Effect of pH on Toxicity of Copper to *Scytalidium* sp., a Copper-tolerant Fungus, and Some Other Fungi

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SUMMARY

Scytalidium sp. grew in acidic media (pH 2.0 to 0.3) saturated with copper sulphate (approx. 1 M) but was sensitive to low concentrations of copper (4×10^{-5} M) near neutrality. Eleven other fungi differed in that they tolerated relatively high concentrations of copper near neutrality; nine tolerated high concentrations from pH 3.0 to 7.0, and six tolerated 4×10^{-3} M-copper sulphate at pH 2.0 to 7.0. Oxine was more toxic to *Scytalidium* sp. at neutrality than it was at an acid pH and in the presence of relatively large amounts of copper. EDTA had no appreciable effect on toxicity of copper. The fungus was relatively tolerant to chlorides of iron, cobalt and chromium at both neutral and acid pH.

INTRODUCTION

Although copper is toxic to most fungi (Horsfall, 1956), some fungi are remarkably tolerant to it. Cultures of *Penicillium* and *Aspergillus* species can grow in media saturated or nearly saturated with copper sulphate (Trabut, 1895; Pulst, 1902; Bedford, 1936; Hubert, 1938; Sato, 1939; Jurkowska, 1952). Other fungi can grow in media containing from 1% to 15% copper sulphate (Brooks, 1914) or copper ammonium citrate (Villedieu & Villedieu, 1920). Bacteria are less tolerant to copper, but copper tolerance of the autotrophic sulphur bacteria *Thiobacillus thiooxidans*, *T. ferrooxidans* and *T. concretivorus* is outstanding in that adapted strains withstand up to 0.12 to 0.23 M-copper sulphate (Booth & Mercer, 1963). The first two cultures oxidize copper sulphide ores, so resulting in production of acid, which dissolves the copper (Bryner, Beck, Davis & Wilson, 1954; Marchlewitz, Hasche & Schwarz, 1961; Sutton & Corrick, 1963; Kuznetsov, Ivanov & Lyalikova, 1963).

Starkey & Waksman (1943) reported that two fungi, isolated from acid solutions (pH 0.2 to 0.7) containing 4% copper sulphate, could grow in media saturated with copper sulphate and having a pH close to 0. Studies of the influence of pH on toxicity of copper have been made with one of these cultures, a species of *Scytalidium* (Ellis, 1971) previously designated Fungus D, as well as other fungi. The effect of the chelating agents, oxine (8-hydroxy-quinoline) and EDTA (ethylenediaminetetra-acetic acid) on toxicity of copper to *Scytalidium* sp., and the influence of salts of iron, cobalt and chromium on development of the fungus, have been determined.

METHODS

Cultures of the *Scytalidium* sp. have been deposited in the following culture collections: Commonwealth Mycological Institute, Surrey; Centraalbureau voor Schimmelcultures,

Baarn, Netherlands; American Type Culture Collection, Rockville, Maryland, U.S.A.; Northern Marketing and Nutritional Research Division, A.R.S., U.S.D.A., Peoria, Illinois, U.S.A.; Mold Herbarium and Culture Collection, University of Alberta, Edmonton, Alberta, Canada (no. UAMH 3489). J. W. Carmichael of the University of Alberta identified Fungus D as a new species of the genus *Scytalidium* and intends to publish the description.

The fungus was maintained on the following medium (g/l): glucose, 10; $(\text{NH}_4)_2\text{SO}_4$, 0.5; KH_2PO_4 , 0.5; K_2HPO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; distilled water to 1000 ml. The glucose was sterilized separately. The fungus grew well in this medium with initial pH values of 7.0 to 0.5, and made some growth at pH 0. The medium became increasingly acid during growth; as tolerance to copper was greater at low pH, it was difficult to establish the influence of pH on copper toxicity. To overcome this difficulty, NaNO_3 containing an equivalent amount of nitrogen was substituted for $(\text{NH}_4)_2\text{SO}_4$ in media for cultivation of *Scytalidium* sp. except in the first experiment (Table 1). The copper was added as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. When oxine was used it was dissolved and sterilized in ethanol, and appropriate dilutions in water were added to sterile media. The 125 ml Erlenmeyer culture flasks contained a final volume of 50 ml of medium after all constituents had been added. The media were inoculated with portions of 7- to 14-day-old fungus pellicles grown on the glucose-nitrate solution medium. Preliminary tests provided no evidence that cultivation of *Scytalidium* sp. in medium containing 0.1 M-copper sulphate increased its tolerance to copper. The inoculated media were static during incubation at 28 °C.

