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# Stimulation of Mycelial Growth of *Endothia parasitica* by Heavy Metals[1]

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Of 16 metal cations tested on agar medium, only copper and iron stimulated mycelial growth of *Endothia parasitica* in relatively high concentrations. Similarly enhanced growth was produced in high (32%) glucose concentrations and also when the fungus was grown on cellophane placed over the agar surface. *E. parasitica* secreted large amounts of oxalate that precipitated primarily as calcium oxalate at the periphery of the fungal colony, causing an opaque halo in the medium. Mycelial growth was retarded greatly when calcium oxalate accumulated, but retardation was reversed by copper and iron salts that prevented accumulation of the calcium oxalate crystals. *E. parasitica* grew well on media containing copper oxalate and copper-calcium oxalate but grew poorly with calcium oxalate as the carbon source and was inhibited by sodium oxalate in the medium. The specificity by which only copper and iron salts stimulated mycelial growth suggested that the metal and oxalate ions interact to form specific oxalate complexes that reverse the inhibition of simple oxalate salts. This probably accounts for enhanced growth in the presence of otherwise toxic levels of metals and oxalate. The stimulation did not occur in liquid cultures.

During an investigation of the toxicity of copper salts to various fungal species, we noted that mycelial growth of *Endothia parasitica* (Murr.) And. was stimulated by high concentrations of $CuSO_4$ that inhibited *Aspergillus niger*, a copper-tolerating fungus. Stimulation at high copper concentrations precludes a micronutrient function for copper. Although fungi can develop resistance to metal toxicants, adaptation generally requires continual culture on sublethal levels of the toxicant and does not account for growth stimulation.

Stimulation of *E. parasitica* by high concentrations of $CuSO_4$ and the relatively poor growth of this fungus on standard culture media suggest that stimulation may be due to interaction of the $CuSO_4$ with a self-inhibiting fungal metabolite in the medium, such that both are inactivated. The present study was designed to test this hypothesis and to determine the influence of other metals on the growth of *E. parasitica*.

## MATERIALS AND METHODS

The influence of metal salts on the growth of *E. parasitica* was determined on potato-dextrose-agar (PDA) prepared from the infusion of 200 g of potatoes, 20 g of dextrose, and 20 g of agar (Difco) in 1 liter of distilled water. In certain experiments, the potato infusion was filtered through Whatman no. 1 paper and then centrifuged at 4,080 × *g* for 0.5 hr. The potato broth was sterilized by autoclaving with the dextrose and agar or by filtering through a Millipore HA filter (0.45 μm) before addition to a sterile dextrose-agar solution.

Tubes containing 19 ml of molten PDA were cooled to 45 C, and the desired *p*H was obtained by adding 0.1 N HCl or NaOH. Unless otherwise specified, the *p*H was adjusted to 5.0. Metal salts were added in 1.0 ml of an aqueous solution, and the medium from each tube was poured into a petri plate. Each plate was inoculated with a 7-mm disc cut from the periphery of a colony of *E. parasitica* on PDA and incubated at 23 C.

Mycelial growth of the fungus on solid media was estimated by linear or dry weight measurements, or by both methods. In each experiment, average colony diameters were obtained from two perpendicular measurements made daily from the time when the most rapidly growing colonies were about 20 mm in diameter until they reached the edge of the petri plates. During this period, the daily growth rate was relatively constant.

Dry weights of the mycelium from nutrient agar cultures were obtained by first melting the agar in an autoclave and collecting the mycelial mat on Whatman no. 4 filter paper. The mats were dried to constant weight at 80 C.

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To determine whether growth stimulation occurs in liquid culture, *E. parasitica* was grown on potato broth medium (PDA without agar). Erlenmeyer flasks (250 ml) containing 30 ml of the broth medium were autoclaved; the medium was adjusted to *p*H 5.0 and inoculated with a 7-mm plug of *E. parasitica*. The cultures were incubated at about 23 C for 7 days. The mycelial mats were then collected on glass filter paper and dried to constant weight at 60 C.

The copper content of culture media was determined by atomic absorption analysis and was $2.5 \times 10^{-6}$, $3.2 \times 10^{-6}$, and $5.7 \times 10^{-6}$ M for PDA, PDA with a cellophane cover, and PDA with 320 g of dextrose/liter, respectively. In all cases, the copper content of the media from impurities in the components was insufficient to stimulate growth of *E. parasitica*.

