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Melanin Production by a Filamentous Soil Fungus in Response to Copper and Localization of Copper Sulfide by Sulfide-Silver Staining

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***Gaeumannomyces graminis* var. *graminis*, a filamentous soil ascomycete, exhibited enhanced cell wall melanin accumulation when exposed to as little as 0.01 mM $CuSO_4$ in minimal broth culture. Because its synthesis was inhibited by tricyclazole, the melanin produced in response to copper was dihydroxynaphthalene melanin. An additional hyphal cell wall layer was visualized by electron microscopy when hyphae were grown in the presence of copper and fixed by cryotechniques. This electron-dense layer was between the outer cell wall and the inner chitin layer and doubled the total wall thickness. In copper-grown cells that were also treated with tricyclazole, this electron-dense layer was absent. Atomic absorption spectroscopy demonstrated that up to 3.5 mg of Cu per g of fungal mycelium was adsorbed or taken up by hyphae grown in 0.06 mM $CuSO_4$. A method for silver enhancement was developed to determine the cellular location of CuS. CuS was present in cell walls and septa of copper-grown hyphae. Electron microscopy of silver-stained cells suggested that CuS was associated with the melanin layer of cell walls.**

Gaeumannomyces graminis var. *graminis* is a filamentous soil ascomycete that colonizes root and crown tissue of many members of the Poaceae (grass family). At least some *G. graminis* var. *graminis* strains invade host vascular tissue, causing disease in rice or ornamental turfgrasses (47, 54). Previous studies suggested that copper-based fungicides fail to control diseases caused by *G. graminis* (3), and our goal was to determine the mechanism(s) of copper tolerance and/or binding by this fungus.

Although several metallic elements play essential roles in metabolism, overabundance of trace elements leads to excessive cellular accumulation and consequent toxicity or death (reviewed in reference 18). Many fungi bind or sequester metals and could be exploited as heavy metal bioremediators (reviewed in reference 54). Some fungi secrete chelating compounds that sequester metal ions extracellularly. Others secrete organic acids, such as oxalic acid, that form extracellular complexes or crystals with metal ions. Metal ions are also actively transported into fungal cells, where they may bind specific proteins, such as the metallothioneins, or possibly become sequestered in organelles (reviewed in reference 31). *Saccharomyces cerevisiae* generates H_2S that traps metal ions as insoluble sulfides either intracellularly, extracellularly, or in the periplasm (1, 8, 30, 33, 34, 45). In addition, fungal cell walls, even those of killed hyphae or yeast, bind significant amounts of metal ions (15, 36, 53). Previous studies suggested that purified chitin or chitosan, polysaccharide cell wall components of many fungi, bind metal ions, but chitin is probably not the major metal-binding compound in fungi (discussed in reference 36).

Fungal pigments may also bind metals and could serve to protect cells from metal toxicity (reviewed in reference 36). For example, the yeast *Debaromyces hansenii* produces a metal-binding yellow pigment, identified as a riboflavin-type compound, upon exposure to toxic metal concentrations (20). In addition, fungal melanin(s) could bind heavy metal ions. Melanins are dark, pigmented polymers that often protect organisms in stressful environments (27, 28, 37 [reviewed in reference 4]). Fungi produce different types of melanin, including polymers of L-dihydroxyphenylalanine (L-DOPA), catechol, glutaminyl-3,4, dihydroxybenzene, and 1,8 dihydroxynaphthalene (1,8 DHN). *G. graminis* apparently produces 1,8 DHN melanin, because its synthesis is inhibited by low concentrations of cerulenin and tricyclazole (11a, 24a, 54a). Electron micrographs comparing albino and wild-type fungal hyphae indicate that melanins are located in hyphal cell walls as a layer exterior to chitin or as electron-dense granules distributed in various patterns in the fungal cell wall (reviewed in reference 4). Electron-dense material, presumed to be melanin, was also seen in the septa of *Aureobasidium pullulans* (38). In all previous studies of melanized hyphae, conventional chemical fixation was used prior to visualization by electron microscopy (EM). In this study, we visualized *G. graminis* melanized hyphae after performing a cryofixation technique that is known to improve the preservation of cellular structures (25, 26).

Several studies demonstrate that either synthetic or extracted L-DOPA or tyrosine-derived melanins bind metal ions (14, 29, 43, 55). Extracted fungal melanins of unknown chemical structure also adsorb metal ions (19, 42). For example, Gadd demonstrated that *Cladosporium resinae* and *A. pullulans* culture supernatant fluids containing extracellular melanin and probably other extracellular material bind Cu (19). Moreover, *A. pullulans* melanin, reportedly of the DHN type (46), is produced in response to Cu, Co, Pb, Hg, Cd, Fe, Mn, Ag, Al, and Ni but not Mg or Zn (17). Rowley and Pirt also noted that melanization of *Aspergillus nidulans* greatly increased in medium containing iron (41). The ectomycorrhizal fungi *Suillus pictus* and *Suillus granulatus* produce more pigmentation in response to Cu (24). This pigment is probably L-DOPA melanin, because an enzyme that cross-links L-DOPA, tyrosinase,

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also either increased in activity or amount when hyphae were grown in copper (24).

Although several reports suggest that fungal melanins bind metal cations in vivo (23, 38, 40), direct evidence for this finding is lacking. Energy-dispersive X-ray microanalysis indicated that a significant amount of iron was associated with electron-dense (presumably melanin) granules of *A. pullulans*, but data were not shown (38). Melanized *Armillaria* rhizomorphs, specialized fungal structures with several hyphal layers, also adsorb high concentrations of metal cations, and energy-dispersive X-ray microanalysis demonstrated that metals are in the melanized cortex of the rhizomorph (40).

Cytological studies of metals and melanin are complicated because many nonmetallic and nonmelanized structures are electron dense, depending on fixation and staining procedures. Several analytical EM techniques are useful for general localization of metal ions within cells, if they are in high enough concentrations (reviewed in reference 50). Atomic absorption spectroscopy (AAS) is useful for quantifying low concentrations of metal ions, but samples are destroyed in the process and localization of metals within cells is not possible.

