TOLERANCE OF *WOLFIPORIA COCOS* ISOLATES TO COPPER IN AGAR MEDIA

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ABSTRACT

Several wood preservatives, especially some newer waterborne systems, use copper (II) as a deterrent to wood-decay fungi. However, several brown-rot wood-decay fungi exhibit tolerance to copper. Among these is the genus *Wolfiporia,* which was at one time included within the taxon *Poria* (Pres. ex Gray). This research investigated the variations of tolerance to copper among isolates using agar-screening trials. The basis for the characterization of isolates as high, medium, and low copper tolerant is the minimum inhibitory concentration (MIC) of copper that deters growth of the isolate. We also compared growth rates among isolates at sublethal levels of copper. The variations of tolerance to copper among isolates of *W. cocos* in agar media indicate that the difference in copper tolerance is statistically significant among isolates. Interpretative analysis of the radial growth indicates that there is not a strong correlation between rate of growth and tolerance to copper. The copper tolerance of various isolates can have a significant practical impact on the long-term performance of treated-wood products or the bioprocessing of spent wood materials treated with copper-based preservatives.

Several brown-rot, wood-decaying fungi that were once closely aligned or included within the genus *Poria* (Pers. ex Gray) are tolerant of copper (11,12,24, 27,30,33). This is of practical concern because some wood preservatives, especially several of the newer, waterborne systems, are copper based (16). Whether isolates of a given species have comparable or different tolerances to specific metals may also be of importance in characterizing biosequestration and remediation processes. Although the existence of copper-tolerant fungi has been known for some time, the potential variation, among isolates of the same species, has not been extensively studied (10). Collett (10) determined that isolates of *Antrodia vaillantii* (DC.; Fr.) Ryv., a brown-rot fungus recognized for its copper tolerance, differed significantly in their tolerance to copper. In this study, we investigated the variations of tolerance to copper among isolates of the copper-tolerant fungus (15,28) *Woljiporia cocos* (Schw syn. *Poria cocos),* which is widely distributed on hardwoods and conifers throughout the United States (12,20).

In this paper, we report our results from agar screening trials. Future work will evaluate capabilities of isolates to attack wood treated with different formulations of copper-based preservatives. These studies will contribute to better laboratory methods for predicting long-term performance of treated-wood products and to technologies for bioprocessing spent wood products that are treated with a copper-based preservative.

The few direct comparisons that can be made from published literature on relative tolerance of specific cultures of decay fungi to copper when assayed in agar media and wood substrates indicate a greater tolerance of copper by fungi in wood than in agar (2,13) and show a general (28) correlation of results between the two methods.

MATERIALS AND METHODS

FUNGAL ISOLATES

Twenty isolates of *W. cocos* were obtained from the culture collection in the Center for Forest Mycology at the USDA Forest Service, Forest Products Laboratory. These isolates had been isolated from wood substrates at various locations within the United States (Table 1). The tolerance to copper on agar media was determined with all isolates. Prior to testing, the isolates were maintained on a 2 percent malt extract medium containing 20 g malt-extract (Oxoid), 15 g agar (Difco), and 1.0 L of water demineralized by a reverse osmosis process.

AGAR MEDIA

Unbuffered, malt-extract agar medium was supplemented with copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) at concentrations of 0.003M, 0.005M, 0.0lM,

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TABLE 1. — Isolates of Wolfporia cocos.

Culture ID no.		Wood type	Decay	Location of isolation	Collector	Identified by
4178	Pnel-B-25	*Abies balsamea*	Brown cubical buttrot	Ralph, Michigan	D.O. Prielipp	H.H. McKay
4179	H+E-45	*Pinus contorts*	Brown cubical buttrot	Targhee National Forest, Idaho	T.E. Hinds	F.F. Lombard
4180	Log-WH-45-A	*Tsuga heterophylla*		Siskiyou National Forest, Oregon	E.R. Wright	E.R. Wright
4181	L(61)1-8-A	*Carya*	White pocket buttrot	Ohio	F.F. Lombard	F.F. Lombard
4182	MD-104-R	*Pinus* spp.		Gainesville, Florida	W.R. Smith	F.F. Lombard
4183	MD-106-R	Alnus		Oregon	R.D. Graham	F.F. Lombard
4184	MD-215	Pine, southern yellow		Florida	M. Hudson	F.F. Lombard
4185	MD-275	*Pinus contorts*		Corvallis, Oregon	R.D. Graham	F.F. Lombard
4187	FP-71054	*Quercus velutina*		Willow Springs, Missouri	N.L. Noecker	W.A. Campbell
4188	FP-71284-A	*Acer*	Tree rot	Pennsylvania	W.A. Campbell	W.A. Campbell
4189	FP-71691-R	*Fagus* spp.		State College, Pennsylvania	W.A. Campbell	W.A. Campbell
4190	FP-71692-R	*Betula papyrifera*		Irving, Massachusetts	W.A. Campbell	WA. Campbell
4191	FP-71693-T	*Quercus* roots		Philadelphia Pennsylvania	K.D. Doak	K.D. Doak
4192	FP-71730-T	Kitchen floor		Harold Harbor, Maryland	J.A. Stevenson	J.A. Stevenson
4193	FP-90850-R	*Pinus echinata*	Brown mottled rot	Alabama	E.R. Roth	L.O. Overholts
4194	FP-90850-SP	*Pinus echinata*	Brown mottled rot	Little leaf plot 20, Alabama	E.R. Roth	L.O. Overholts
4195	FP-90886-T	*Pinus echinata*		Walhalla, South Carolina	W.A. Campbell	W.A. Campbell
4197	FP-97438-SP	*Pinus monticola* (log)		Coeur d'Alene, Idaho	R. W. Davidson	J.L. Lowe
4199	FP-104264-SP	Pine, southern yellow		Gainesville, Florida	J.L. Lowe	J.L. Lowe
NA	MD-104	unknown			F. Lombard	F. Lombard