The effect of copper on growth of the following filamentous fungi was determined also: *Aspergillus flavus*, *A. niger*, *A. oryzae*, *Penicillium notatum*, *P. simplicissimum*, *Trichoderma koningi*, *Fusarium oxysporum*, *F. culmorum*, *Monilinia fruticola*, *Stemphylium sarcinaeforme* and *Rhizopus nigricans*. The cultures of *M. fruticola* and *S. sarcinaeforme* were generously provided by J. G. Horsfall of the Connecticut Agricultural Experiment Station, New Haven, Connecticut, U.S.A. The other fungi came from the culture collection of the Department and most had been isolated from soil. Some of these fungi grew poorly or produced acid when grown in the glucose-nitrate medium. The following changes in medium composition resulted in good growth, and the pH of the media increased during growth. In the medium for *A. niger* and *R. nigricans* 0.1 % (w/v) L-asparagine was substituted for nitrate and 1.0 % (w/v) Na-lactate and 0.5 % (w/v) glucose were the sources of carbon. For *M. fruticola* 0.1 % (w/v) L-asparagine was used as the source of nitrogen, 1.0 % (w/v) Na-succinate and 0.5 % (w/v) glucose were the sources of carbon and 0.05 % (w/v) yeast extract was added. For *S. sarcinaeforme* 0.1 % (w/v) L-asparagine was used as the source of nitrogen.

Since addition of relatively large amounts of copper sulphate increased the acidity of the media, alkali was added to adjust the pH. Much of the copper in media with more than 4×10^{-5} M-copper sulphate and having pH 5.0 to 7.0 became precipitated as copper hydrate, and there was some precipitate at pH 4.0 to 5.0. To avoid interference from the precipitate when the mycelia were harvested, 3 to 10 ml of 1 M-HCl was then added to the culture medium to dissolve the precipitate. The mycelium was collected on tared filter paper and was thoroughly washed before being dried to constant weight at 110 °C. The values for cell weight are averages of closely agreeing duplicate cultures. The cultures were generally incubated for 4 to 6 weeks.

RESULTS

Effect of nitrogen sources on changes in pH and copper toxicity

Scytalidium sp. grew at all the pH values tested in the absence of copper, and in the media with ammonium nitrogen that contained copper (Table 1). The metal was somewhat toxic

Table 1. Influence of source of nitrogen on change in reaction resulting from growth of *Scytalidium* sp., and its effect on toxicity of copper sulphate

The cultures were incubated 50 days.											
(NH ₄) ₂ SO ₄ nitrogen						NaNO ₃ nitrogen					
No copper			10 ⁻² M-CuSO ₄			No copper			10 ⁻² M-CuSO ₄		
pH		Growth (mg dry wt)	pH		Growth (mg dry wt)	pH		Growth (mg dry wt)	pH		Growth (mg dry wt)
Initial	Final		Initial	Final		Initial	Final		Initial	Final	
6.5	3.0	98	6.5	5.0	28	6.9	7.6	71	7.0	6.7	0
5.2	2.4	140	5.8	3.9	156	5.5	7.0	108	5.9	5.7	0
4.5	2.3	152	3.8	3.2	190	4.9	7.2	135	4.4	4.4	0
3.9	2.3	143	2.9	2.6	188	4.1	7.3	160	3.1	3.9	50
2.8	2.1	142	2.7	2.2	190	3.0	7.1	172	2.8	3.7	85
1.7	1.5	155	2.0	1.7	181	2.0	2.3	121	2.1	2.8	147

Table 2. *Influence of reaction on toxicity of copper sulphate to Scytalidium sp.*

NaNO₃ was the nitrogen source. The cultures were incubated 34 days.