## RESULTS

**Influence of metal salts on growth of E. parasitica.** The growth of *E. parasitica* on PDA was significantly stimulated by $CuSO_4$ over a wide range of concentrations, as estimated by either mycelial growth rate or dry weight accumulation after 7 days of growth (Fig. 1A). The mycelial growth rate was enhanced by $CuSO_4$ as the reaction increased from *p*H 4.0 to 8.0, but when copper was absent growth was relatively poor over this *p*H range (Table 1). The growth on PDA with no copper added was generally arrested when the colony attained a diameter of about 40 mm and, thus, never reached the edge of the petri plate.

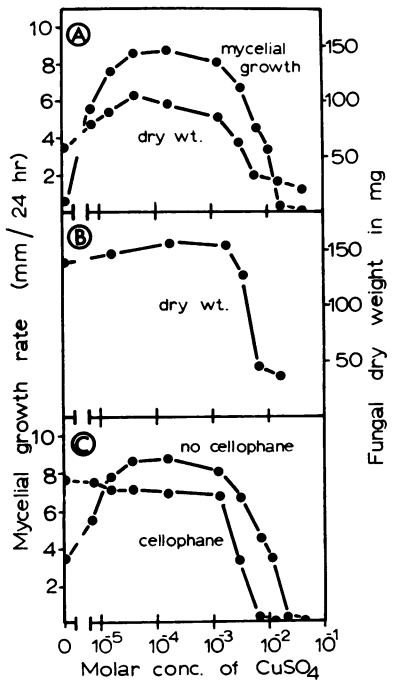


FIG. 1. *Growth of E. parasitica on media with various amounts of $CuSO_4$ added. (A) Mycelial growth rate and dry weight accumulation on PDA; (B) dry weight accumulation in potato-dextrose broth cultures; (C) mycelial growth rate with and without cellophane over PDA.*

TABLE 1. *Effect of pH on the growth rate of E. parasitica on PDA containing $CuSO_4$*

| *p*H of the culture medium | Mycelial growth rate (mm/24 hr) at various concn of $CuSO_4$ added to the medium | | | |
|---|---|---|---|---|
| | 0[a] | $5 \times 10^{-5}$ M | $5 \times 10^{-4}$ M | $5 \times 10^{-3}$ M |
| 3.0 | 1.5 | 2.2 | 1.4 | 0.7 |
| 4.0 | 2.6 | 4.4 | 5.9 | 4.9 |
| 5.0 | 2.4 | 9.4 | 9.2 | 7.1 |
| 6.0 | 1.1 | 9.4 | 9.3 | 5.1 |
| 7.0 | 1.4 | 8.9 | 9.7 | 6.1 |
| 8.0 | 2.0 | 5.9 | 8.1 | 7.2 |

[a] Copper content of PDA was $2.5 \times 10^{-6}$ M.

TABLE 2. *Effect of metal salts on the growth rate of E. parasitica*

| Metal salt | Mycelial growth rate (mm/24 hr) at two metal salt concn[a] | |
|---|---|---|
| | $5 \times 10^{-4}$ M | $5 \times 10^{-3}$ M |
| $CuSO_4 \cdot 5H_2O$ | 9.3 | 6.0 |
| $CuCl$ | 9.3 | 8.6 |
| $FeCl_2 \cdot 4H_2O$ | 9.9 | 3.7 |
| $FeCl_3 \cdot 6H_2O$ | 10.2 | 4.1 |
| $ZnSO_4 \cdot 7H_2O$ | 2.8 | 1.5 |
| $NiCl_2 \cdot 6H_2O$ | 0.9 | 0.1 |
| $MgSO_4 \cdot 7H_2O$ | 1.1 | 1.3 |
| $CoCl_2 \cdot 6H_2O$ | 2.1 | 1.5 |
| $MnSO_4 \cdot H_2O$ | 1.2 | 1.8 |
| $CaCl_2 \cdot 2H_2O$ | 1.5 | 2.2 |
| $KCl$ | 1.1 | 1.1 |
| $BaCl_2 \cdot 2H_2O$ | 1.7 | 1.7 |
| $AgNO_3$ | 1.4 | 0 |
| $PbCl_2$ | 0.5 | 1.0 |
| $CrCl_3 \cdot 6H_2O$ | 0.7 | 0 |
| $CdCl_2$ | 1.8 | 0 |
| $HgCl_2$ | 0 | 0 |

[a] On media without metal salts added, *E. parasitica* had an average growth rate of 1.5 mm/24 hr.