TABLE 2. — Spearman correlation of colony diameter at 14 days.

	M Cu			
M Cu	0.00	0.003	0.005	0.010
0.000	1.0	0.24	0.30	0.13
0.003	0.24	1.0	0.89	0.59
0.005	0.30	0.89	1.0	0.41
0.010	0.13	0.59	0.41	1.0

TABLE 3. — Minimum inhibitory concentration of copper.

Isolate ID	MIC (M Cu)
MD-104	0.04
L(61)1-8-A	0.04
Priel-B-25	0.04
Log-WH-45-A	0.03
FP-71692-R	0.03
MD-275	0.03
MD-104-R	0.03
FP-71691-R	0.03
MD-215	0.02
FP-90886-T	0.02
FP-71730-T	0.02
FP-71054	0.02
FP-90850-SP	0.02
FP-90850-R	0.02
MD-106-R	0.02
FP-71693-T	0.02
FP-97438-SP	0.02
FP-104264-SP	0.02
FP-71284-A	0.02
H+E-45	0.02

0.02M, 0.03M, and 0.04M copper. Copper sulfate solutions and malt-extract media were prepared separately. The latter, containing 80 percent of the final volume of water, was autoclave at 121 °C for 20 minutes; the remaining water was used to dissolve the copper sulfate. The copper solutions were then filter sterilized using 0.45-mm membrane filters (Whatman) and added to cooled agar media. Malt-extract medium with no added copper was used as the control.

Sterile medium was dispensed in 20-mL volumes into 15- by 100-mm petri dishes and inoculated with 5-mm discs cut from the periphery of a 7-day-old colony. Each isolate was inoculated onto three plates and incubated at 28°C, 70 percent relative humidity. Mycelial growth of the isolates was determined by linear measurements of colony radius. Average radial growth was determined from six measurements, i.e., two per plate made at right angles to each other. Measurements were taken at 3,7,10,14, and 21 days after inoculation.

Comparisons

Tolerance to copper was characterized by the minimum inhibitory concentration (MIC) of copper. The data set was not adequate to support a useful 50 percent inhibition dose-response analysis, but we were able to make statistical comparisons among isolates in growth at specified concentrations of copper and periods of incubation. Spearman correlations between the diameters of mycelia were used to examine if there was a parallel response among isolates in growth rate to increasing concentrations of copper sulfate at concentrations of 0.01M Cu and below. In addition, we compared the diameters of all 20 isolates at 14 days on media containing 0.01M Cu using Tukey multiple comparisons on Normal scores. Diameters of isolates that tolerated 0.02M Cu were compared at 21 days of incubation. In these comparisons, the diameter was the average of the two perpendicular measurements. Those instances where the inoculum died (deter mined through attempts to reculture following the experiment) were treated as missing data in the statistical analysis. Interpretive analysis of the radial growth of 20 *W. cocos* isolates on

TABLE 4. — Tukey's multiple comparison test of Normal scores for colony diameters of 20 isolates of W. cocos after 14 days incubation on media containing 0.01M Cu.

Isolate	Normal scores test[a]
FP-71054	A
Log WH-45-A	A B
MD-104	A B C
FP-90886-T	A B C
L(61)1-8-A	A B C D
FP-90850-SP	A B C D E
MD-106-R	A B C D E
FP-71692-R	A B C D E
FP-90850-R	A B C D E
FP-71730-T	A B C D E
Priel-B-25	A B C D E
FP-71691-R	A B C D E
MD-275	B C D E
H+E-45	B C D E
MD-215	B C D E
FP-71693-T	C D E
FP-71284-A	D E
FP-97438-Sp	D E
FP-104264-Sp	E
MD-104-R	E

[a]Isolates with the same letter in the column are not significantly different. The isolate with the most growth is at the top of the list; the isolate with the least growth is at the bottom of the list.

agar with different molar concentrations of copper was also made from inspection of actual observations.