The sulfide-silver technique, or autometallography, is commonly used to locate copper and other heavy metals in animal tissues (9–11, 16). This method depends on a physical developer that consists of silver ions (i.e., silver lactate) and a reducing agent (i.e., hydroquinone). Water-insoluble metallic particles (i.e., metal sulfides) catalyze the reduction of silver ions to metallic silver when reducing molecules are present. Silver shells form around metal particles, increasing their size. This technique reveals only metal sulfides and not metals bound to organic molecules such as proteins or carbohydrates. Although there are colorimetric methods to stain copper in cells and tissues, none can be used in conjunction with EM. We utilized the silver enhancement technique to visualize CuS in fungal hyphae by light and electron microscopy, and to our knowledge, this is the first use of silver staining to localize metal ions in fungi.

MATERIALS AND METHODS

Fungal strain and culture conditions. Wild-type *G. graminis* var. *graminis*, a homothallic ascomycete, was isolated from soybeans (*Phaseolus vulgaris* L.). This strain was deposited with the National Center for Agricultural Research (accession no. 25027) in Peoria, Ill. Fungal mycelia were produced in defined liquid minimal medium (pH 5.5) containing the following (grams per liter): saccharose, 3; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4$, 0.05; KCl, 0.05; $CaCl_2$, 0.1; $FeCl_3$, 0.0005; and trace elements [$ZnSO_4 \cdot 7H_2O$, 0.0125; $Fe(NH_4)(SO_4)_2 \cdot 6H_2O$, 0.0025; $CuSO_4 \cdot 5H_2O$, 0.0006; $MnSO_4 \cdot H_2O$, 0.0001; H_3BO_3, 0.0001; $NaMoO_4 \cdot 2H_2O$, 0.0001; biotin, 0.001; and thiamine, 0.001]. Stock trace elements were filter sterilized and diluted 1:4,000 into autoclaved medium. Cultures were grown in 200 ml of medium in 1-liter flasks on a rotary shaker (50 cycles per min) at 25°C with a photoperiod of 12 h at 3,000 lx. Medium was supplemented with $CuSO_4$ to produce concentrations of 0.0224, 0.0424, 0.0624, or 0.0824 (referred to as 0.02, 0.04, 0.06, and 0.08 hereafter) mM $CuSO_4$. The trace $CuSO_4$ concentration was 2.4 μM. Cupric sulfate (Sigma) was dissolved in distilled, deionized water (40 mM stock solution) and filter sterilized before addition to autoclaved media. Tricyclazole, or 5-methyl-1,2,4-triazolo-(3,4-*b*)-benzothiazole (98% active ingredient; Eli Lilly Research Laboratories, Greenfield, Ind.), was dissolved in 100% ethanol (2.5-mg/ml stock solution) and added to autoclaved medium. Ethanol used to dissolve tricyclazole was less than 0.6% of the culture volume.

After 9, 14, 18, and 24 days, cultures were collected through filter paper (Whatman no. 1) and then freeze-dried to determine their mycelial dry weight. Nine- and 18-day cultures were collected for EM, silver development, and AAS. Transmission EM of silver development was conducted in similar defined medium but without trace elements and with 0.01 g of $FeSO_4$ per liter.

EM. The ultrastructure of *G. graminis* var. *graminis* hyphae grown in minimal media at different concentrations of $CuSO_4$ and/or tricyclazole was examined after specimens were processed for freeze substitution (25, 26). Fungal hyphae were allowed to attach to a cellophane membrane (PUDO-193; DuPont Co.) for 2 h. They were frozen in liquid propane at −190 to −193°C, substituted in 2% osmium tetroxide and 0.05% uranyl acetate in acetone (high-performance liquid chromatography grade), and embedded between two microscope slides in Epon 812-Araldite 6005 epoxy resin. Cells were examined for freeze damage by phase-contrast optics at ×1,000. Undamaged cells were mounted onto epoxy stubs, thin sectioned longitudinally, and stained with 2% aqueous uranyl acetate for 15 min and lead citrate (39) for 2 min. Specimens were examined with a JEOL JEM 100CX transmission microscope operated at 80 kV. At least 15 to 30 cells from each treatment from two to four separately executed experiments were examined. Only cell segments with a straight, double-plasma membrane were measured to determine wall layer thickness. Standard deviations and the number of cells measured are indicated in the Results section.

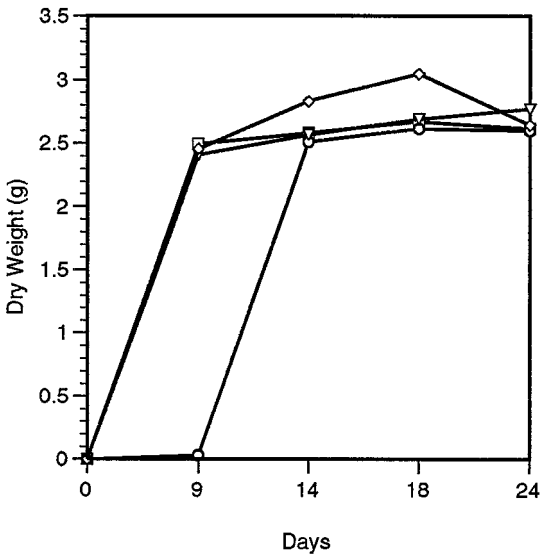


FIG. 1. Growth curves of *G. graminis* var. *graminis* in minimal medium. Hyphae were grown under the following conditions: □, trace amount (2.4 μM) of $CuSO_4$; ○, 0.04 mM $CuSO_4$; ▽, 0.04 mM $CuSO_4$ and 10 μg of tricyclazole per ml; and ◇, 10 μg of tricyclazole per ml. Each point is the average of three separate experiments.

