ASSESSING BIOCIDE EFFECTS ON PROTOPLASTS OF WOOD DECAY FUNGI[1]

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ABSTRACT

Hyphal fragments and protoplasts of two wood-degrading fungi, *Postia placenta* and *Trametes versicolor*, were used to examine the effects of two fungicides, copper sulfate and azaconazole, on fungal health (as measured by oxygen consumption, glucose utilization, potassium levels, cell regeneration, and copper uptake). Although both cell systems responded to the presence of fungicide, there were some differences in response. Protoplasts tended to absorb higher levels of copper and were less sensitive to copper sulfate than were mycelial fragments. In general, however, protoplasts provided uniform cells for evaluating the effects of fungicides on cell health.

Keywords: Biocides, protoplasts, basidiomycetes, copper sulfate, azaconazole, *Postia placenta, Trametes versicolor.*

INTRODUCTION

The effects of biocides on the activity of wood-degrading fungi are generally assessed by exposing wood treated with the biocide to the test fungus either in soil or on agar (ASTM 1992). Although these trials produce information on the relative effectiveness of a given biocide, they provide no data on the mechanism by which the biocide effected control. That information can be obtained indirectly by growing the fungus in liquid media containing the biocide and then measuring various growth properties, but that method has several drawbacks.

Filamentous hyphae growing in liquid cultures contain hyphae of varying ages, which have different degrees of physiologic activity and potential sensitivity to toxicants. In many fungi, the most physiologically active portion is the hyphal tip, which makes up a relatively small percentage of the material present in a liquid culture. Because an effective biocide should prevent extensive microbial growth, the hyphal tips may be an important target. Methods that use mycelial masses to evaluate the effects of biocides may measure only averages, which are dominated by the higher percentage of less active hyphae. These older hyphae may mask the effects of biocides on actively growing hyphal tips.

An alternative to the use of mycelial masses is the use of protoplasts of the test fungi. Protoplasts lack a cell wall and can be produced from liquid cultures of filamentous fungi treat-

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ed with cell-wall lytic enzymes (Anderson and Millbank 1966; Benitez et al. 1975; Davis 1985; Collings et al. 1989). Protoplasts should be more uniform than mycelial masses in their response to toxicants, and more representative of hyphal tips. There is little data on the effects of biocides on protoplasts produced from cultures of wood degrading fungi. In this report, we compare the effects of two fungicides on the activity of mycelium and protoplasts of two common wood degrading fungi.

MATERIALS AND METHODS

Protoplasts were produced from actively growing cultures of *Postia placenta* (Fr.) M. Lars. & Lomb. (Isolate Madison FP 94267R), U.S. Forest Products Laboratory (Madison, Wisconsin) and *Trametes versicolor* (Fr.:Fr.) Pilat (Isolate Madison, R105) through a method described in Rui and Morrell (1993). Eight-day-old cultures of the test fungi were grown in a liquid medium containing 1% glucose, 1% malt extract, and 0.1% yeast extract. The mycelial mass was filtered through sterile filter paper and washed twice with sterile distilled water followed by two rinses in 50 mM maleic-NaOH buffer (pH 5.5) with 0.5 M mannitol. The mycelium was then resuspended in 3 ml of 50 mM maleic-NaOH buffer that contained 0.4% Novozyme 234 (Industria A/S., Bagsaverd, Denmark) and 0.5 M mannitol. The mycelium was exposed to the enzyme for 2 h, then filtered through 8 layers of cheesecloth to remove larger mycelial fragments. The filtrate was centrifuged for 20 min at 2,500 × g, and the pelleted protoplasts were used for assays.

Hyphal fragments were prepared from 8-day-old cultures grown on the same medium. The mycelium was filtered through a coarse sintered glass filter and washed with 50 mM maleic-NaOH buffer (pH 5.5). The mycelium was resuspended in 200 ml of the same buffer and homogenized for 4 sec in a blender. The suspension was filtered through cheesecloth to remove larger fragments, then centrifuged for 20 min at 2,500 × g. The pelleted hyphal fragments were used in all assays.