Of 16 other metal compounds tested in PDA, only salts of $Cu^+$, $Fe^{2+}$, and $Fe^{3+}$ enhanced mycelial growth to a similar degree at relatively high concentrations (Table 2). Zinc sulfate slightly increased the growth rate at a concentration of $5 \times 10^{-4}$ M, but the other metal salts failed to enhance growth significantly at this concentration.

Relative to the corresponding controls to which no copper was added, growth in liquid medium was slightly stimulated at the lower copper con-

centrations but was inhibited at high concentrations that were stimulatory on solid medium (Fig. 1B). Thus, enhanced growth at relatively high copper concentrations appears to depend on components in the agar or is characteristic of growth on solid media.

*E. parasitica* was grown on several natural and synthetic solid media to determine whether stimulation is unique on PDA. Copper sulfate was added at a final concentration of $1.6 \times 10^{-4}$ M to 2% water-agar; filter-sterilized PDA; autoclaved PDA; Campbell's medium (3) containing agar, salts, biotin, thiamine, dextrose, and asparagine; and Leonian's medium (11) containing agar, salts, peptone, maltose, and malt extract.

When no copper was added, the fungus grew poorly on all of the above media, but when copper was present growth was stimulated about ninefold. Thus, stimulation by copper is not unique on PDA and is unaffected by the method of medium sterilization.

*E. parasitica* grows rapidly on cellophane placed over PDA (12). Its growth on PDA containing various $CuSO_4$ concentrations with or without a cellophane cover was therefore measured to determine the possible relationship between enhanced growth over cellophane and stimulation by copper. Cellophane discs (DuPont 215 PD) 8.5 cm in diameter were autoclaved, moistened with sterile water, placed wrinkle-free on PDA in petri plates, and inoculated with 7-mm agar discs of *E. parasitica*. Mycelial growth was estimated by linear measurements.

In the absence of copper, the fungus grew rapidly on cellophane placed over PDA. Rather than being stimulated by relatively high copper concentrations that enhance growth on PDA without cellophane, its growth was inhibited (Fig. 1C). Cellophane without copper present appears to stimulate mycelial growth, and, in the presence of cellophane, the toxicity of $CuSO_4$ was significantly increased.

**Influence of dextrose on growth of E. parasitica.** The concentration of dextrose in PDA was varied to determine whether the mycelial growth rate could be enhanced by increasing the amount of carbon in the medium. At concentrations of 160 g/liter and greater, dextrose significantly stimulated mycelial growth (Table 3). Growth of most fungi is inhibited by sugar levels above 150 g/liter (4), and the remarkable stimulation of *E. parasitica* at such high dextrose concentrations suggests that in this case a non-nutrient role of

TABLE 3. *Effect of dextrose on the growth rate of E. parasitica on PDA*

| Dextrose (g/liter)[a] | Mycelial growth rate (mm/24 hr) |
|---|---|
| 20 | 1.2 |
| 80 | 1.6 |
| 160 | 4.2 |
| 240 | 5.7 |
| 320 | 5.2 |

[a] The copper content of PDA ranged from $2.5 \times 10^{-6}$ to $5.7 \times 10^{-6}$ M in media with 20 and 320 g of dextrose/liter, respectively.