RESULTS

None of the isolates tested was able to grow at the highest molar concentration (0.04M Cu) of $CuSO_4 \cdot 5H2O$, but all were able to tolerate a concentration of 0.01M Cu and below. Even with the range of 0.003 to 0.01 M Cu, the correlation among isolates between dose and colony diameter at 14 days was not strong **(Table 2).** In most instances, radial growth decreased with increased copper concentrations, but with 7 of the 20 isolates, radial growth increased in the presence of low concentrations of copper. These seven isolates were the following: MD-104 **(Fig. lC);** FP-71054 **(Fig. 3A);** MD-106-R **(Fig. 4A);** FP-71693-T **(Fig. 4B);** FP-90850-R **(Fig. 4C);** FP-97438-SP **(Fig. 5C);** FP-104264-Sp **(Fig. 5D).**

The 20 isolates of *W. cocos* that were tested were divided into three groups (high, medium, and low), according to the inhibitory concentration of copper **(Table 3).** The three isolates in the high tolerance group (MIC = 0.04M) were Priel-B-25, L(61)1-8-A, and MD-104 **(Fig. 1).** Five isolates in the medium tolerance group (MIC = 0.03M) were FP-71691-R, MD-104-R, MD-275, FP-71692-R, and Log WH-45-A **(Fig. 2).** The remaining 12 isolates fit into the low tolerance group (MIC = 0.02M) **(Figs. 3-5):** H+E-45; FP-71284-A; FP-104264-Sp; FP-97438-Sp; FP-71693-T MD-106-R; FP-90850-Sp; FP-71730-T FP-90850-R; FP-71054; FP-90886-T; MD-215.

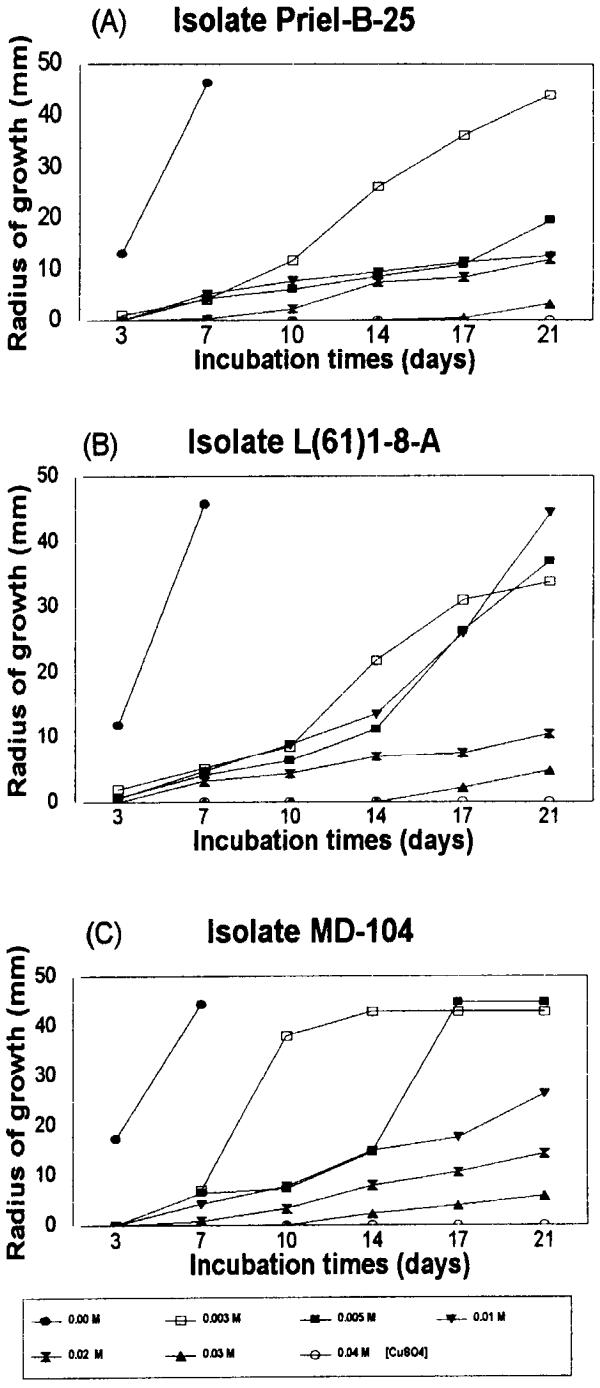

Figure 1. — Radial growth of *Wolfiporia cocos* isolates with a high tolerance to copper in agar plates.