Chemical exposures

Hyphal fragments or protoplasts were exposed to either copper sulfate or azaconazole ((1-[2-(2,4-dichlorophenyl)-1,3-dioxolan-2-methyl]-1 H-1,2,4-triazole) (Janssen Pharmaceutical, Washington Crossing, New Jersey). Copper compounds have an array of potential effects against cells (Ross 1975; Gadd et al. 1984, 1987; Gadd and White 1985). Azaconazole is a triazole that affects sterol synthesis (Leclercq 1983; Valcke and Messina 1985). Aqueous copper sulfate stock solutions were prepared at concentrations of 10, 25, 50, 75, 100, 125, and 150 mM; azaconazole was prepared at 22, 55, 110, 165, 220, and 330 μg/ml of water. The fungicide stock solutions were sterile-filtered through a 0.45-μm diameter membrane (Millipore, Bedford, Massachusetts) and stored at 5 C until needed.

Regeneration

The ability to regenerate to produce normal mycelial cultures can provide a measure of the relative physiologic capabilities of a given fungus. Regeneration was evaluated for protoplasts of both fungi by suspending the cells in 2 ml 50 mM maleic-NaOH buffer amended with 0.5 M mannitol to produce a final cell density of approximately 2×10^7 cells/ml. Hyphal fragments were suspended in the same buffer (without mannitol) to produce approximately 3.7 mg cell (dry weight)/ml. Previous studies had shown that these concentrations produced comparable oxygen consumption levels between hyphal fragments and protoplasts of the fungi evaluated (C. Rui unpublished data). The cells were exposed to concentrations of 200, 600, 1,000, 1,600, 2,000, or 4,000 μM copper sulfate or 0.44, 1.10, 2.20, 3.30, 4.40, or 8.80 μg/ml of azaconazole.

Regeneration was assessed by adding 20 μl of diluted protoplast or hyphal fragment solution to a 1.5-ml vial that contained 980 μl of malt-yeast-glucose solution (MYG). Protoplast preparations were supplemented with 0.5 M mannitol as an osmotic stabilizer. The preparations were poured over the surface of

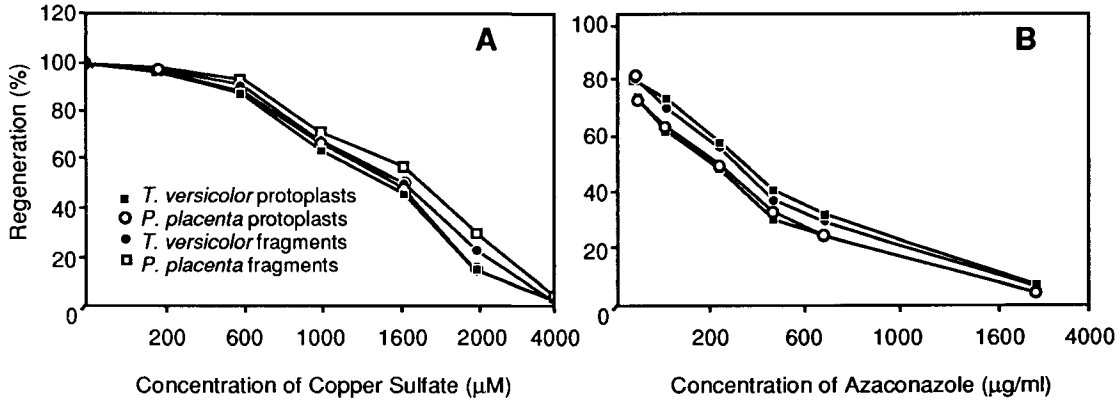

FIG. 1. Regeneration of *T. versicolor* and *P. placenta* protoplasts and hyphal fragments after 2-h exposure to copper sulfate or azaconazole. For plating efficiency of protoplasts and hyphal fragments, values are expressed relative to controls. Plating efficiencies of control samples for *T. versicolor* and *P. placenta* protoplasts were 21.3% and 10.8%. Corresponding values for *T. versicolor* and *P. placenta* hyphal fragments were 90.5% and 87.4%, respectively.

