechanism or that copper acts by a different mechanism to inhibit germination of spores of the two species.

The response of Aspergillus and Stemphylium to a two-hour copper treatment appears to be intermediate between the other two fungi. Neither Aspergillus nor Stemphylium have the pronounced polymodal curve of Monilinia or the more linear curve of Fusarium.

The method used for the two-hour treatment of spores has not been extensively used and the polymodal dosage-response curves could be a product of short term treatments and not solely characteristic of copper. Thus, the response of the parent Monilinia strain to other fungicides (captan, dichlone, methylisothiocyanate and zinc sulfate) was determined using the two-hour treatment. The parent strain of Monilinia was used because it had the most pronounced polymodal curve.

A polymodal dosage-response curve was obtained for the inhibition of spore germination only with the metal salts (Figure 3), and it was concluded that the polymodal response was not a characteristic of the treatment method. The dosage-response curve for zinc was similar to that for copper but zinc was less toxic.

The pH of the treatment solutions affects the toxicity of metals probably because of competition between hydrogen ions and metal ions for binding sites on the spores (12). Since the spore treatment solutions were not buffered, experiments were performed to determine the effect of pH and buffers on the inhibition of germination of the parent Monilinia strain spores by copper.

The spores were treated for two hours as previously described except buffers were included in the treatment solutions or the pH was adjusted to two pH levels with HCl or NaOH in unbuffered solutions. The buffers used were 0.067 M phosphate, citrate and acetate at pH 5.8.

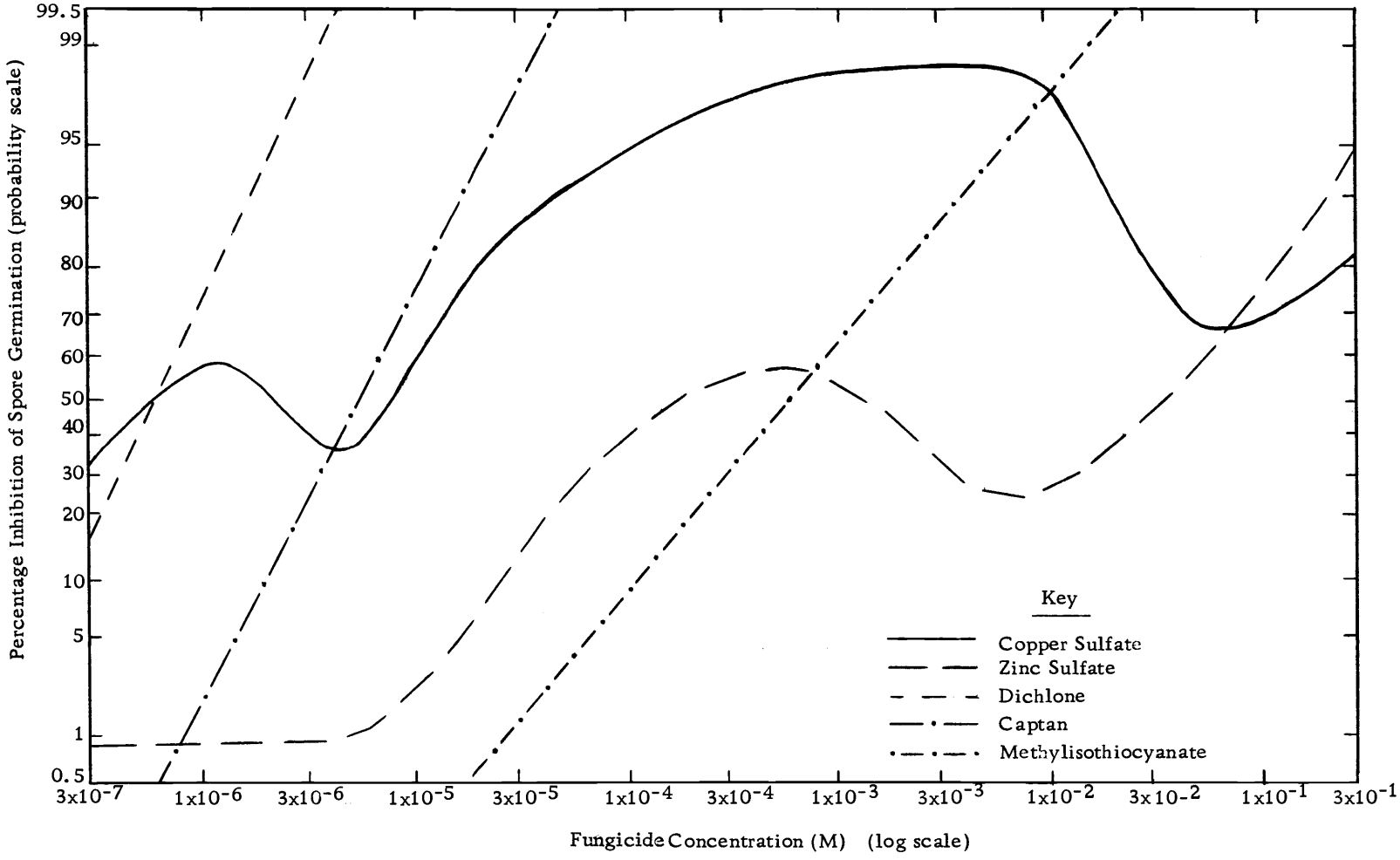


Figure 3. The effect of a two-hour treatment of spores of the parent Monilinia strain with various fungicides.

Dense precipitates formed with the phosphate buffer at high copper concentrations. Unbuffered treatment solutions were adjusted to pH 3.8 and 5.8 which represented the extremes of pH in treatment solutions with no pH adjustment.

The buffer solutions drastically altered the response of the spores to copper (Figure 4). Toxicity in phosphate buffer was greatly enhanced at low levels of copper. In citrate and acetate buffers, spore germination was significantly inhibited only at the high copper concentrations.

The enhanced toxicity of copper in phosphate buffer at low copper concentrations may be due to the formation of copper phosphate which could be more readily taken up or more toxic than the cupric ion, or phosphate may act independently on the spore to increase its uptake of cupric ions. However, at high copper concentrations a dense precipitate forms and the copper and phosphate become unavailable, and inhibition of germination is reduced. The anions of the metal salt are generally considered to have little effect on the toxicity of the metal except in cases where the salt is insoluble or the metal is bound so firmly that receptor sites cannot take it up (22).