A significant difference in colony diameters was detected among all 20 isolates on media containing 0.01 M Cu and among those isolates that grew on media containing 0.02 M Cu **(Table 4).** The relative growth rate of isolates on media without copper **(Table 5)** was not a good

predictor of growth rates in the presence of copper. Furthermore, the growth rate at concentrations of 0.01 M Cu or less does not seem to be related to the MIC of individual isolates, witness the ability of the slower growing isolate MD-104-R to tolerate more copper than some isolates that grew faster at 0.01 M Cu in the media. At 21 days of incubation on media containing 0.01 M copper, total linear growth of several isolates that were inhibited by 0.02 M copper was greater than that of some isolates that could tolerate 0.03M copper.

Patterns of growth response that are difficult to describe in a single statistic were observed. Generally, the lag period for growth that occurred was increased by the presence of copper. One exception to this was with isolate FP-71693-T at 0.003 M copper, the lowest concentration of copper that was used. With many isolates, the post-lag period growth rate on media with 0.003 and 0.005 M copper sulfate approximated those of the controls.

DISCUSSION

It is generally recognized that microorganisms with tolerance to copper are a part of the natural environment. Results from microbial ecosystem-oriented studies that used agar media have led to the development of a general, but not universal, perception that the introduction of

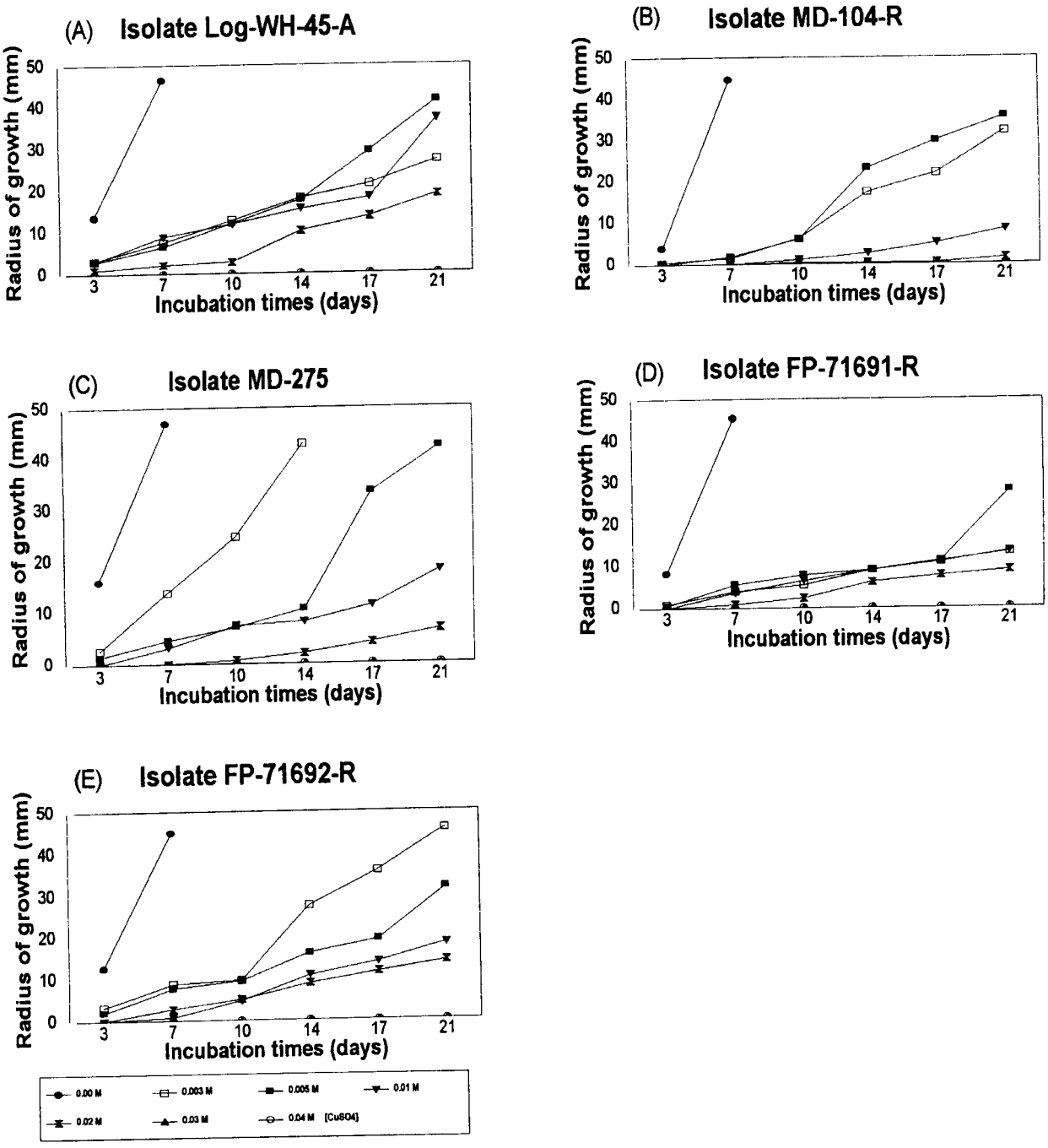

Figure 2. — Radial growth of *Wolfiporia cocos* isolates with a medium tolerance to copper in agar plates.