Copper sulphate (molarity)	pH		Growth (mg dry wt)
	Initial	Final	
0	6.8	7.7	151
4×10^{-6}	6.9	7.5	125
4×10^{-5}	6.7	6.8	11
4×10^{-4}	6.7	6.7	0
0	3.7	7.0	136
4×10^{-7}	3.9	7.0	90
4×10^{-6}	3.9	6.6	55
4×10^{-5}	3.9	6.3	26
4×10^{-4}	4.0	4.7	0
0	2.9	7.5	158
4×10^{-5}	2.8	7.4	133
4×10^{-4}	3.0	4.7	21
4×10^{-3}	2.9	4.2	28
4×10^{-2}	2.8	3.1	10
0	2.1	2.3	103
4×10^{-2}	1.9	2.0	89

near neutrality in the ammonium medium. The fungus did not grow at pH 4.4 or above in the nitrate media containing copper and its growth was restricted at initial pH values of 2.8 and 3.1, but here growth resulted in an increase in pH to nearly 4.0, which may have prevented further development.

Effect of pH on toxicity of copper to Scytalidium sp.

Copper tolerance of this fungus is not lost readily, as there was no apparent change in resistance to high concentrations of copper in more than 20 years of cultivation on a medium devoid of added copper. A survey by Ashida (1965) indicated that acquired resistance to copper is relatively stable but that some cultures lost resistance when cultivated in absence of copper.

Scytalidium sp. was more resistant to copper in acid media (Table 2). The pH increased during growth in the nitrate-containing media except at initial pH values close to 2.0. Copper concentrations greater than 4×10^{-6} M were strongly toxic or inhibitory near neutrality, although under these conditions much of the copper was precipitated, reducing the amount of soluble copper.

Toxicity of copper to various fungi

The effect of pH on copper toxicity for other fungi differed from that for *Scytalidium sp.* Whereas *Scytalidium sp.* failed to grow at neutrality or pH 4.0 in media containing 4×10^{-4} M-copper, all of the following fungi grew under these conditions (Table 3): *Aspergillus flavus*, *A. niger*, *Penicillium notatum*, *P. simplicissimum*, *Trichoderma koningi*, *A. oryzae*, *Fusarium culmorum*, *F. oxysporum*, *Rhizopus nigricans*, *Monilia fructicola*, *Stemphylium sarcinaeforme*. The first five cultures grew in media containing 4×10^{-4} to 4×10^{-2} M-copper and having pH values of 2.0 to 7.0, except *P. notatum* that failed to grow at pH 2.0. The other six fungi were somewhat less tolerant to copper. The amount of growth decreased with increasing concentrations of copper sulphate. As with *Scytalidium sp.*, the pH rose appreciably during growth in media having initial pH values of 7.0, 4.0 and 3.0.

Table 3. Influence of copper sulphate and pH on growth (mg dry wt) of various fungi
The average incubation period was 38 days.

Initial pH	Copper sulphate (molarity)	Penicillium												Stemphylium sarcinae-forme	
		<i>Aspergillus flavus</i>	<i>Aspergillus niger</i>	<i>Penicillium notatum</i>	<i>Penicillium simplicissimum</i>	<i>Trichoderma koningi</i>	<i>Aspergillus oryzae</i>	<i>Fusarium culmorum</i>	<i>Fusarium oxysporum</i>	<i>Rhizopus nigricans</i>	<i>Monilinia fructicola</i>				
7.0	0	114	115	55	100	163	150	128	113	93	62	165			
	0.0004	126	107	96	106	146	155	126	127	108	76	149			
	0.004	103	100	87	98	153	127	90	106	80	80	16			
	0.04	97	28	91	56	121	65	0	0	16	0	0			
4.0	0	135	152	94	96	142	150	125	108	119	82	125			
	0.0004	112	157	92	107	134	141	135	88	106	104	163			
	0.004	123	121	84	95	114	0	0	31	56	80	0			
	0.04	87	28	65	38	112	0	0	0	0	0	0			
3.0	0	136	173	65	96	154	146	105	96	128	57	157			
	0.0004	134	177	85	99	117	119	59	90	110	110	0			
	0.004	75	112	107	94	102	35	18	0	67	50	0			
	0.04	72	65	40	130	106	0	0	0	0	0	0			
2.0	0	53	153	0	88	134	112	0	70	0	20	0			
	0.0004	53	158	0	83	132	106	0	53	0	31	0			
	0.004	48	154	0	74	144	81	0	0	0	21	0			
	0.04	34	140	0	65	76	0	0	0	0	0	0			

Table 4. *Influence of oxine and copper sulphate on growth (mg dry wt) of Scytalidium sp.*

The cultures were incubated 41 days.