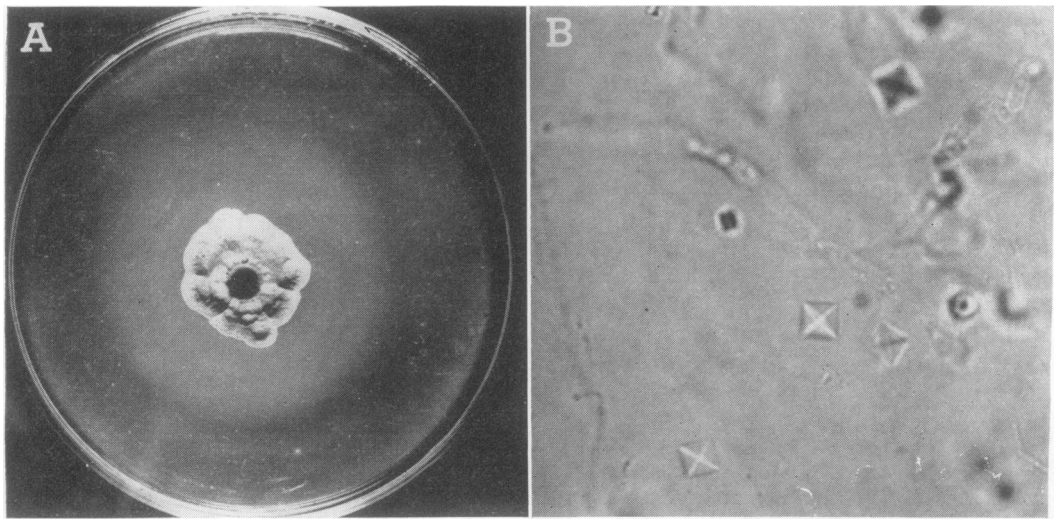


FIG. 2. (A) *Opaque halo surrounding a colony of E. parasitica growing on PDA; (B) crystals of calcium oxalate trihydrate in the medium that cause the opaque halo* (× *1,640*).

in dextrose might result in an action similar to that of copper on growth.

**Accumulation of calcium oxalate in cultures of E. parasitica.** Stimulation of mycelial growth by relatively high concentrations of copper and the relatively poor growth of *E. parasitica* on standard culture media suggest that enhanced growth might result from interaction of the metal toxicant with a self-inhibiting fungal metabolite such that both are inactivated in the medium. PDA cultures of this fungus contain a distinct opaque halo around the periphery of the colony (Fig. 2A). The halo was found in all cultures on solid media on which *E. parasitica* grew poorly but was absent in media containing copper or iron salts where growth was enhanced. Media from the halo region contained many bipyramidal octahedral shaped crystals that were in close proximity to the hyphae (Fig. 2B) and caused the opaqueness.

To obtain crystals for analysis, *E. parasitica* was grown on 2% water-agar to avoid the problem of separating the crystals from the starch granules in PDA. Since the crystals were relatively insoluble in most solvents, a disc of nylon screen (38 mesh) was placed in a Büchner funnel, and the media from 10 petri plate cultures were placed on the screen. The stem of the funnel was placed in a centrifuge bottle (250 ml), and the bottle and centrifuge head were steamed in an autoclave for 4 min. The melted agar was centrifuged at 7,970 × g for 2 min at 45 C, the supernatant liquid was decanted, and the bottle was rinsed three times with distilled water. The pelleted crystals were suspended in distilled water, poured into an evaporating dish, and dried at 80 C.

Analysis of the partially purified crystals showed them to be slightly soluble in hot water and 1 N NaOH, and highly soluble in 1 N HCl, $H_2SO_4$, and $HNO_3$; they were insoluble in cold water, 6 M acetic acid, 6 M $NH_3OH$, 6 M $NH_3Cl$, ethanol, benzene, anhydrous ether, acetone, hydrogen peroxide (10%), carbon tetrachloride, ethyl acetate, *N*,*N*-dimethylformamide, acetonitrile, dioxan, and *N*,*N*-dimethylacetoacetimide. The crystal structure was altered at 160 to 170 C, and the crystals decomposed at 350 C without melting. Infrared absorption (KBr pellet) showed major peaks at 2.95, 3.4, 6.2, 7.25, 7.45, 10.5, and 12.8 $\mu$m. The X-ray diffraction pattern (CuK $\alpha$ radiation and nickel filter) had major peaks at 0.619, 0.594, 0.365, 0.296, 0.278, 0.250, 0.241, 0.235, 0.224, 0.202, and 0.196 nm. On the basis of this analysis, the substance was identified as calcium oxalate (1, 6). Calcium was the only metal found in a sufficient amount (13.5%, w/w) to be theoretically a component of the crystals. The calcium required for formation of these crystals probably came from the agar, which contains about 0.13% calcium (*personal communication*, D. G. Erwin, Difco Laboratories, Detroit, Mich.).

Three hydrates of calcium oxalate occur naturally (9), but only the trihydrate has the bipyramidal octahedral shaped crystals typical of those isolated from cultures of *E. parasitica*. The gradual addition of oxalic acid to an excess of calcium ions approximated the conditions under which calcium oxalate is formed in cultures of *E. parasitica*, and resulted in crystals identical to those deposited in PDA by the fungus.