additional metals to soil reduces diversity of microbial species with a resultant reduction in antagonistic effects (3,17,18, 21,31). Sometimes this causes an increase in populations of microorganisms that colonize remains of microfauna, which succumb to the additional metals in their environment (22). Arguments for constitutive tolerance in populations of organisms isolated from metal-contaminated soils are based on the isolation of tolerant microflora from unaffected sites.

One mechanism for copper tolerance in some wood-decay fungi is attributed to their ability to immobilize copper by precipitating copper oxalate, but whether this is the sole mechanism for tolerance or some additional metal-binding proteins are involved is a subject for consideration. One hypothesis is that the hyphae are surrounded by an extracellular mucilaginous sheath containing dissolved oxalic acid, which in turn extracts or reacts with cations, such as calcium and/or copper, that are precipitated and deposited around the hyphae (25,26,28).

Recognition that the composition of the microbiological growth media can significantly affect the apparent toxicity of metals to fungi has lead to a guarded acceptance of results from preservative screening trials with agar media (7,8). Performing comparative laboratory tests (27,29) using strains of *Serpula lacrymans* (Schum. ex Fr.), S.F. Gray demonstrated that the ability of some fungi to decay wood is stimulated by low levels of copper in the wood and that the relative tolerance of strains to copper-containing preservatives varies with the formulation of the preservative. In those studies, the same strains of *S. lacrymans* were used in agar plate assays against copper chromate arsenate (CCA) and in agar block tests against copper naphthenate. There was a difference in relative tolerance of the isolates against the two preservatives, but the difference in methods and potentials for interactive effects of chromium and arsenic in one study does not allow more detailed analysis of these data. in studies dealing with the copper toxicity of fungi and yeast, pH has seldom been considered (6,10,26); however, the pH dependency of copper uptake has been noted by others who suggest its importance in determining tolerant behavior (17,18,25). Most fungal species that tolerated high percentages of copper in agar adjusted to pH 2 (32) also decayed pine sapwood treated to a retention of 0.175 pcf (2.8 kg/m^3) (15).

The threshold level, as determined in the soil-block test within a specified period, is essentially an MIC value. Regarding laboratory test procedures for preservative efficacy, the American-type soil-block tests (4,5), in which the preservative-treated assay block is challenged with a pure monoculture of fungus growing on a wood strip in soil, is regarded as being more severe than the European agar-block tests, in which the assay block is challenged with fungi growing on agar (9,23). Still, the possible interactions of components in natural media and resultant microbial tolerance to metals are not well understood. The type of soil used in laboratory soil-block procedures can affect the amount of decay caused in untreated wood by either brown- or white-rot fungi (1), but there is