Hyphae were chemically fixed and gold-conjugated wheat germ agglutinin (WGA-CG) labelled to determine which wall layer was composed of chitin (6). Cells were fixed for 1 h at room temperature in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.7) and postfixed for 1 h in 2% osmium tetroxide. Acetone dehydration was followed by infiltration in Quetol 651. Thin sections were labelled for 2 h at room temperature in 1:10 WGA-CG solution. Sections were incubated in drops of WGA-CG in 30 mM *N*,*N*′,*N*″-triacetylchitotriose (Sigma) for hapten controls (5).

Sulfide-silver amplification. Specimens were silver enhanced with the commercial silver enhancement kit HQ SILVER (Nanoprobes, Inc., New York, N.Y.). The silver solution was prepared immediately before use, and all manipulations were undertaken in a darkroom. Different regimens of fixation were

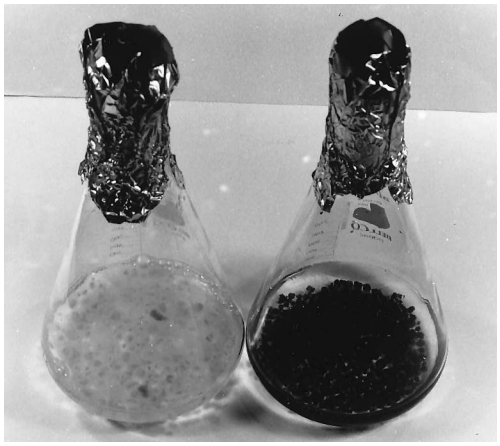
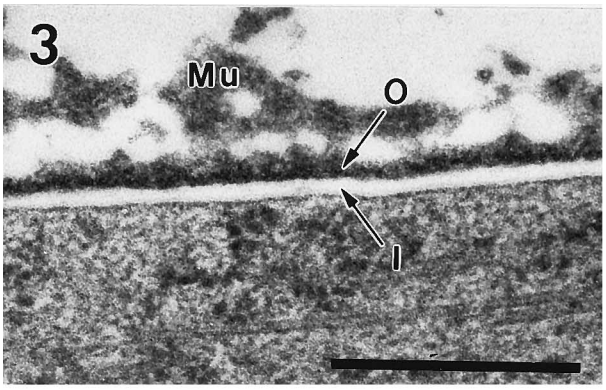


FIG. 2. Eighteen-day-old cultures. Hyphae grown in 0.04 mM $CuSO_4$ were heavily melanized compared with hyphae grown in a trace amount (2.4 μM) of $CuSO_4$ (left).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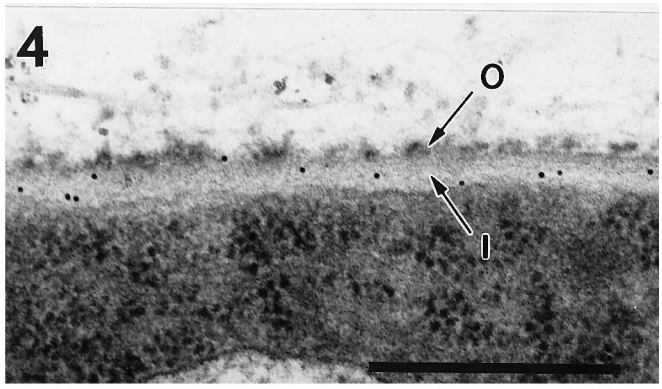
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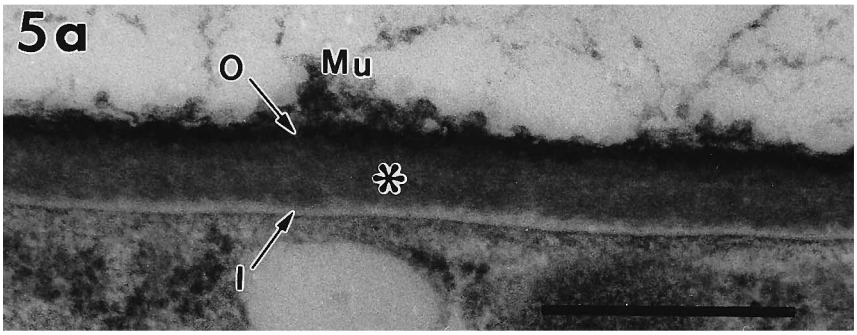
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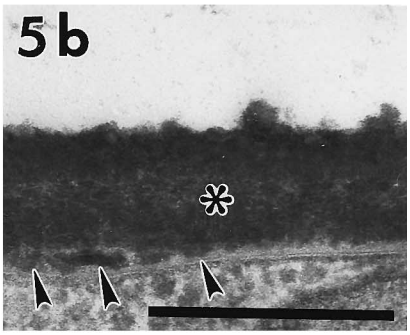
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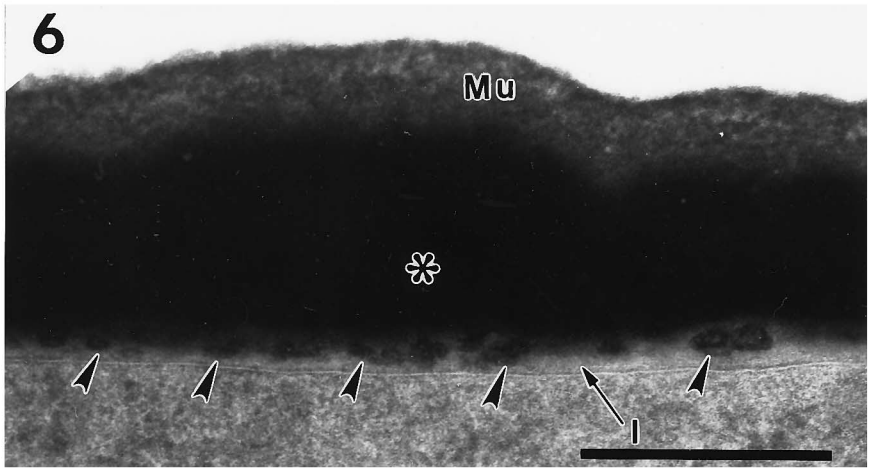
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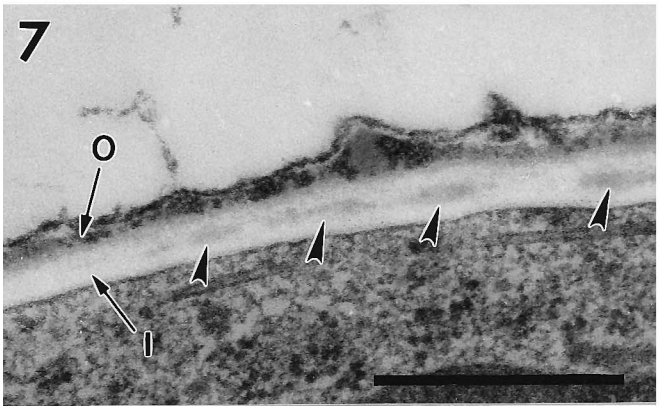
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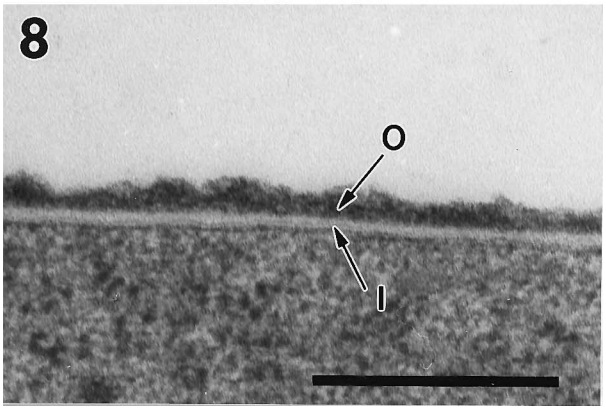
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tested to optimize cellular preservation and detection of CuS in copper-grown cultures. Fixations included formaldehyde and/or glutaraldehyde (9). Because silver amplification is time dependent, a series of development times from 30 to 15 min were tested to find the optimal silver particle size and intensity of signal (7). The following procedure was finally adopted. Mycelia were fixed for 30 min in 4% formaldehyde (prepared fresh from *p*-formaldehyde) in 0.2 M cacodylate buffer (pH 5.5) at 25°C. Cells were washed three times with cacodylate buffer for 15 min each and then rinsed twice with deionized water for 5 min each. Development of specimens in HQ SILVER was performed for 10 min at 25°C with constant agitation in a darkroom under a safelight. Specimens were rinsed with deionized water until supernatant fluids became completely clear. Specimens were mounted on glass slides and observed immediately with a Nikon Microphot FXA optical microscope with bright-field optics.