The reaction of copper with acetate and citrate forms compounds that may be less toxic to Monilinia spores than copper, or are not readily taken up, so germination is not greatly inhibited until the copper concentration becomes very high.

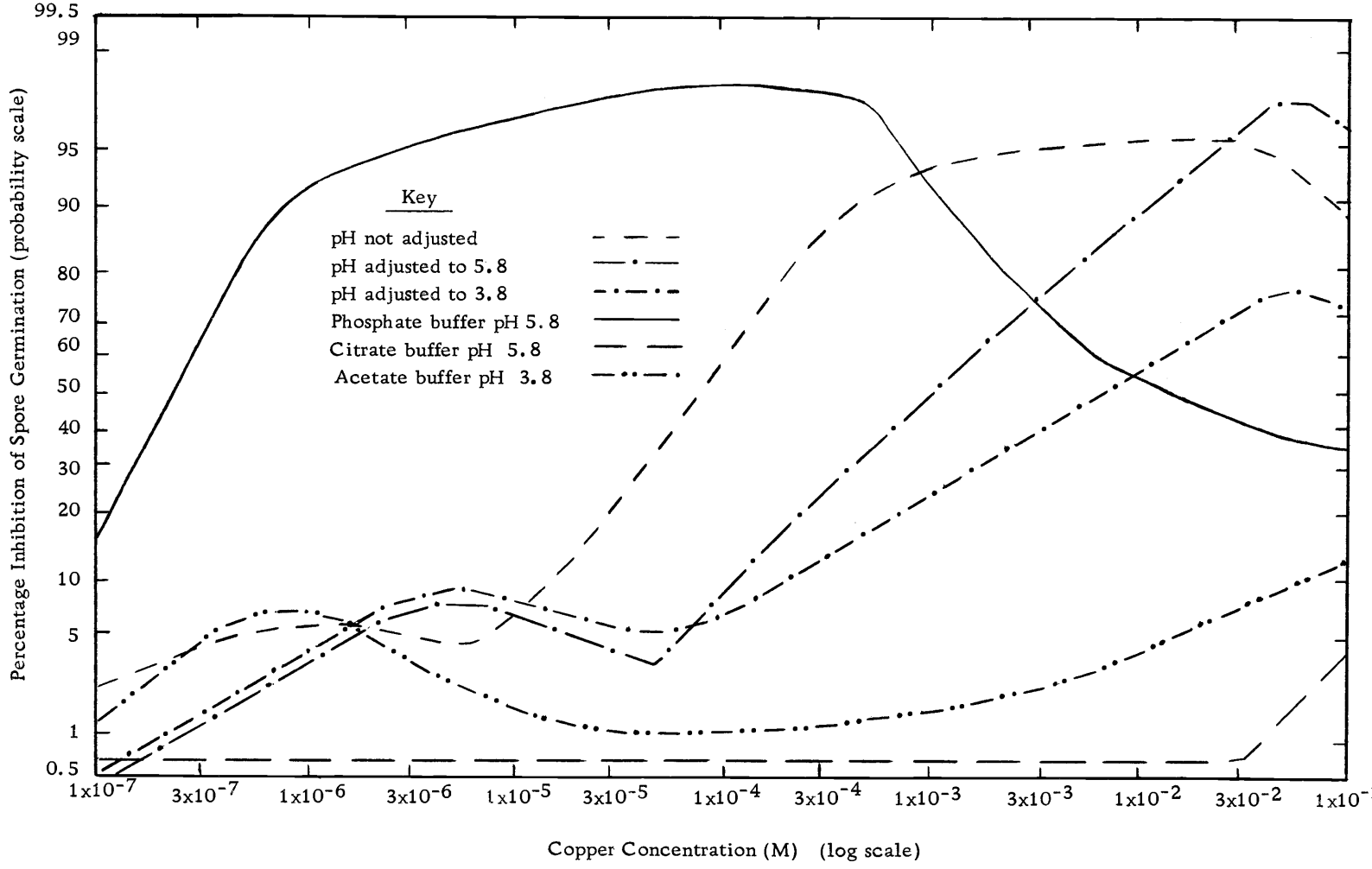


Figure 4. The effect of pH and buffers on inhibition of germination of spores of the parent Monilinia strain by copper sulfate.

Martin, Wain and Wilkinson (17) tested various copper compounds for their ability to inhibit spore germination and found that the LD_{50} concentration of cupric phosphate was not significantly different from that of Bordeaux mixture or copper sulfate. This was different than the enhanced toxicity with phosphate observed here. Their method of treatment and the fungi they used were different than in the buffer experiments, but the cupric organic acids were less toxic than copper sulfate in both cases. Miller (20) observed that citrate or orange juice reduced the toxicity of copper to fungal spores. Organic acids similarly reduced the inhibition of spore germination by silver (23).

Lin (15) observed that increasing the concentration of neutral salts, such as calcium nitrate, calcium chloride, magnesium nitrate and magnesium chloride, in a copper sulfate solution produced a bimodal dosage-response curve for the inhibition of spore germination with M. fructicola. However, the dosage-response curve with increasing copper concentrations was not bimodal. He also found that addition of mono- or di-basic potassium phosphate, sodium acetate, and acetic acid antidoted copper toxicity within certain concentration ranges, which he attributed to the effect of pH. In combination, mono- and di-basic potassium phosphate were less effective than either alone. There was little similarity between the methods used by Lin and the methods used in the buffer experiments reported here.

In Lin's experiments, spores were in continual contact with the toxicant and the concentrations of chemicals and spores were much lower than the concentrations used here. In the experiments reported here, spores were in contact with buffered copper sulfate for two hours and no change in pH was observed during this time.

Spores treated at pH 3.8 and 5.8 behaved differently than those treated in a copper sulfate solution in which the pH was unadjusted, but the polymodality of the dosage-response curves was still evident (Figure 4). It was concluded that within the range of pHs tested, pH was not responsible for the shape of the dosage-response curves. Since differences brought about by adding buffers or adjusting the pH of the treatment solutions were so great in comparison with the unadjusted treatment solutions in 1% glucose, it was considered that effects of competition between hydrogen ions and metal ions in the unbuffered treatment solutions must be small.