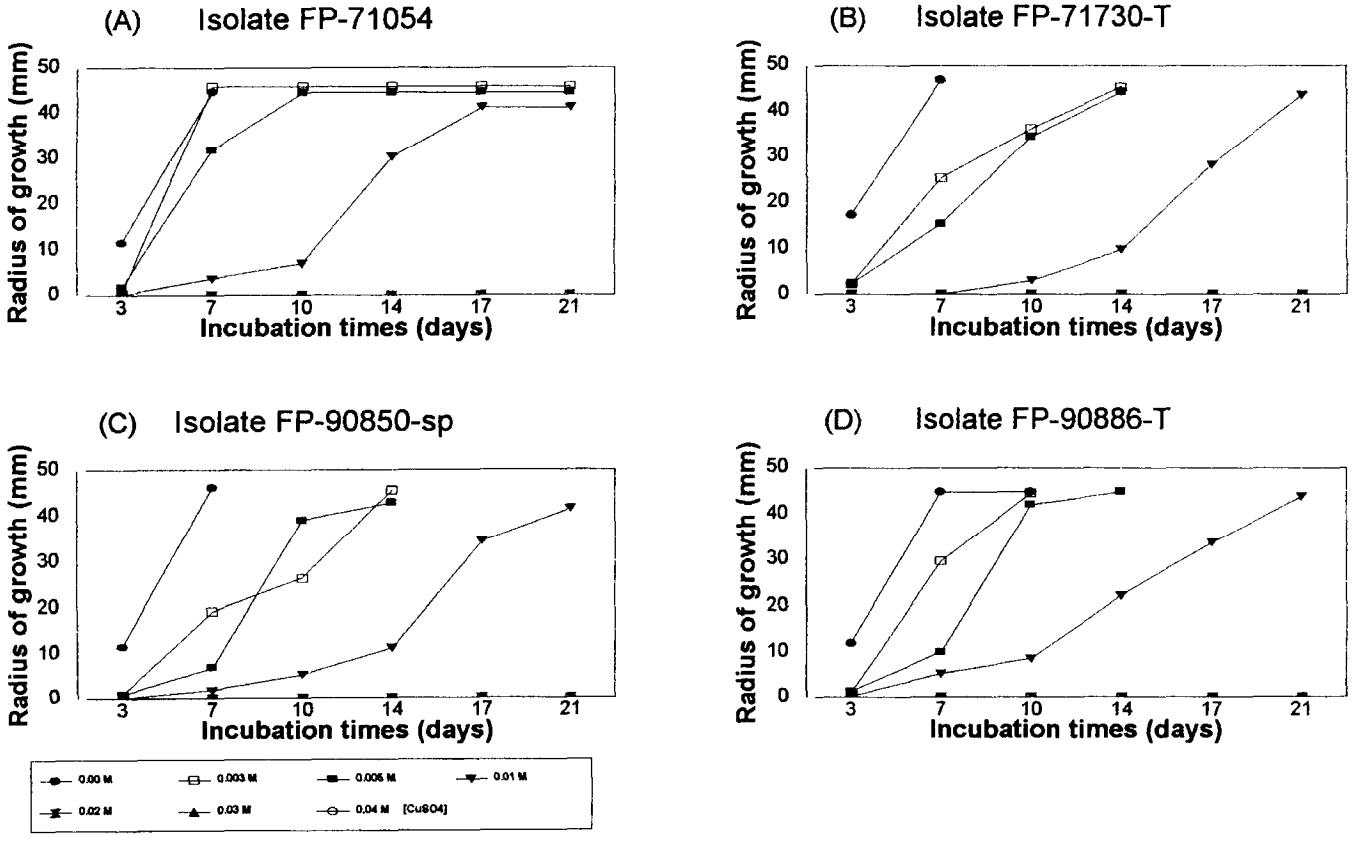

Figure 3. — Radial growth of fast growing isolates of *Wolfiporia cocos* with a low tolerance to copper in agar plates.