Copper sulphate (molarity)	Initial pH	Oxine (molarity)				
		0	7×10^{-7}	7×10^{-6}	7×10^{-5}	7×10^{-4}
0 to 4×10^{-4}	7.0	105	0	0	0	0
0	4.0	135	166	0	0	0
4×10^{-6}			153	0	0	0
4×10^{-5}			34	0	0	0
4×10^{-4}			0	0	0	0
0	3.0	143	175	146	0	0
4×10^{-6}			173	104	0	0
4×10^{-5}			160	49	0	0
4×10^{-4}			20	30	0	0
0	2.0	83	101	103	86	68
4×10^{-8}			105	58	83	41
4×10^{-7}			101	56	90	54
4×10^{-6}			98	54	87	42
4×10^{-5}			95	66	84	25
4×10^{-4}			96	43	93	0

Unlike *Scytalidium* sp., all of the other fungi grew at neutrality in media with 4×10^{-3} M-copper and six of the eleven developed at neutrality with 4×10^{-2} M-copper. None showed increased tolerance to copper with increase in acidity of the media. They were less tolerant to acidity than was *Scytalidium* sp., which grew in media as acid as pH 0 in absence of copper sulphate and at pH 0.3 in a medium saturated with the salt (Starkey & Waksman, 1943). None of the other fungi grew at pH 1.0 even in the absence of copper, and 4 cultures failed to develop at pH 2.0.

Effect of oxine on copper toxicity for Scytalidium sp.

Oxine was toxic in the absence of copper sulphate (Table 4), more so at higher pH values. Comparison of the data in Tables 2 and 4 indicates that oxine toxicity was dominant where both oxine and copper were present. Wherever there was growth, the pH rose as in previous experiments. Based on chelation of one atom of copper by 2 molecules of oxine (1:2 complex), there was more than sufficient oxine to chelate all of the copper at one or more concentrations of copper sulphate at each oxine level.

Tests were made also with the chelating agent EDTA with both Na-EDTA and Cu-EDTA at concentrations of 3×10^{-3} and 3×10^{-4} M. There was no evidence of reduction of copper toxicity by EDTA for *Scytalidium* sp. in media containing 4×10^{-3} M-copper and having pH values of 0.5, 2.0, 3.0 and 6.0. Furthermore, there was no evidence of toxicity of the chelating agent at these pH values in the absence of copper sulphate except that 3×10^{-3} M-Na-EDTA inhibited growth at pH 3.0.

Effects of salts of other heavy metals on Scytalidium sp.

The fungus was grown in the glucose-nitrate medium containing several concentrations of the following compounds and at pH 7.0, 4.0, 3.0, 2.0 and 1.0: $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$. In the media with high concentrations of ferric and chromic chlorides, there was considerable precipitation except at the most acid pH values. Table 5 shows that the

Table 5. Growth (mg dry wt) of *Scytalidium* sp. in media with salts of heavy metals

Metal salts were added to the medium as indicated. The cultures were incubated 28 days.

Concn of metal salt (molarity)	Initial pH				
	7.0	4.0	3.0	2.0	1.0
0	135	95	182	80	115
FeCl ₃ ·6H ₂ O					
5 × 10 ⁻⁴	129	150	196	103	147
5 × 10 ⁻³	113	216	235	167	61
5 × 10 ⁻²	41	28	45	142	27
CoCl ₂ ·6H ₂ O					
5 × 10 ⁻⁷	129	148	161	133	92
5 × 10 ⁻⁶	91	130	178	122	60
5 × 10 ⁻⁵	11	46	75	131	101
5 × 10 ⁻⁴	0	0	0	0	0
CrCl ₃ ·6H ₂ O					
5 × 10 ⁻⁷	119	121	123	53	116
5 × 10 ⁻⁶	82	90	125	40	112
5 × 10 ⁻⁵	65	43	149	43	108
5 × 10 ⁻⁴	48	41	158	69	122
5 × 10 ⁻³	49	30	102	153	121

fungus was tolerant to relatively high concentrations of salts of these metals at all pH values. Cobalt was more toxic than iron or chromium. As in previous experiments growth in media with initial pH values of 3.0, 4.0 and 7.0 resulted in marked increase in pH.