The possibility was considered that differences in copper uptake at various copper concentrations was responsible for the shape of the dosage-response curve, since the method of treatment and pH differences were not responsible. Spores of the parent Monilinia strain and both strains of Fusarium were treated for two hours then removed from the treatment solution and the copper content of the spores determined as described earlier. Larger quantities of spores were treated for the determination of copper than for spore

germination tests, but the dosage-response relationship was the same.

The uptake of copper by all three fungi was linear (Figure 5). The uptake curves obtained with Monilinia spores should have been polymodal if differences in uptake were responsible for the polymodal dosage-response relationship. Since the uptake of copper was linear, some other factor must be responsible for the polymodal response.

Significantly more copper was taken up by the spores of the copper-tolerant Fusarium strain than by the parent strain. In yeasts, the copper-resistant strains are able to bind more copper than their parent strains (35). Similarly, the tolerant Fusarium strain may be able to bind more copper than its parent strain, such that copper would not be as available for toxic reactions. If copper were precipitated outside the cell by some substance or substances released by the spores, the precipitate could have been carried along with the spores during filtration and expressed as copper uptake. Whether the copper was bound inside the cell or precipitated outside the cell, the copper-tolerant strain would be protected from the toxicity of copper but would show a higher copper concentration in the spores.

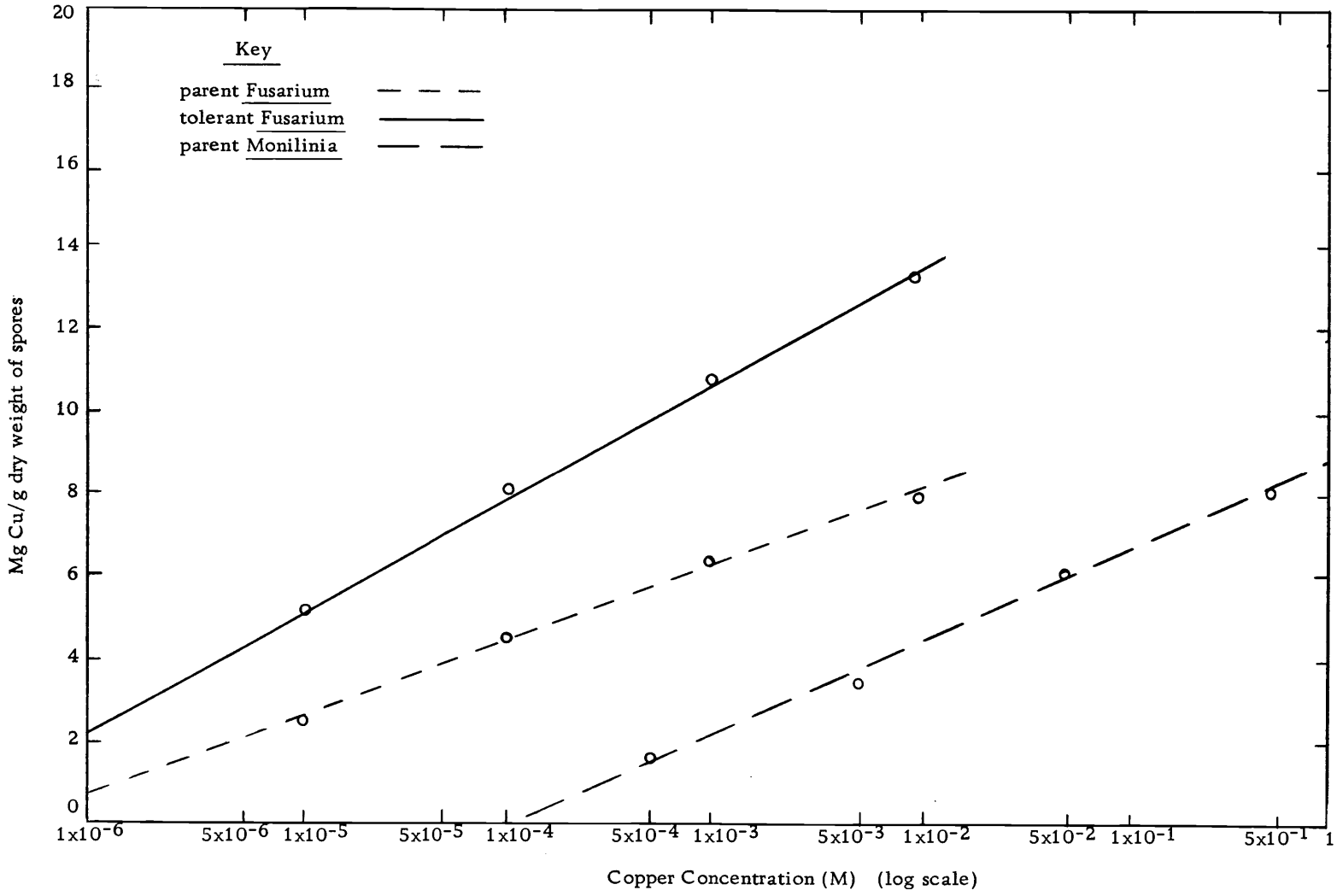


Figure 5. The amount of copper taken up during a two-hour exposure to copper sulfate by spores of the Fusarium strains and the parent Monilinia strain.

Physiological Studies

Spore Exudates

Experiments have shown that spore exudates and metabolic products excreted from fungal mycelia play a role in the resistance of copper in fungi (7, 12). The solution used to collect spores from fungal colonies growing on PDA could contain substances released from the spores or mycelium as well as soluble substances extracted from the medium. Different substances or different amounts of substances released by the spores or mycelium of the parent and tolerant strains could account for the resistance of the tolerant strains so experiments were conducted to determine the effect of the exudates on the inhibition of spore germination by copper.

The exudate solutions were prepared by filtering the spore suspensions collected from fungal colonies on PDA through 0.8μ Millipore filters. The spores on the filters were resuspended in the exudate solution and the concentration adjusted to 100,000 spores/ml. This spore suspension was added in a 1:1 ratio to a copper sulfate solution and mixed for two hours then refiltered. The treated spores were suspended in 1% glucose, placed on nutrient agar to determine inhibition of germination.