MYG agar in petri dishes and incubated for 10 days at 28 C. Viable fungal colonies were counted for each treatment, which provided a measure of the ability of hyphal fragments or protoplasts to regenerate.

Oxygen consumption

Oxygen consumption provides a relative measure of cell activity in response to biocides. Oxygen uptake was measured for *T. versicolor* by placing 1.8 ml of either protoplast or fragment suspension in the electrode chamber of a YSI 5357 Clark-type polargraphic oxygen probe with a model 5300 Oxygen monitor (YSI, Inc., Yellow Springs, Ohio). At the beginning of the test, the solution contained approximately 0.80 μM of dissolved oxygen at 30 C. For each test, 36 μl of 50 mM glucose was added to the electrode chamber along with enough fungicide stock solution to produce concentrations of 150, 1,500, 4,000, or 10,000 μM of copper sulfate or 0.44, 4.40, or 13.40 μg/ml of azaconazole and a final concentration of 1 mM glucose. Oxygen consumption was recorded over the exposure period.

Glucose depletion

Glucose consumption provides a measure of the cells' ability to actively metabolize sugars and produce energy. Chemicals that affect steps in the glycolysis or electron transport should eventually inhibit glucose consumption. Glucose consumption was evaluated by combining 3.84 ml of the *T. versicolor* fragment or protoplast suspension with 80 μl of 55 mM glucose and 80 μl of the appropriate fungicide stock solution in 10-ml test tubes to produce biocide concentrations of 200 and 1,500 μM of copper sulfate and 0.44 and 4.40 μg/ml of azaconazole and glucose concentrations of 200 μg/ml. The mixtures were incubated at 28 C for 15, 30, and 72 h. At each time point, 0.5 ml of solution was withdrawn and centrifuged for 8 min at 10,000 rpm. The supernatant was then analyzed for residual glucose with the O-Toluidine assay (Sigma Chemical Co., St. Louis, Missouri).

Potassium levels

Many biocides act upon cell-wall membranes, either by inhibiting active transport or by increasing cell permeability, which leads to loss of electrolytes. The latter effect can be detected by measuring changes in potassium ion concentration. Protoplasts or hyphal fragments of *T. versicolor* and *P. placenta* were suspended in 50 mM maleic-NaOH buffer (pH 5.5) containing 1 mM glucose and copper sulfate or azaconazole at the desired levels. Copper sulfate was evaluated at 200, 1,000, and

TABLE 1. *Effect of copper sulfate and azaconazole on oxygen uptake of* T. versicolor *protoplasts and hyphal fragments in the presence of 1 mM glucose.*

Fungicide	Concentration	Time (min)[1]		O_2 saturation %
		Protoplast	Fragment	
Copper sulfate	150 μM	31.2	30.6	0
	1,500 μM	34.8	29.5	0
	4,000 μM	44.6	26.3	0
	10,000 μM	71.0	51.4	64
Azaconazole	0.44 μg/ml	30.8	30.7	0
	4.40 μg/ml	30.2	31.4	0
	13.40 μg/ml	63.0	61.0	64
Control		30.5	30.1	0

[1] Time required to decrease 100% oxygen saturation to corresponding saturation.

1,500 μM; azaconazole was tested at 0.0, 0.44, 1.10, 2.20, 3.30, and 4.40 μg/ml. The mixtures were incubated for 2 h at 28 C; then 1.5 ml of the mixture was centrifuged for 5 min at 10,000 rpm and the supernatant was removed and retained for analysis of copper (below). The pellet was extracted in 1 ml of 6 N HNO_3 for 30 min at 95 C. That solution was centrifuged for 5 min at 10,000 rpm; then 0.8 ml of supernatant was diluted with 0.8 ml of distilled water and 0.8 ml of 3 N HNO_3. The diluted supernatant was analyzed for potassium with a Perkin Elmer 4000 Atomic Absorption Spectrophotometer with an air-acetylene flame and a wavelength of 324.8 nm. Control samples without fungal material were included for comparison (data not shown).