When a solution of $CuSO_4$ ($10^{-2}$ M) was added to freshly prepared calcium oxalate crystals, they dissolved. Depending on the metal and the concentration of oxalate and metal ions, simple salts or metal oxalate complexes with oxalate groups coordinated to the metal ions can form (10). Most metal oxalates are only sparingly soluble, but their solubility can be increased through complex formation by the addition of other metal ions (10). For example, the ferric ion is frequently used to form a complex with calcium oxalate, thereby increasing the solubility of the oxalate (13). Thus, the dissolution of calcium oxalate crystals by a $CuSO_4$ solution is probably attributable to complex formation and may account for the lack of calcium oxalate crystals in *E. parasitica* cultures containing high levels of $CuSO_4$.

**Growth of E. parasitica on media containing metal oxalates.** *E. parasitica* was grown on potato infusion-agar with various metal oxalates in place of dextrose to determine whether the form of the oxalate could influence growth of the fungus. Calcium, copper, and copper-calcium oxalates were obtained by precipitation from solutions of $Ca(NO_3)_2$, $CuSO_4$, or both after addition of oxalic acid. These oxalates were sterilized dry at 80 C for 24 hr, and were then dissolved or suspended at three concentrations in the medium. The medium was adjusted to *p*H 5.0, poured into petri plates (ca. 20 ml/plate), and inoculated. Mycelial growth rate versus

TABLE 4. *Growth of E. parasitica on media containing metal oxalates*

| Metal oxalate at $10^{-2}$ M | Mycelial growth rate (mm/72 hr) |
|---|---|
| Copper oxalate | 24.0 |
| Copper-calcium oxalate | 22.2 |
| Calcium oxalate | 9.6 |
| Sodium oxalate | 2.4 |
| Control (potato infusion-agar) | 4.8 |
| Control (PDA) | 9.4 |

oxalate concentration was plotted, and the growth rates at $10^{-2}$ M were chosen for comparison because they were indicative of results at the other concentrations.

When copper oxalate and copper-calcium oxalate were present, fungal growth was significantly stimulated (Table 4) as on PDA containing $CuSO_4$. On the calcium oxalate medium, the growth rate was twice that on potato infusion-agar without oxalate and was about equal to growth on the medium containing dextrose (PDA). Although calcium oxalate accumulates when growth is inhibited, these results suggest that calcium oxalate is not inhibitory per se; the toxicity of sodium oxalate, however, suggests that high levels of oxalate ions may be inhibitory.

## DISCUSSION

Because of the general toxicity of heavy metal ions to fungal cells and the relatively high concentrations of copper that stimulate growth of *E. parasitica*, it is most probable that the stimulatory action of copper is initiated outside the cells in the culture medium.

Limited growth of *E. parasitica* on natural and synthetic media and the concomitant accumulation of calcium oxalate at the colony edge suggest a possible causal relationship between poor growth and oxalate accumulation. Growth stimulation by copper is accompanied by a substantial reduction in the accumulation of calcium oxalate crystals, further indicating a positive correlation between poor growth and calcium oxalate accumulation.

On the basis of this study, the following working hypothesis is offered to explain the relationship of metal salts and oxalate to the growth of *E. parasitica* on solid culture media. Oxalate accumulates and inhibits growth either by direct toxic action or by blocking the uptake of needed nutrients (e.g., calcium). When copper or iron salts are present at stimulatory concentrations, complexes are formed that detoxify the oxalate and the heavy metal.

Detoxification mechanisms have been postulated to explain the growth of some fungi in the presence of metal toxicants (2). For example, *Poria vaporaria* produces oxalic acid in sufficient quantities to detoxify $CuSO_4$ in treated wood (8) and other copper salts in an agar medium (7), but only if growth has occurred prior to the addition of the copper salt. No reports have been found, however, of enhanced growth by *P. vaporaria* on media containing copper salts.

Calcium oxalate fails to accumulate in stimulated cultures of *E. parasitica* on PDA containing excessive dextrose concentrations and when the fungus is grown on cellophane over the PDA medium. High levels of dextrose may stimulate growth by solubilizing oxalate as it is formed in the culture medium (5), and cellophane may influence growth either by adsorbing oxalate or by dispersing oxalate over the entire surface of the PDA, thus preventing its concentration around the fungal colony.

The precise physiological mode of action of oxalates and metals on the growth of *E. parasitica* is not known. However, the specificity by which only copper and iron salts stimulate mycelial growth of *E. parasitica* suggests the formation of specific metal-oxalate complexes that reverse toxicity of the simple oxalate salts.

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