The ED_{50} concentration of copper for inhibition of spore germination was higher for all fungi tested when the copper was added to

solutions in which the spores were collected than when the copper was added to a fresh 1% glucose solution (Table 3). Spores from the parent strains were protected from copper more by exudates from the tolerant strains than by their own exudates. Exudates from the tolerant Fusarium strain were also effective in protecting spores of the parent Monilinia strain but not as effective as exudates from either Monilinia strain.

Table 3. The effect of spore exudates on the ED_{50} concentration of copper for inhibition of spore germination.

Source of spore exudate solutions	ED_{50} concentration of copper (μM)		
	Source of spores		
	parent Fus.	tolerant Fus.	parent Mon.
1% glucose	4	100	8
parent Fusarium	230	---	---
tolerant Fusarium	1,300	2,200	700
parent Monilinia	---	---	3,000
tolerant Monilinia	---	---	15,000

These results suggest the presence of a substance or substances in the exudate solutions that provides protection to spores. Either different substances or greater quantities of the same substances may be produced by the tolerant strains. No protection occurred if the spores were treated with the exudates either before or after a copper treatment; thus, the substance or substances probably act by combining with copper outside the cell to make it unavailable to the spores.

Miller (23) showed that the solids in filtrates from suspensions of conidia of several fungal species retarded uptake of fungicides by the conidia of the same or other species. The substances from A. niger were more effective than those from other fungi in retarding uptake of silver. The substances in the exudate solutions from Monilinia and Fusarium could retard uptake of copper in a similar manner. Miller demonstrated that organic acids were an important component of these materials, but their removal did not eliminate the retardation; it only reduced it.

To determine differences in the exudates between parent and tolerant strains, the solutions were concentrated and the components separated by paper chromatography. One-dimensional ascending paper chromatography using the bromphenol blue indicator-solvent system was used for determination of the organic acids. Two acids were detected in the exudates from the Monilinia strains and their concentrations were about equal in both strains. The same two acids were present in smaller amounts in the tolerant Fusarium strain exudates, but none were detected from the parent Fusarium strain exudates.

Glucose was the main component detected on the chromatograms of the simple sugars in the exudates. A ratio of the size of the glucose spot (length x width) between the parent and tolerant strains was calculated. Equal quantities would give a value of 1.0. The glucose

concentrations were about equal in exudates from the Monilinia strains with a calculated value of 0.90, but the exudates from the tolerant Fusarium strain contained more glucose than the parent strain as indicated by the value of 0.25.

More differences between strains were apparent in the amino acids of the exudates than in the simple sugars or organic acids, especially for Fusarium. Nine compounds were separated in the exudates from the tolerant Fusarium strain but only two compounds were detected from the parent strain exudates. These two compounds were present in exudates from both strains. Fifteen compounds were detected in the exudates from the tolerant Monilinia strain and 14 in the exudates from the parent strain. There appeared to be higher concentrations of certain compounds in exudates from the tolerant strains.

Cultures Grown in Liquid Medium

Growth and sporulation. Because isolation of substances produced by fungi would be easier from liquid cultures, further studies were conducted with fungi grown in the defined liquid medium containing copper. The Monilinia strains grew poorly in the liquid medium, so only the Fusarium strains were studied.

Two mycelial plugs of the parent Fusarium strain or three plugs of the tolerant strain growing on PDA were used to inoculate 100 ml

of defined liquid medium containing copper at 0, 0.1, 0.5, 1.0 and 2.0 mM in 250 ml Erlenmeyer flasks. The cultures were grown at room temperature on a rotary shaker. Maximum growth and sporulation of the parent strain occurred in seven days but the copper-tolerant strain did not reach maximum growth until 11 days after inoculation. The cultures were harvested at these times.

Cultures of the parent Fusarium strain produced an average dry weight of mycelium and spores of 0.8686 g/flask and an average of 3,000,000 spores/ml at maximum growth. The corresponding dry weight and spore concentration for the tolerant strain were 0.5644 g/glask and 475,000 spores/ml, respectively.

Total fungal growth of the strains as measured by dry weight was not greatly inhibited by copper at concentrations lower than 1mM (Figure 6), but the tolerant Fusarium strain grew at concentrations exceeding 2mM while there was no growth of the parent strain at 2mM. Sporulation of the tolerant strain was more severely inhibited at lower copper concentrations than the parent strain but the tolerant strain maintained a low level of spore production at copper concentrations that completely inhibited growth of the parent strain. The decreased sporulation of the tolerant strain may be beneficial by allowing nutrients needed for spore production to be utilized for mycelial growth or production of substances that can bind with copper.