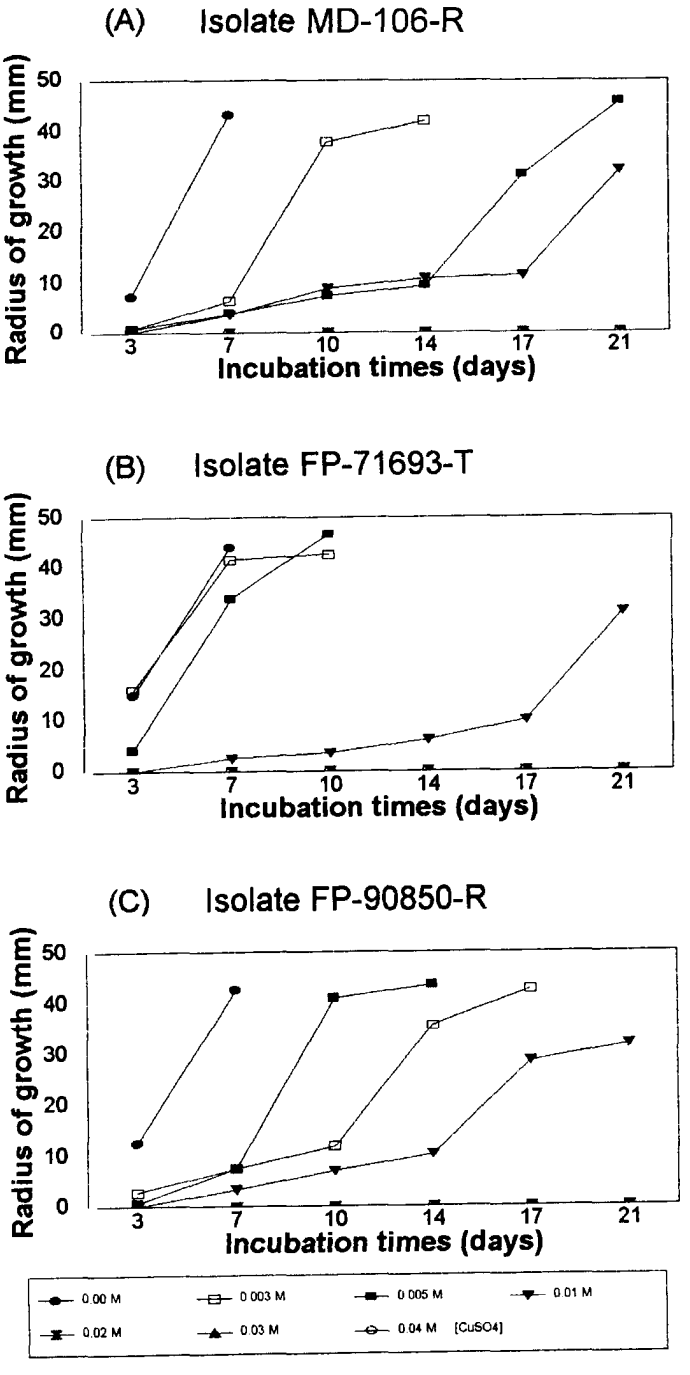

Figure 4. — Radial growth of average growing isolates of *Wolfiporia cocos* with a low tolerance to copper in agar plates.

TABLE 5. — Tuky's multiple comparison test of Normal scores for colony diameters of 20 isolates of W. cocos after 3 days incubation on meida with no copper added. (Control).

Isolate	Normal scores tests[a]
H+E-45	A
MD-104	A B
FP-71730-T	A B
FP-71284-A	A B
FP-97438-Sp	A B C
MD-275	A B C
FP-71693-T	A B C D
Log-WH-45-A	A B C D E
Priel-B-25	A B C D E
FP-90850-R	A B C D E
FP-71692-R	A B C D E
FP-71054	B C D E
L(61)1-8-A	B C D E
FP-90850-Sp	B C D E
FP-90886-T	B C D E
FPP-71691-R	C D E F
FP-104264-Sp	D E F
MD-106-R	D E F
MD-104-R	E F
MD-215	F

[a]Isolates with the same letter in the column are not significantly different. The isolate with the most growth is at the top of the list; the isolate with the least growth is at the bottom of the list.

little evidence that difference in soil nutrient levels will change threshold values in such tests (14). However, the fixed, time-domain aspect of the standard soil-block test may prevent accurate long-terrn assessment of slow-growing, wood-degrading fungi that can ultimately tolerate high copper loadings. The role of such fungi in the natural ecosystem needs to be further defined.

CONCLUSIONS

We conclude from this work with *Wolfiporia cocos* and from work previously completed by Collett (10) that even though copper tolerance in wood-decay fungi may be a species effect, the level of tolerance to copper differs significantly among isolates of those species. Furthermore, with *W. cocos,* the maximum concentration of copper that can be tolerated in a growth medium is not linked with rate of hyphal growth on copper-free media or on media containing levels of copper below the growth-limiting concentration. This can have significant practical impacts relative to the competitiveness of various isolates at sublethal levels of copper in a copper-enriched soil or in a wood product that is treated with a copper-based preservative.

LITERATURE CITED

1. Amburgey, T.L. 1978. Soil effect on soil-block wood decay tests. Material und Organismen 13/4:245-251.
2. Archer, K., D.D. Nicholas, and T.P Schultz. 1995. Screening of wood preservatives: Comparison of the soil-block, agar-block, and agar-plate tests. Forest Prod. J. 45(1): 86-89.
3. Arnebrant, K., E. Baath, and A. Nordgren. 1987. Copper tolerance of microfungi isolated from polluted and unpolluted forest soil. Mycologia 79(6):890-895.
4. American Society for Testing and Materials. 1996. D 1413-76 (1994). Standard test method for wood preservatives by laboratory soil-block cultures. ASTM Annual Book of Standards. Vol. 4.10 Wood. ASTM, West Conshohocken, Pa. pp. 207-213.
5. American Wood-Preservers' Association. 1996. E10-91. Standard method of testing wood preservatives by laboratory soil-block cultures. Book of Standards. AWPA, Granbury, Tex. pp. 305-315.