Specimens for EM were fixed with 2.5% glutaraldehyde in cacodylate buffer (pH 5.5) overnight at 4°C and then washed with cacodylate buffer (three times for 15 min each), dehydrated in ethanol, and embedded in Epon-Araldite. Epon polymerization occurred for 1 week at 45°C (9). Ultrathin sections were collected on single-slot grids by surface tension and then incubated on a drop of HQ SILVER solution for 2 min in a safelight box. Sections were washed three times for 5 min each with deionized water and deposited on Formvar-coated single-slot grids. Unstained sections were immediately observed with a JEOL 100CX transmission EM operated at 80 kV.

AAS. Hyphae were grown in 200 ml of medium, from which 10 ml was removed before inoculation to determine copper content. Mycelia were harvested by filtration after 18 days, washed with distilled, deionized H_2O, filtered, and freeze-dried. Culture supernatant fluids were also sampled for copper content. The amounts of copper in samples were determined with a Perkin-Elmer model 403 atomic absorption spectrophotometer equipped with a graphite furnace attachment. Prior to analysis, mycelial samples were digested at 80°C in 15 ml of a 1:1 solution of nanopure water and Baker Instra-Analyzed nitric acid. Liquid medium samples were analyzed directly without further modification. All samples (1 μg/ml) were run in triplicate. Copper standards were prepared from metallic copper dissolved in a 1:1 solution of HPLC-grade HNO_3 and nanopure water.

RESULTS

Effects of copper on growth of *G. graminis* var. *graminis*. Wild-type *G. graminis* var. *graminis* was grown in defined minimal medium with or without added $CuSO_4$ (0.04 mM) and tricyclazole (10 μg/ml), an inhibitor of DHN melanin biosynthesis (4 [Fig. 1]). Copper sensitivity was expressed immediately after inoculation, with a lag period of approximately 9 days that was not observed in cultures with only a trace amount (2.4 μM) of $CuSO_4$. However, by 14 days, the biomass of copper-grown cultures was approximately the same as that of cultures with trace concentrations of copper. The growth rates of cultures grown with tricyclazole or with elevated levels of copper and tricyclazole were similar to those of cultures grown in trace amounts of $CuSO_4$.

Melanization of hyphae in response to copper. Cultures grown in minimal medium supplemented with $CuSO_4$ (0.02 to 0.08 mM) became dark compared with cultures grown in trace amounts (2.4 μM) of $CuSO_4$ (Fig. 2). The cell walls of 9-day-old hyphae grown in 2.4 μM $CuSO_4$ exhibited two distinctive layers: an electron-translucent inner layer (mean thickness, 45 ± 8 nm; $n = 22$) adjacent to the plasma membrane and an irregular, fuzzy, electron-opaque outer layer (mean thickness, 66 ± 12 nm; $n = 21$) (Fig. 3). Hyphae had an electron-dense, amorphous, extracellular mucilage that either adhered to the outer wall layer or was proximal to the cell surface. Hyphae treated with WGA-CG, which binds to terminal and internal *N*-acetylglucosamine residues (5), revealed that only the inner cell wall layer contained chitin (Fig. 4).