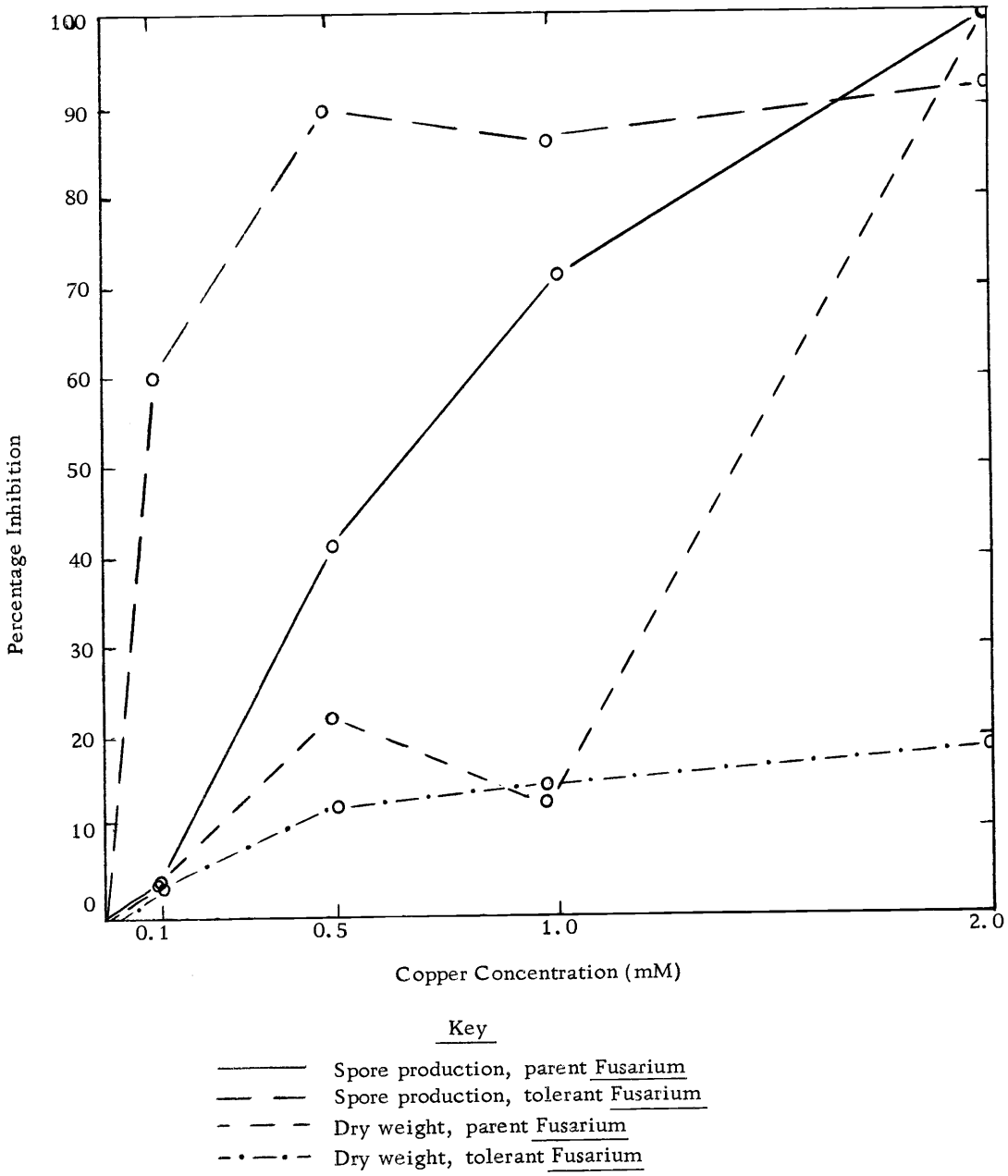


Figure 6. The effect of copper sulfate on dry weight of spores and mycelium and spore production of the Fusarium strains in liquid culture.

Culture filtrates: The culture filtrates obtained by removal of spores and mycelium from the Fusarium liquid cultures were dried and fractionated into two portions, one soluble in 80% ethanol and a water-soluble portion. Ethanol was used to separate amino acids from proteins.

The two fractions were tested for their ability to protect spores of the parent strain from inhibition of germination by copper. All four fractions reduced the toxicity of copper by a significant amount, but more reduction occurred with the fractions from the tolerant strain than the parent strain (Figure 7). The slope of the dosage-response curves with the water-soluble and ethanol-soluble fractions were different. More protection was provided by the water-soluble fraction. The dosage-response curves with the ethanol-soluble fractions were similar to those obtained with spore exudates.

The ultraviolet absorption spectrum for each of the four fractions from the culture filtrates was determined (Figure 8). The ethanol-soluble fraction from the copper-tolerant strain had the most distinctive spectrum with peaks at 265, 270, and 276 mμ. The spectrum for the water-soluble fraction of the filtrate from the tolerant strain contained the same peaks, but in less quantity which suggests that the substance or substances responsible for the peaks were not completely extracted with 80% ethanol. The fractions from filtrates of the parent strain had no peaks at these wavelengths. The spectra