Copper analysis

While it was not possible to determine azaconazole uptake, copper uptake was determined from the first supernatant collected from the incubated protoplast and hyphal fragment suspensions. The solutions were analyzed for residual copper with the same spectroscopy unit used for potassium analysis (operated at a wavelength of 766.5 nm). The difference between solutions with and without fungal protoplasts or hyphal fragments provided a measure of the copper that was either transported into the cell or bound to the cell wall or membrane.

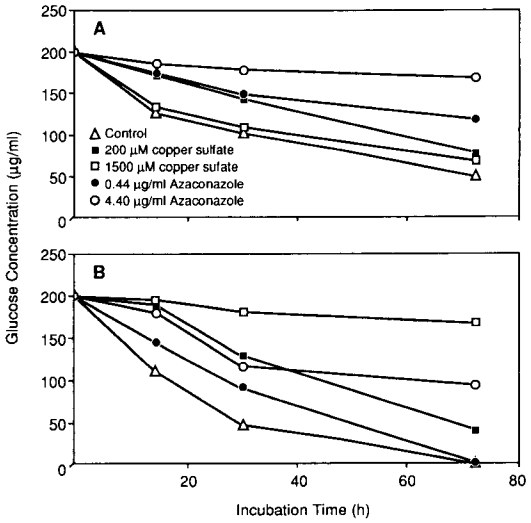

FIG. 2. Effect of copper sulfate or azaconazole on glucose consumption of *T. versicolor* protoplasts (A) or hyphal fragments (B).

RESULTS AND DISCUSSION

Cell regeneration

The effects of both fungicides on regeneration of protoplasts or hyphal fragments increased with increasing concentration (Fig. 1). Regeneration ceased when cells were exposed to 4,000 μM of copper sulfate or 8.80 μg/ml of azaconazole. In general, protoplasts and hyphal fragments reacted similarly to the presence of either fungicide, which suggests that residual cell walls on the hyphal fragments did not interfere with fungicide uptake or activity. This was perplexing because cell walls could bind the fungicide, thereby reducing the amount available, or act as a physical barrier to its diffusion. The two species of fungi also reacted similarly to copper sulfate and azaconazole. *Postia placenta* is well known for its copper tolerance (Zabel 1953; Cowling 1957), but showed no substantive difference in response. Copper tolerance by brown rot fungi is believed to be related to the production of oxalic acid (Levi 1969). It is possible that oxalic acid production was minimal during the test period or that the test conditions were not suitable for the production of significant quantities of this metabolite.

TABLE 2. *Potassium levels of protoplasts and hyphal fragments of* T. versicolor *and* P. placenta *after 2-h exposure to copper sulfate and azaconazole in 50 mM maleic-NaOH buffer of pH 5.5 and 1 mM glucose at 28 C.*

Fungicide	Concentration	Potassium level[1]			
		T. versicolor		*P. placenta*	
		Protoplasts	Fragments	Protoplasts	Fragments
Copper sulfate	200 μM	0.873	0.913	0.890	1.009
	1,000 μM	0.544	0.613	0.801	0.839
	1,500 μM	0.602	0.662	0.635	0.551
Azaconazole	0.44 μg/ml	0.877	0.776	0.920	1.029
	1.10 μg/ml	0.867	0.776	0.856	1.009
	2.20 μg/ml	0.901	0.841	0.886	1.003
	3.30 μg/ml	0.897	0.820	0.901	0.999
	4.40 μg/ml	0.941	0.790	0.944	0.979
Control (initial)	0.0	1.105	1.103	1.100	1.050
Control (final)	0.0	0.925	0.915	0.905	0.970

[1] Units for protoplasts and hyphal fragments were 2×10^7 cells/ml and 3.7 mg dry weight/ml, respectively. At that level, hyphal fragments-per-ml gave an oxygen uptake equivalent to that of protoplasts-per-ml.