Hyphae were dark after 1 day of growth in elevated $CuSO_4$ concentrations (0.01 to 0.08 mM). Moreover, the metal had a striking effect on the ultrastructure of hyphal cell walls (Fig. 5). After 9 days, a third intermediate wall layer (122 ± 15 nm; $n = 15$) was detected between the inner wall layer, adjacent to the plasma membrane, and the outer layer (Fig. 5a). The intermediate layer and the outer layer were distinguishable because the intermediate layer was somewhat less electron dense than the outer layer. Irregular dense granules were often visible in the intermediate layer (arrows, Fig. 5b). This intermediate layer approximately doubled the cell wall thickness of hyphae grown in elevated concentrations of copper compared with hyphae grown in trace concentrations of copper. The inner and outer wall layers of copper-grown hyphae were as thick as those of hyphae grown in trace concentrations of copper.

After 18 days in the presence of an elevated copper concentration, the inner translucent layer was the only distinguishable wall layer. The remaining (intermediate and outer) layers were too dense to resolve (Fig. 6). Again, amorphous granules were present along the boundary of the inner and intermediate layers (arrow, Fig. 6). At this stage of development, it was difficult to determine cell wall thickness with precision because fuzzy, extracellular mucilage was often present and appeared undulated and closely associated with cells.

The effect of copper on cell wall morphology was mediated via its action on the DHN melanin pathway. Cultures grown in elevated levels of copper and tricyclazole became reddish-brown and the electron-opaque intermediate layer seen in copper-grown hyphae was mostly absent (Fig. 7). Only a vestige of the intermediate layer (arrows, Fig. 7) was visible in cells grown under these conditions. Cultures grown for 9 days with tricyclazole in the presence of trace amounts of copper became yellow-white, and hyphal cell walls (Fig. 8) exhibited wall structures similar to those of hyphae grown in trace amounts of copper without tricyclazole (Fig. 3); that is, they lacked the intermediate dense layer.

Quantification of copper binding by AAS. AAS showed a gradual increase in copper binding to hyphae grown in increasing concentrations of $CuSO_4$ (Fig. 9A). For example, after 18 days, 3.5 mg of copper per g of fungal mycelium was adsorbed or taken up by hyphae grown in 0.06 mM $CuSO_4$, whereas copper was undetected in mycelia grown in trace concentrations of copper. Cultures grown in the presence of copper (0.04 mM) and tricyclazole also had considerable amounts of copper bound to mycelia, although the cultures were less pigmented (Fig. 9B).

CuS localization by sulfide-silver amplification. A silver-staining method was utilized to demonstrate the presence of

FIG. 3–8. (Fig. 3 and 5–8) Transmission electron micrographs of freeze-substituted *G. graminis* var. *graminis*. |, inner translucent layer; o, outer layer; *, intermediate layer; Mu, extracellular mucilage. For all micrographs, the magnification is ×66,000, and the bar is 0.5 μm.

FIG. 3. Hypha after 9 days of culture in medium with a trace amount of copper. The wall had only two layers: an electron-translucent inner layer adjacent to the plasma membrane and an electron-dense outer layer. Extracellular mucilage was proximal to the cell surface.

FIG. 4. Chemically fixed hypha grown in a trace amount of copper and labelled with WGA-CG. Only the inner wall layer was labelled.

FIG. 5. (a) Hypha grown in 0.04 mM copper sulfate after 9 days. An additional heterogeneous, intermediate layer is visible between the inner electron-transparent wall layer and the outer more-electron-opaque layer. (b) In the proximity of the inner wall layer, various-sized electron-dense granules were often observed and appeared to be part of the intermediate layer (arrows).

FIG. 6. Hypha grown in 0.04 mM $CuSO_4$ after 18 days. The outer layer-intermediate layer boundary was not visible because of its electron density. Dense granules were present along the intermediate layer near the inner wall layer (arrows). Mucilage was often attached to the cell surface, giving it an undulated appearance.

FIG. 7. Hypha grown in 0.04 mM $CuSO_4$ and 10 μg of tricyclazole per ml for 9 days. The third intermediate layer was absent, but some opaque material (arrows) was still observed within the electron-translucent wall layer.

FIG. 8. Hypha grown in 10 μg of tricyclazole per ml for 9 days. The cell wall was similar to that of hypha grown in trace amounts of $CuSO_4$ (FIG. 3).