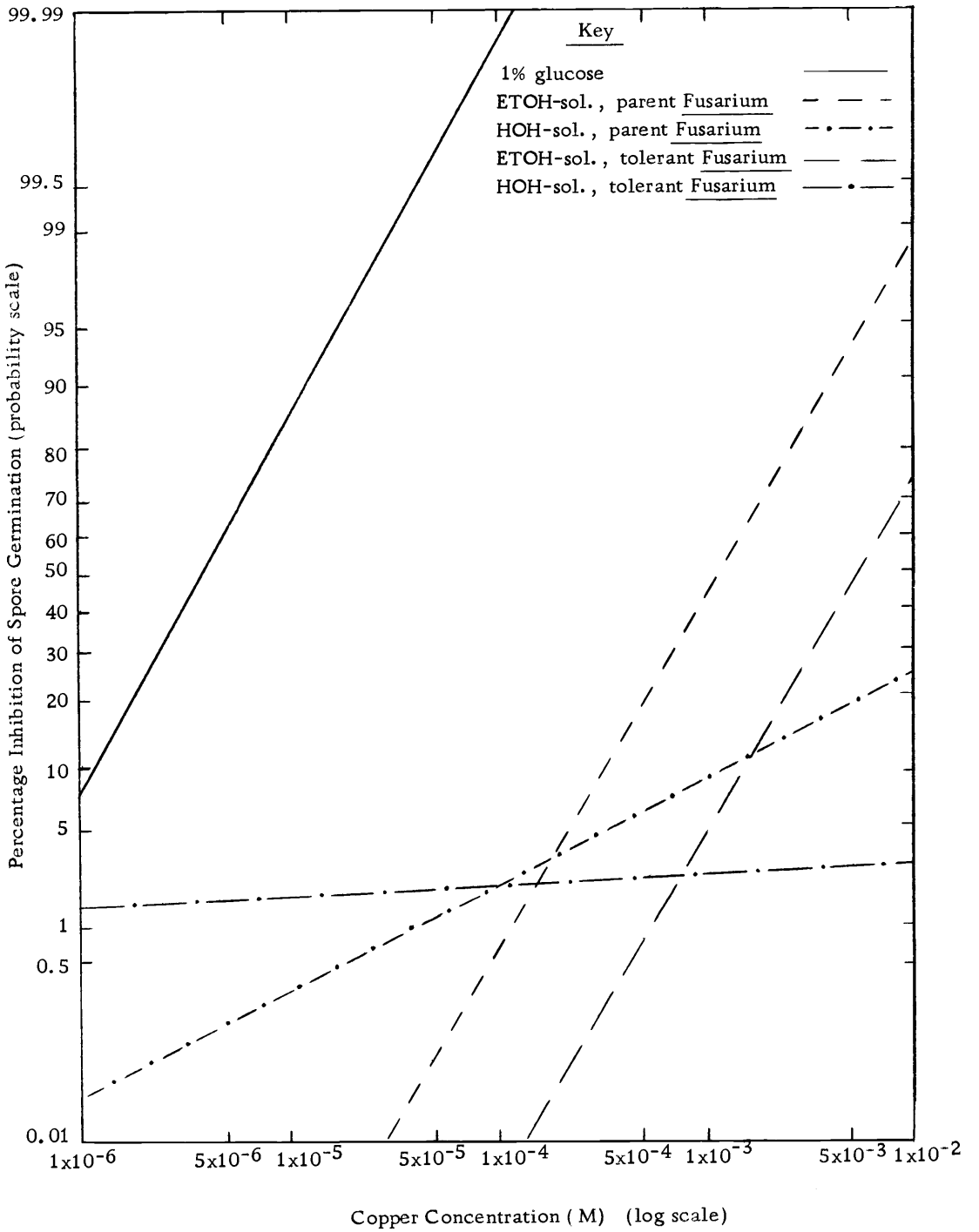
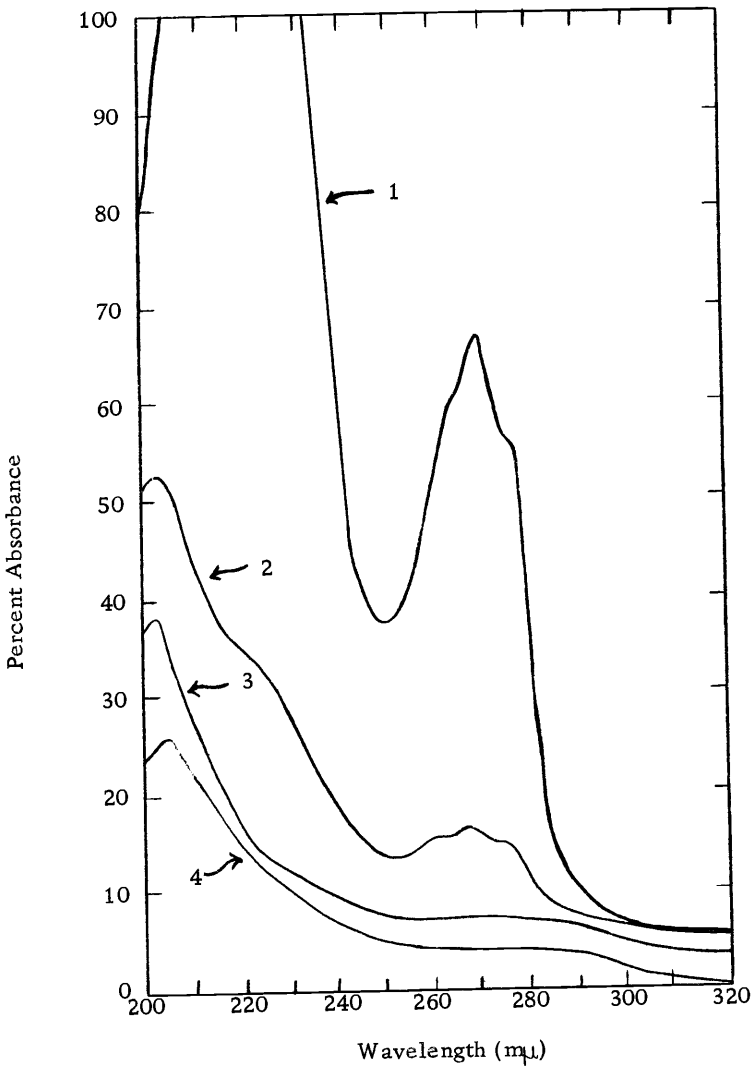


Figure 7. The effects of ethanol- and water-soluble fractions of filtrates from liquid cultures of Fusarium strains on inhibition of spore germination by copper.



Key

1: Ethanol-soluble fraction, tolerant Fusarium
2: Water-soluble fraction, tolerant Fusarium
3: Water-soluble fraction, parent Fusarium
4: Ethanol-soluble fraction, parent Fusarium

Figure 8. Ultraviolet absorption spectra of fractions from liquid culture filtrates of copper-tolerant and parent strains of Fusarium.

show that a different substance or substances are produced by the tolerant strain, but it is not known if these are responsible for protection of the spores from inhibition of germination by copper.

Amino acid determinations: Since amino acids differed greatly in the spore exudate solutions, the amino acid production in liquid culture was investigated. The total amount of amino acids in the culture filtrates and the amount of free and bound amino acids in the mycelium and spores of both strains of Fusarium were determined colorimetrically. The amount of amino acids/g dry weight of mycelium and spores from the tolerant strain was higher than from the parent strain in all cases (Table 4). The amino acid concentration in all fractions was increased by treatment with copper except for a decrease of about 100 mg leucine equivalents in the free amino acid content of the mycelium and spores of the tolerant strain. The free amino acid content remained nearly constant in the parent strain.

Table 4. The effect of copper on amino acid content of culture filtrates and mycelium and spores of parent and copper-tolerant strains of Fusarium.

	Amino acid content (mg leucine equivalents/g dry weight)			
	parent		tolerant	
mM Cu	0	1.0	0	1.0
filtrate (total)	1.0	14.0	9.7	128.5
mycelium and spores (total)	179.1	204.4	420.8	439.4
free amino acids	29.5	30.7	150.6	50.1
bound amino acids	149.6	173.7	270.2	389.3

Paper chromatography was used to follow changes in the amino acid content of untreated culture filtrates during the growth experiments. Culture filtrates were collected at daily intervals, concentrated and the amino acids separated by one-dimensional descending chromatography using the butanol:acetic acid:water (4:1:1) solvent system.

After three days of growth, culture filtrates from the parent strain contained seven substances that produced a yellow color with ninhydrin similar to proline and hydroxyproline, but their Rf values did not correspond to these amino acids. No distinct purple spots were detected. The culture filtrates from the tolerant strain contained four substances which gave a yellow reaction but the concentration was less than in the parent strain filtrates. The parent Fusarium strain produced eight different amino acids in the culture filtrates and no substances with a yellow reaction, but the tolerant strain produced 11 amino acids and four substances with a yellow reaction at the time of maximum growth.