Oxygen consumption

Oxygen consumption decreased as fungicide dosage increased for both protoplasts and hyphal fragments, but hyphal fragments were less sensitive than were protoplasts (Table 1). Inhibition of oxygen consumption was generally slow, and complete inhibition was achieved only with the highest dosages of either chemical. The lag in effect suggests that inhibition of respiration reflects more general cell damage, which eventually is reflected in decreased oxygen consumption, rather than direct inhibition of the electron transport system. Previous studies of other fungi with both copper sulfate and azaconazole support this conclusion (Leclercq 1983; Gadd et al. 1987). The greater sensitivity of protoplasts implies that chemical uptake occurs more rapidly, producing more dramatic effects on oxygen consumption.

Glucose depletion

Both protoplasts and hyphal fragments were capable of glucose utilization during the exposure to copper sulfate and azaconazole, but they differed in consumption patterns (Fig. 2). Protoplasts initially used glucose at a slightly higher rate than did cell fragments, but the rate of consumption declined more rapidly near the end of each test. The use of a blender to produce hyphal fragments may have initially affected glucose transport or respiration, thereby reducing the physiologic capacity of the cells. As expected, glucose consumption in hyphal fragments slowed as dosage of either copper sulfate or azaconazole increased; the effects were reversed for protoplasts. The reasons for this difference are unclear, since both protoplasts and hyphal fragments should be susceptible to increasing concentrations of fungicide.

Potassium levels

Potassium loss from cells has been used to monitor the effects of fungicides on membrane function (Lambert and Hammond 1973; Gale 1974; Lambert and Smith 1976; Cope 1980). Increased cell leakage can profoundly affect many membrane-mediated functions including glucose uptake.

In general, cells treated with copper sulfate were more sensitive to potassium loss than were those treated with azaconazole, but there appeared to be little consistent difference between the responses of protoplasts and hyphal fragments to chemical exposure (Table 2). Copper compounds can bind to cell membranes to interfere with transport and denature proteins. Triazoles such as azaconazole inhibit sterol synthesis, which will eventually disrupt membrane functions (Sancholle et al. 1984). The effect on protoplasts and hyphal fragments would probably be similar, although cell walls

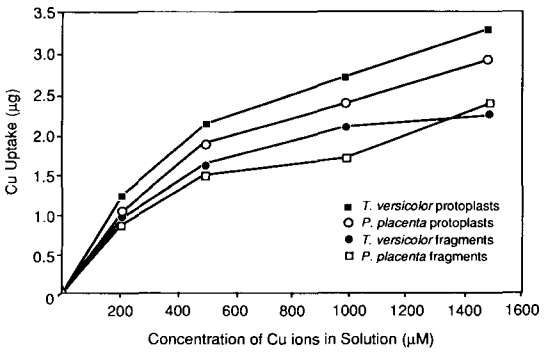

FIG. 3. Copper uptake of protoplasts and hyphal fragments of *T. versicolor* or *P. placenta* after 2-h exposure to copper sulfate in maleic-NaOH buffer (pH 5.5) at an incubation temperature of 28 C.

might sorb some copper, which would reduce the amount available to bind to the membrane. It is likely that the copper binding on cell walls and membranes was slight and may have been masked by the high copper levels tested.

Copper uptake

As expected, copper uptake increased with increasing dosage of copper sulfate (Fig. 3), although the rate of uptake slowed for *T. versicolor* hyphal fragments at the higher levels of copper sulfate. Higher dosages also tended to produce differences between protoplasts and hyphal fragments of a given species; protoplasts sorbed more copper than did hyphal fragments. Cell walls on hyphal fragments can be expected to sorb copper, which would produce higher levels of copper in those cells; however, the presence of an intact cell wall and cytoplasmic membrane also can combine to slow copper uptake into the cytoplasm. Studies that segregate cell-wall material from the cell membrane and cytoplasm prior to copper analysis would help delineate the nature of copper uptake in hyphal fragments.

CONCLUSIONS

Protoplasts and hyphal fragments both appear to be useful for monitoring the effects of fungicides on fungal physiology; however, there were slight differences between these two cell systems in copper uptake and in oxygen consumption. These subtle differences in cell response may be due to the presence or absence of a cell wall, which could affect the mechanisms of chemical uptake or the site of action. Thus, protoplasts may represent an alternative system for studying fungal response to fungicides in the absence of cell walls.

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