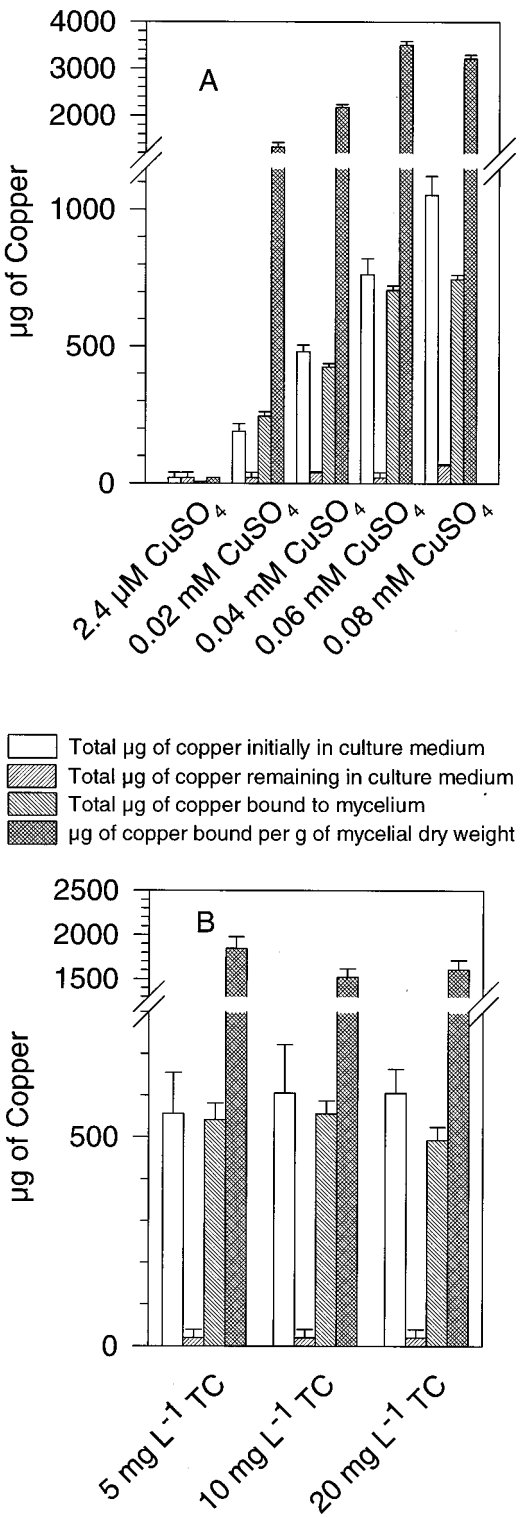

FIG. 9. Distribution of copper in cultures exposed to different concentrations of copper sulfate (A) and different concentrations of tricyclazole (TC) with 0.04 mM $CuSO_4$ (B). All data are the average of two separate experiments, with the exception of the 0.04 mM $CuSO_4$–20-μg/ml tricyclazole data, which were obtained from one experiment.

copper sulfide in hyphae. No silver deposition was observed in 18-day hyphae grown in trace concentrations of copper (Fig. 10), but the cell walls, septa, and cytoplasm of hyphae grown in 0.04 mM $CuSO_4$ were silver stained (Fig. 11). The silver distribution in hyphae grown in elevated Cu concentrations was heterogeneous, and the colors of stained hyphae ranged from light yellow to dark brown in the cytoplasm to dark brown or black in hyphal branches and septa. Silver deposits were observed in subapical regions but not in hyphal tips (Fig. 12). Cultures grown in elevated concentrations of copper (0.04 mM) and tricyclazole (10 μg/ml) stained light yellow without septal staining (Fig. 13). Septal formation was not affected by tricyclazole, because the lengths of intervals between septa in hyphae grown in elevated levels of copper and hyphae grown in elevated levels of copper plus tricyclazole were not significantly different (data not shown).

Thin sections of hyphae grown in trace copper were silver stained and viewed by EM. Silver deposits were not observed in hyphae grown in trace concentrations of copper (Fig. 14), whereas deposition occurred in the septa of hyphae grown in elevated concentrations of copper (Fig. 15). Moreover, small silver grains were observed in or near the intermediate layer between the inner, electron-translucent layers and the outer, electron-dense layers (arrows, Fig. 16).

DISCUSSION

G. graminis var. *graminis* exhibited a growth lag in minimal medium supplemented with 0.04 mM $CuSO_4$, and growth under these conditions yielded heavily pigmented hyphae. Pigment was not released into culture media, and hyphal pigmentation was reversible. That is, when the fungus was transferred after growth in 0.04 mM $CuSO_4$ to a medium with only trace amounts (2.4 μM) of $CuSO_4$, the hyphae became less melanized (data not shown). Hence, pigmentation in response to copper was an adaptive phenomenon and was not caused by mutation.

Melanization in *A. nidulans* occurs in response to slow growth during carbon limitation (41). However, melanization of *G. graminis* was not observed in carbon-limited cultures (3 mg of sucrose per liter), which grew more slowly (data not shown).

The electron micrographs presented in this report are the first of melanized hyphal walls with cryofixation followed by freeze substitution. The dark pigmentation of mycelia grown in elevated levels of copper correlated with formation of an additional, electron-dense layer between the inner and outer cell wall layers. This layer and its associated electron-dense granules were not present in cultures containing elevated levels of copper and tricyclazole. Because tricyclazole specifically inhibits the DHN melanin pathway (4), this intermediate layer is most likely composed of DHN melanin.

Tricyclazole inhibits enzymes that reduce 1,3,6,8-tetrahydroxynaphthalene to scytalone and 1,3,8-trihydroxynaphthalene to vermalone in the DHN melanin pathways of various fungi (4). Although *G. graminis* hyphae treated with tricyclazole alone were white, hyphae grown in tricyclazole and elevated concentrations of copper were reddish-brown. This suggested that tricyclazole suppressed, but did not completely inhibit, melanization in the presence of copper. Alternatively, copper may have induced the melanin biosynthetic pathway, overcoming tricyclazole inhibition.

In other fungi, such as *A. pullulans* (21, 35), *Verticillium dahliae* (12), and *Phomopsis* spp. (13), melanin is associated with electron-dense granules present in a fibrillar matrix that extends outward from the cell wall. Gadd and Griffith (21) also