Some of the compounds were identified by co-chromatography with known amino acids or by comparison with published Rf values (10) (Table 5). Many ninhydrin positive compounds detected on the chromatograms could not be identified by either of these two methods and were not included in the table.

Table 5. Amino acids present in culture filtrates and ethanol extracts and acid hydrolysates of mycelium and spores from the two Fusarium strains.

	filtrate				eth. extract				hydrolysate			
	par.		tol.		par.		tol.		par.		tol.	
Amino acids	1	2	1	2	1	2	1	2	1	2	1	2
cysteine	+	+	+	+	+	+	+	+	+	+	+	-
cystine	-	-	-	-	+	-	+	-	+	+	+	-
asparagine	+	-	+	-	+	+	+	-	-	-	-	-
arginine	+	-	+	+	-	+	-	+	+	+	+	+
aspartic acid	-	-	-	-	+	+	+	+	-	-	-	-
serine-glycine	-	-	+	-	+	+	+	+	+	+	+	+
threonine	-	-	+	-	+	+	+	+	+	+	+	+
alanine-tyrosine	-	-	+	-	+	+	+	+	+	+	+	+
gamma amino butyric acid	-	-	-	-	+	-	-	-	-	-	-	-
valine	-	-	+	+	+	-	+	+	+	+	+	+
leucine	-	-	+	+	+	-	+	+	+	+	+	+
glutamic acid	-	-	-	-	-	-	-	-	+	+	+	+
proline	-	-	-	-	-	-	-	-	+	+	+	+
lysine	-	-	-	-	-	-	-	-	+	+	+	+

par: parent strain
tol: tolerant strain
1: untreated
2: 1.0 mM copper
+: present
-: absent

Besides the decrease in identified amino acids from the untreated parent Fusarium strain, there was a decrease in unidentified compounds from five to two by treatment with copper. In filtrates from the tolerant strain there were in addition to the identified amino acids one unidentified compound not present in the 1mM copper treatment, nine unidentified compounds in the filtrates from the 1mM treatment not present in the filtrates from the untreated cultures,

and two unidentified compounds present in both treated and untreated culture filtrates. The total number of amino acids in the culture filtrates increased in the tolerant strain from 11 to 15 with treatment by 1mM copper.

The total number of free amino acids in the spores and mycelium of both parent and tolerant strains was decreased by treatment with copper. The number of compounds decreased from 14 to 9 in the parent strain and from 16 to 12 in the tolerant strain. Nearly all free amino acids changed in concentration, either decreased or increased, following treatment with copper and the same pattern existed with both strains. The only exception was an increased concentration of aspartic acid in the parent strain and a decreased concentration in the tolerant strain following copper treatment. The parent strain contained large quantities of gamma amino butyric acid when grown without copper, but with 1mM of copper in the medium there was none. Small amounts may have been present in the tolerant strain, but identification was doubtful because of the faintness of the spots. Gamma amino butyric acid is synthesized from aspartic acid, so inhibition of the synthesis of gamma amino butyric acid should result in an increased concentration of aspartic acid.

There was little change in the bound amino acids of either strain. Three unidentified compounds were produced by the parent strain in response to copper treatment. The number of bound amino

acids increased with copper treatment of the parent strain from 13 to 16 but the number in the tolerant strain decreased from 14 to 12.

The greater amount and number of amino acids produced by the tolerant strain than by the parent strain in the culture filtrates suggests the possibility that the amino acids are responsible for protection of the tolerant strain from inhibition of spore germination by copper. Differences in the individual free or bound amino acids were not great, so probably the increased concentration is of more importance for resistance to copper than are the individual amino acids.

Determination of simple sugars: The culture filtrates obtained at daily intervals during growth studies of the two Fusarium strains were chromatographed to determine if any differences could be detected in the utilization of glucose or the release of simple sugars into the medium. The simple sugars were separated by one-dimensional descending chromatography in butanol:acetic acid:water (4:1:1). No differences could be detected and the chromatograms only documented the fact that glucose was utilized by the fungi. At the time of maximum growth only traces of glucose remained in the culture filtrates.

The total amount of simple sugars in the filtrates from cultures treated with copper sulfate increased with increasing concentrations of copper. The filtrates from the untreated parent strain contained 30 mg of glucose equivalents and 67 mg of glucose equivalents with the 1mM treatment. The tolerant strain had 68 mg in the filtrates

without copper and 104 mg in the filtrates from the 1mM treatment. These figures probably only reflect the amount of growth of the fungi and do not indicate any change in metabolism of glucose as the result of copper treatment.

Determination of copper: The concentration of copper in the culture filtrates and the ethanol extracts and acid hydrolysates of the spores and mycelium of the two Fusarium strains was determined to detect possible differences in uptake of copper between the two strains. The copper concentration remaining in the culture filtrates of both strains was nearly equal with the 1mM treatment; 4.69 mg for the parent strain and 4.71 mg for the tolerant strain. However, the amount of copper extracted with the free amino acids at this treatment level was nearly twice as much for the tolerant strain as for the parent; 3.50 mg versus 1.80 mg/g dry weight. This suggested that copper was bound to soluble compounds within the cell rather than to cell structures or enzymes. The reverse was probably true of the copper in the parent strain since more copper, 2.96 mg/g dry weight, was contained with the bound amino acids from the 1mM treatment than with the same treatment of the tolerant strain which contained 2.17 mg copper/g dry weight. Although the amount of copper taken up by the fungi was nearly equal, the distribution of the copper within the spores and mycelium was different between the two strains. Because of the greater concentration of free amino acids in

the tolerant strain, it would seem likely that the copper is bound by the free amino acids in the cell, and thus contributes to its tolerance to copper.

DISCUSSION

Ashida (2) suggested two ways to measure the resistance which fungi develop toward a toxic agent. First, there can be an increase in the toxicant concentration to obtain a standard response, and second, a standard concentration may be less toxic. Toxic action can be measured by any of several fungal responses (e. g. , killing of mycelium or spores, inhibition of growth or spore germination, inhibition of some growth habit such as sporulation, or the inhibition of metabolic activity). The strains of Fusarium and Monilinia that developed tolerance to copper were resistant in nearly all of these ways. The copper concentration had to be increased to kill mycelium and spores, and growth and spore germination occurred at higher copper concentrations than with the parent strains.