DISCUSSION

The unusual tolerance of *Scytalidium* sp. to copper was confined to acid media, as it grew in media saturated with copper sulphate at pH 2.0 to 0.3 (Starkey & Waksman, 1943), but it made practically no growth in media containing 4×10^{-4} M-copper with initial pH values of 4.0 or above. In media of pH 3.0 containing 10^{-4} M-copper sulphate, the pH rose to less than 5.0, but at lower concentrations of copper the pH rose much higher and there was more growth. The pH range of 4.2 to 5.0 appears to be the critical one for copper toxicity and possibly reflects the effect of pH on the uptake of copper, so that above pH 4.2 to 5.0 copper could penetrate the cell but at the lower pH values copper uptake is reduced or ceases. Among the factors responsible for development of copper tolerance by fungi and yeasts, Ashida (1965) mentioned reduction in permeability, production of copper-binding compounds such as cysteine, and changes in certain metabolic and enzymatic activities. Possibly the reported tolerance of copper by the acidophilic cultures of sulphur bacteria of the genus *Thiobacillus* (Booth & Mercer, 1963) is also caused by a lack of copper absorption at low pH levels.

The effect of pH has seldom been considered in studies of toxicity of copper to fungi. Bedford (1936) reported that a species of *Penicillium* was not affected appreciably by high concentrations of copper sulphate between pH 2.5 and 6.4 but that there was no growth in alkaline media. I have found that various fungi, other than *Scytalidium* sp., tolerated high concentrations of copper in neutral and acid media. On the basis of development of pregrown mats of *Penicillium lycopersici*, Scott (1929) concluded that toxic cations, such as copper, were most inhibitory at pH greater than 5.5. From such results, Scott (1929) and Horsfall

(1956) concluded that low pH lowers absorption of copper by fungi and so increases tolerance.

It is generally agreed (Martell & Calvin, 1952; Albert, Gibson & Rubbo, 1953; Albert, Hampton, Selbie & Simon, 1954; Block, 1955; Horsfall, 1956; Albert, 1958, 1968) that copper exerts its toxic effects within the cell, and Somers (1963, 1966) concluded that it enters the cell in combination with a carrier. His observation that pretreatment of conidia of *Neurospora crassa* with 0.1 M-HCl reduced uptake and toxicity of copper, supports this conclusion. Unlike *Scytalidium* sp., all of eleven other fungi grew at neutrality at relatively high concentrations of copper sulphate. If copper enters the cell by means of a carrier, as proposed by Somers (1963, 1966), it is possible that the carrier(s) of the eleven fungi was not the same as that in *Scytalidium* sp.

According to Martell & Calvin (1952) toxicity of oxine is due to its combining with the metal attached to cellular enzymes and thus inactivating them. Others (Mason, 1948; Vicklund, Manowitz & Bagdon, 1954; Block, 1955; Nordbring-Hertz, 1955) noted that oxine increases the toxicity of copper. My results show that, over a wide range of pH (3.0 to 7.0), oxine failed appreciably to increase copper toxicity to *Scytalidium* sp. and I found no evidence that Na- or Cu-EDTA at 3×10^{-3} or 3×10^{-4} M reduced toxicity of copper at pH 0.5 to 6.0. However, Horsfall (1956) has reported that EDTA can reduce copper toxicity.

The toxicity of oxine and copper sulphate to *Scytalidium* sp. was lower at lower pH values. Copper forms one of the most stable of the metal oxinates (Irving & Williams, 1948), but the complex becomes ionized at low pH, when oxine and copper oxinate are least toxic (Fleck & Ward, 1933; Zentmyer, 1944; Martell & Calvin, 1952; Block, 1955; Horsfall, 1956). Nordbring-Hertz (1955) stated that toxicity of both oxine and copper oxinate decreased with drop in pH and that the decrease was pronounced between pH 5.0 and 4.0.

Albert *et al.* (1953, 1954) and Albert (1958, 1968) concluded that oxine serves to transport the copper across the cell membrane as the 1:2 complex which persists in the presence of excess oxine but breaks down to the toxic 1:1 complex where there is no excess. However, the data in Table 4 show no consistent decrease in toxicity where there was excess oxine.

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