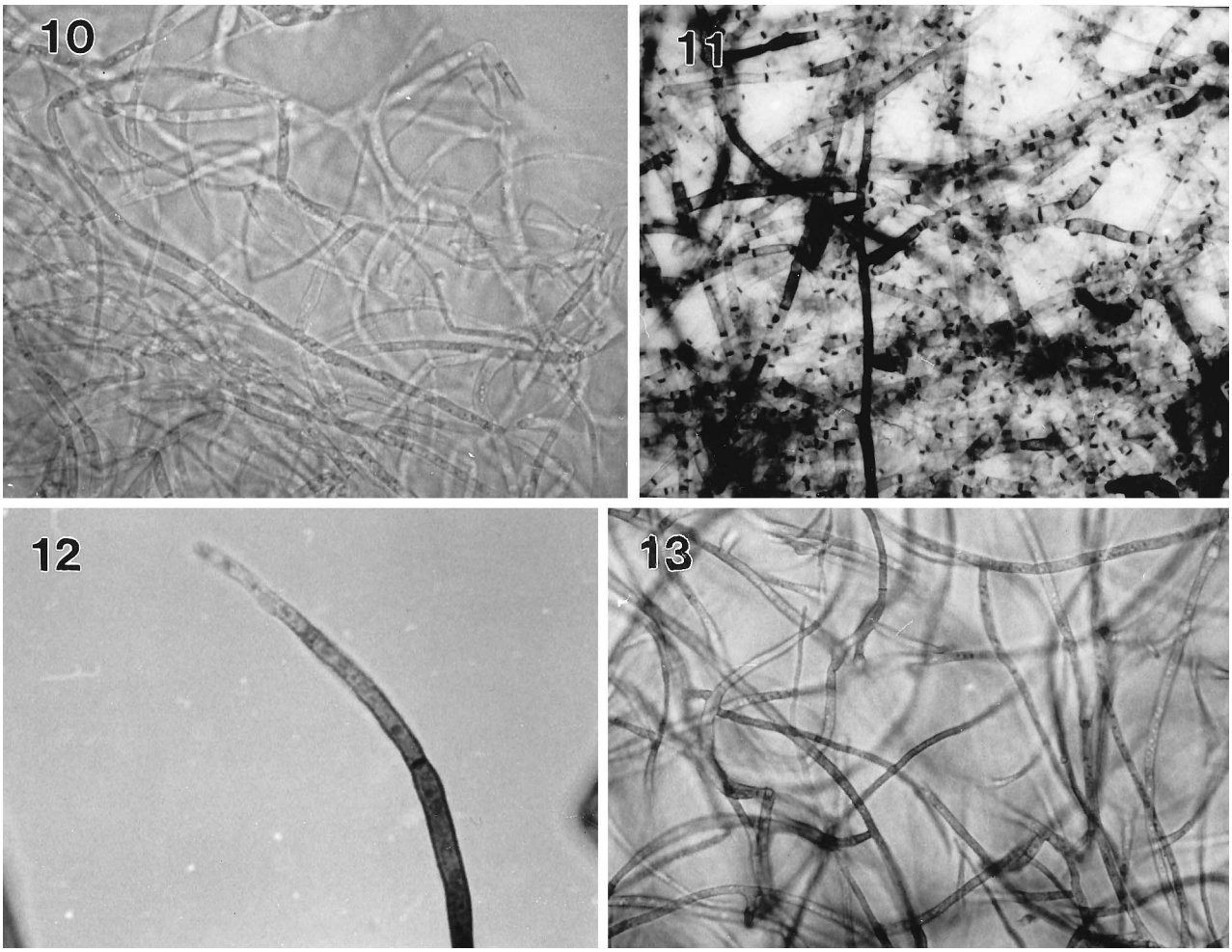

FIG. 10–13. Copper localization with the sulfide-silver technique for light microscopy of 18-day-old cultures.
FIG. 10. No metal was detected in hyphae grown in trace amounts of $CuSO_4$. Magnification, ×2,500.
FIG. 11. Hyphae grown in 0.04 mM $CuSO_4$. All septa and cell walls were silver stained, and some hyphal branches were heavily stained. Magnification, ×2,500.
FIG. 12. Unstained hyphal tip. Silver deposition occurred in the subapical region. Magnification, ×6,250.
FIG. 13. Hyphae grown in 0.04 mM $CuSO_4$ and 10 μg of tricyclazole per ml. The cytoplasm of the hyphae was lightly silver stained, but no silver deposits were visible in septa. Magnification, ×2,500.

reported the presence of melanin granules at the outer surface of cell walls of *A. pullulans* grown in copper. In *G. graminis*, an electron-dense layer was present on hyphal exterior surfaces, but it was also present in unpigmented, tricyclazole-grown cultures that had white hyphae; hence, it was probably not melanin or another pigment.

Animal melanin (DOPA melanin) is synthesized as cytoplasmic granules within specialized organelles, or melanosomes (2, 44). How fungal hyphae become melanized is unknown. Is melanin formed in the cell wall or in the cytoplasm then deposited into the cell wall? Electron micrographs of freeze-substituted *G. graminis* hyphae did not reveal exocytosis of melanin granules through the plasma membrane or any vesicles containing melanin near the plasma membrane.

The mechanism of increased *G. graminis* melanin in response to copper is also not known. The activity of laccase, a secreted enzyme that cross-linked 1,8 DHN in vitro, also increased in response to copper (23a). Others suggest that fungal laccases polymerize melanin precursors in vivo (reviewed in reference 4). It is possible that increased laccase activity is responsible for increased melanization in hyphae grown in elevated levels of copper. Electron-dense granules observed in the cell wall could be the product of DHN cross-linked by laccase.

AAS confirmed that *G. graminis* biomass absorbed most of the supplemented copper within 18 days. Silver staining suggested that hyphal cell wall melanin trapped CuS, because silver deposits were observed in the intermediate cell wall layer. DHN has two hydroxyl groups that could bind metal cations, and at least some of these hydroxyl groups would still be available in the cross-linked DHN polymer. However, little is known of how DHN is cross-linked, and it has not been demonstrated that pure DHN melanin binds metal ions.

Although melanin is protective in general, direct evidence for melanin protection from heavy metal exposure is lacking. Melanin may act as a physical barrier to toxic levels of metal ions, and several heavily melanized organisms are also metal tolerant (22). Because low, nontoxic concentrations of copper provoke melanization of *G. graminis* hyphae, melanization could serve to sequester essential metal ions for later metabolic use. Surprisingly, AAS showed that cultures grown in 0.04 mM $CuSO_4$ and tricyclazole bound almost as much Cu as