The mechanism of tolerance to copper may be different in Monilinia and Fusarium. The difference in the type of dosage-response curves for the inhibition of spore germination with a two-hour copper treatment, (polymodal for Monilinia and nearly linear for Fusarium), strongly suggests this. Exudates from spores may affect the toxicity of copper (7, 12) and differences in the amino acid content of exudates were observed between the species and between the strains of Monilinia and Fusarium. More copper tolerance was developed by Fusarium than by Monilinia even though Monilinia was originally more tolerant to copper.

Horsfall (12) stated that fungi that secrete acid into the medium on which they grow, (e.g., Monilinia and Aspergillus), are less susceptible to copper than a fungus like Stemphylium that does not. From this, Stemphylium should be inhibited at lower copper concentrations than the parent strain of Monilinia, but when spores were continuously treated with copper there was little difference in the resistance to the fungistatic action of copper by the parent Monilinia strain and Stemphylium. However, Stemphylium was more tolerant of the fungicidal action of copper than Monilinia.

Excretion of organic acids into the growth medium would probably affect fungistatic action most. If fungistasis occurs at the cell surface (42), competition between hydrogen and metal ions would be greatest at this site. There was no difference in the pH of treatment solutions during the two-hour treatment period, and thus, it is unlikely that enough acid was produced in this time to affect the adsorption of copper. Furthermore, there was no difference in the resistance of Stemphylium and the parent strain of Monilinia when they were grown on copper-containing medium. Apparently, organic acid production is not the reason for differences in tolerance to copper with these fungi.

Miller and McCallan (24) published data showing the percentage germination of M. fructicola spores treated with copper for varying lengths of time. The inhibition of germination presented in their

data for a two-hour treatment was similar to the data obtained here when converted to comparable units (μg Cu/g spores), but their experiments only covered the lower part of the curve between 0.1 and 1μM. Therefore, they missed the polymodal region that occurs at higher concentrations. In the same experiments, Miller and McCallan determined that there was an increased amount of phosphorus released from the spores in response to copper. This was interpreted as an indication of increased membrane permeability of the spores. Since the conditions of their experiments and the results of inhibition of spore germination they obtained were similar to the work reported here, phosphorus may also have been released in a similar manner. Their data showed that the maximum amount of phosphorus released (3.1%) occurred at a concentration of 800 μgCu/g spores, but at copper concentrations of 50 and 1,600μg Cu/g spores only 1.1% of the phosphorus was released. Perhaps the variation noted in phosphate release with increasing copper concentrations can occur with other compounds released by spores. If these compounds can react with copper to form compounds of different toxicity, this might account for the polymodal curve of Monilinia.

Most researchers working with the fungitoxicity of copper and other metals have apparently assumed that only a linear dosage-response relationship was possible and they have not used a wide enough concentration range to detect the polymodal curve. However,

there are indications that a bimodal dosage-response curve could be possible with metal toxicants. The dialkyl dithiocarbamates and oxine have polymodal dosage-response curves that are due to differences in solubility and toxicity of their 1:1 and 1:2 complexes with copper or other metals (7, 37). Polymodal dosage-response curves are characteristic of most chelating agents, although they may not be obtained in some cases because of high toxicity, insolubility, instability, or some other property of the chelating agent (7).

If chelating agents produce polymodal curves, then the metal should induce a similar response when combined with cell metabolites that chelate copper (7). This could explain the polymodal dosage-response curves obtained with Monilinia, Aspergillus, and Stemphylium.

The numerous enzyme systems and the many different compounds within fungal spores with which copper could react make it extremely difficult if not impossible to determine the specific reactions of copper in fungal cells. At the relatively high copper concentrations required for significant inhibition, the copper interferes with the determination of copper compounds in cells and in compounds released from the cell, and when spores or mycelium are disintegrated, releasing the cell contents, copper can be exchanged between compounds such that identification of the original compounds is impossible.

Martin, Wain and Wilkinson (17) investigated the fungitoxicity of a large number of copper compounds and found that the dosage-response curves were not always linear. Some compounds had tendencies toward a double slope. This double slope was also observed by Parker-Rhodes (39) and McCallan, Wellman and Wilcoxon (19). This suggested to them that compounds were formed which were either more toxic or less toxic than the cupric ion. Lin (15) observed that the toxicity of copper to the spores of M. fructicola was altered by the addition of neutral salts to the copper treatment solution. A bimodal response occurred when the salt concentration was increased with a constant copper concentration. However, the response to increasing copper concentrations was not bimodal.

Water-soluble substances capable of reducing the toxicity of copper were released into the growth medium by Monilinia and Fusarium. Copper-amino acid compounds tested by Martin and coworkers (17) and Parker-Rhodes (39) were generally less toxic than copper sulfate, so with a substantial concentration of amino acids in the treatment solution, protection of the spores from inhibition by copper would occur. The enhanced release of amino acids in the exudates probably was responsible, at least in part, for the resistance of the tolerant strains to copper.

More copper was extracted from the mycelium and spores of the tolerant strain of Fusarium with ethanol than from the parent

strain. This may indicate that more copper can be bound by the high levels of free amino acids in the tolerant cells than in the cells of the parent strain, and thus, less copper would be available to inhibit enzymes or combine with essential metabolites. It can be concluded that the combination of increased amino acids in the culture filtrates and in the mycelium and spores could be responsible for the resistance of Fusarium to copper.

The conclusions of Siegel and Crossan (41), Tandon and Chandra (45), and Murayama (27, 28) were that copper interferes with the ability of organisms to replenish the amino acids in the amino acid pool. Murayama concluded that copper-resistant yeast could maintain the concentration of free amino acids even in the presence of copper. A decrease in the numbers of amino acids in the amino acid pool occurred with the parent Fusarium strain following copper treatment, but the tolerant strain failed to give this response, and thus, the same conclusions could apply. The tolerant strain could be more resistant because it can maintain the level of amino acids required or because it produces an excess of amino acids that are capable of binding copper and preventing its toxicity.

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