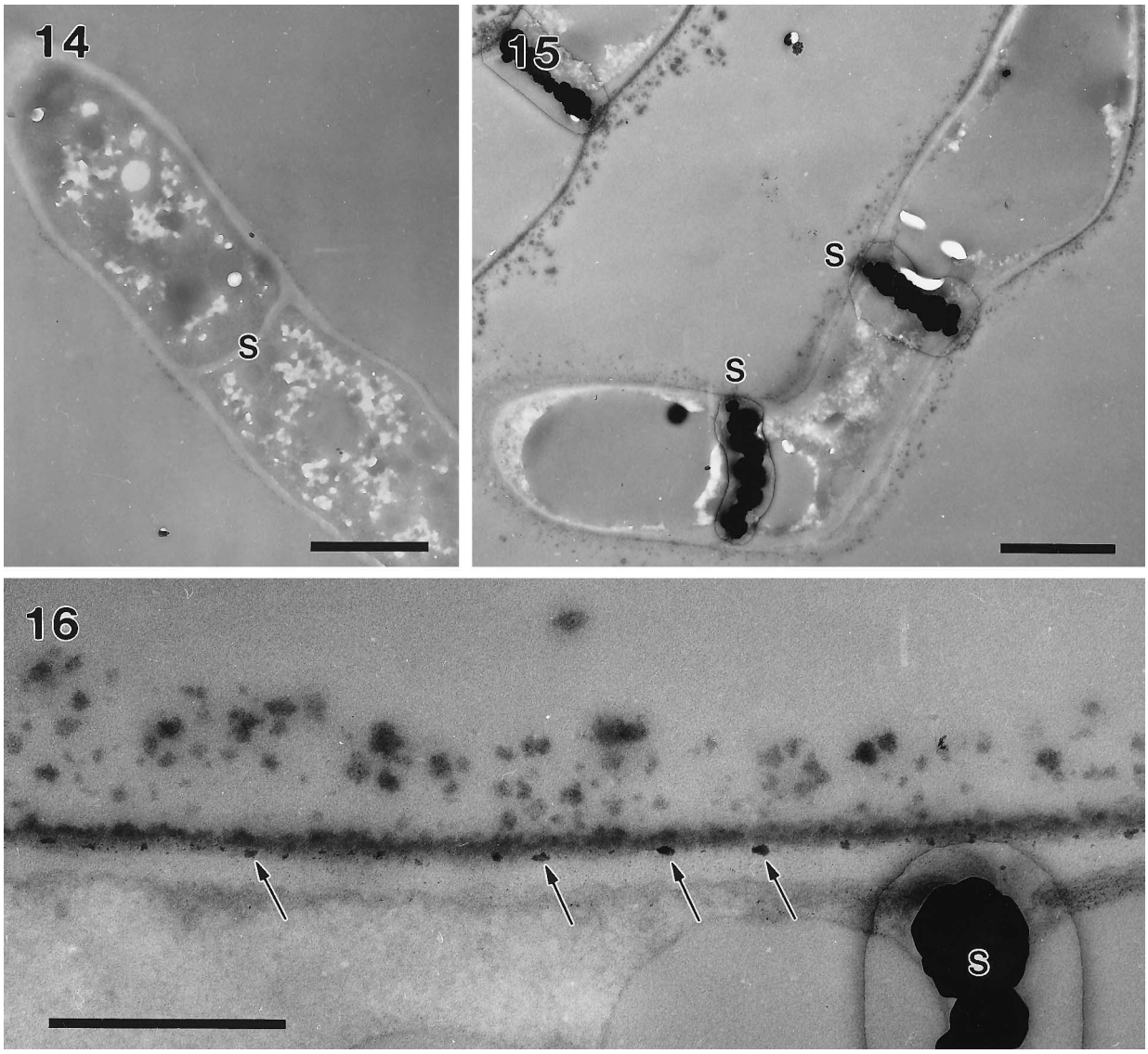

FIG. 14–16. EM of sulfide-silver-stained thin sections from chemically fixed, 9-day cultures grown in minimal medium with trace amounts of copper or 0.04 mM $CuSO_4$. S, septum. For all micrographs, the bar is 0.5 μm.

FIG. 14. Hypha grown in trace amounts of copper had no silver stain in the septum or cell wall. Magnification, ×26,000.

FIG. 15. Silver staining of hypha grown in 0.04 mM $CuSO_4$ revealed large silver precipitates on septa. Magnification, ×26,000.

FIG. 16. Magnified view of a hypha grown in 0.04 mM $CuSO_4$ showing silver grains between the inner and outer cell wall layers (arrows). Magnification, ×66,000.

cultures grown in $CuSO_4$ alone. Perhaps Cu in tricyclazole-grown cultures was distributed throughout the cells as suggested by silver staining. Cu may also have bound melanin pathway precursors such as flaviolin and 2-hydroxyjuglone that are known to accumulate in hyphae treated with tricyclazole (4, 32, 48, 49, 51, 52). Treatment of cultures with tricyclazole concentrations necessary to completely inhibit melanization in the presence of copper also inhibited fungal growth (data not shown). The finding that 0.04 mM $CuSO_4$ completely inhibited growth in 30 μg of tricyclazole per ml, a concentration that did not inhibit growth of hyphae grown in trace amounts of copper, also suggested that melanin protected the fungus from copper toxicity.

Only a few insoluble metal ions are required to nucleate silver precipitation by the silver-enhancement method (9–11). Detection of copper sulfide in melanized cell walls and septa suggested that fungal melanin plays a role in sequestration of copper. Cells grown in elevated levels of $CuSO_4$ plus tricyclazole had less silver deposition in their cell walls, which supported the hypothesis that melanin bound or trapped CuS. Copper sulfide was also observed in the septa of hyphae grown in 0.04 mM $CuSO_4$ but not in hyphae grown in 0.04 mM $CuSO_4$ plus tricyclazole. Septa in hyphae grown in elevated levels of copper may contain melanin that sequesters copper sulfide. Electron-dense granules were observed in the septa of *A. pullulans* hyphae (38). The absence of stain in hyphal tips, which are unmelanized, also suggested that melanin was responsible for CuS binding.

Sulfide-silver staining demonstrated the presence of CuS in hyphal walls, which supports the hypothesis that fungi produce hydrogen sulfide (H_2S) that reacts with metal ions to form insoluble metal sulfides that can be trapped in cell walls (33). Such sequestration of copper sulfide is observed in *Saccharomyces ellipsoideus* grown in elevated concentrations of copper

in which copper sulfides were found between the chitin layer and the outer wall layer (1). Sulfide generation in *S. cerevisiae* promotes cell wall Cu deposition (33). H_2S production in *S. cerevisiae* can cause cells to become slightly more brown (45), but this yeast is not known to synthesize melanin. Therefore, the mechanisms of CuS binding in yeasts and filamentous ascomycetes may be different.

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