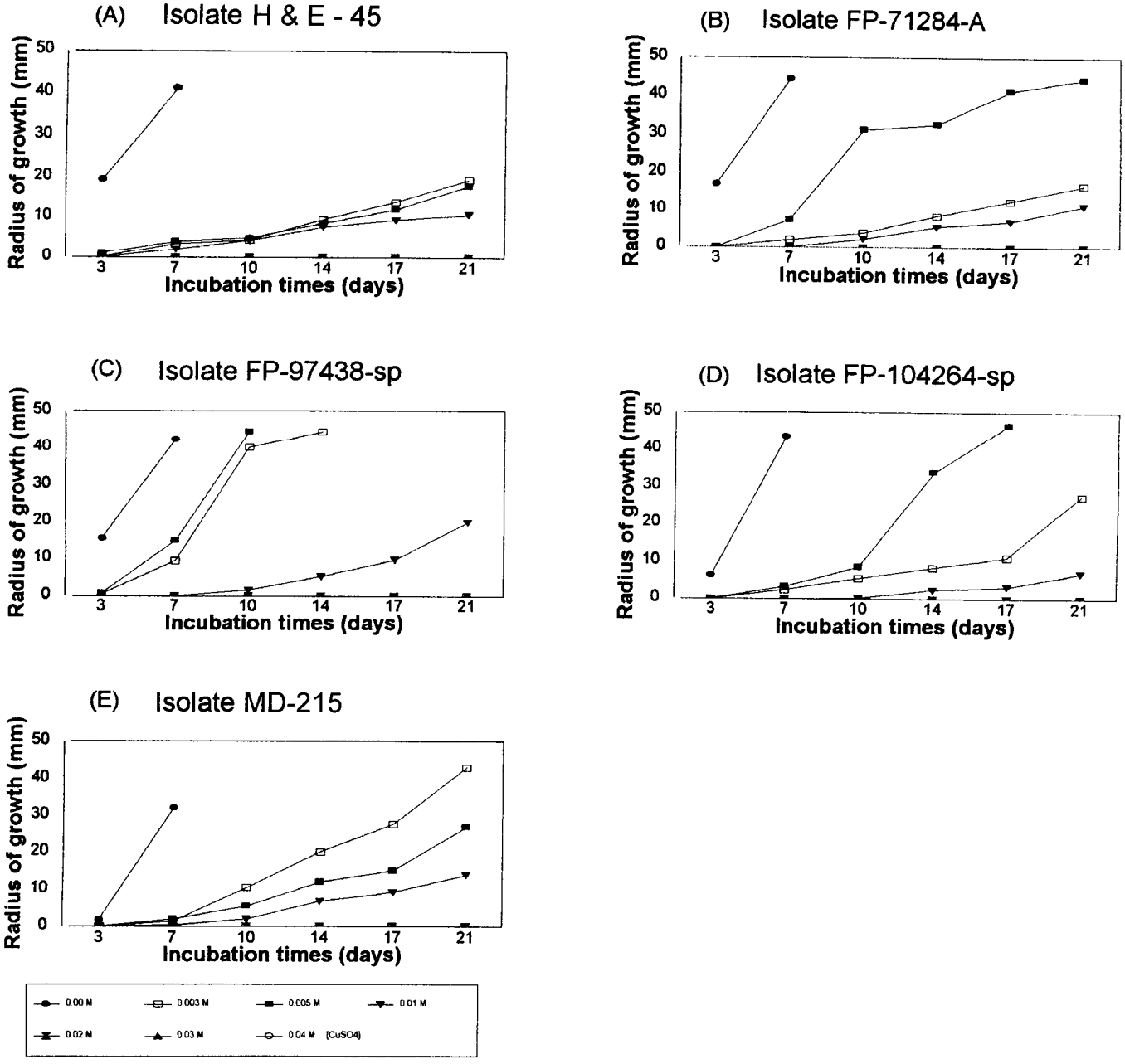

Figure 5. — Radial growth of slow growing isolates of *Wolfiporia cocos* with a low tolerance to copper in agar plates.

6. Brady, D.G. and J.R. Duncan. 1993. Copper tolerance in *Saccharomyces cerevisiae*. Letters in Appl. Microbiology 18:245-250.
7. Bravery, A.F. and J.K. Carey. 1977. Development of an improved technique for rapid screening of wood preservative fungicides. Part I. Selection of test substrate. Int. Biodeterior. Bull. 13(1):18-24.
8. Brown, M.T. and I.R. Hall. 1990. Metal tolerance in fungi. *In*: Heavy Metal Tolerance in Plants: Evolutionary Aspects. J. Shaw, ed. CRC Press, Boca Raton, Fla. pp. 95-104.
9. Cockroft, R. 1974. Evaluating the performance of wood preservatives against fungi. J. Inst. of Wood Sci. 6(6):2-8.
10. Collett, O. 1992. Comparative tolerance of the brown-rot fungus *Antrodia vaillantii* (DC.:Fr.) Ryv. isolates to copper. Holzforschung 46:293-298.
11. Cowling, E.B. 1957. The relative preservative tolerances of 18 wood-destroying fungi. Forest Prod. J. 7(10):355-359.
12. Da Costa, E.W.B. and R.M. Kerruish. 1964. Tolerance of *Poria* species to copper-based wood preservatives. Forest Prod. J. 14(3): 106-112.
13. Davidson R.W. and W.A. Campbell. 1954. *Poria cocos*, a widely distributed wood-rotting fungus. Mycologia 46:234-237.
14. Duncan, C.G. 1953. Soil-block and agar-block techniques for evaluation of oil-type wood preservatives: Creosote, copper naphthenate and pentachlorophenol. USDA Forest pathology special Release No. 37. Beltsville, Md. 39 pp.
15. _____. 1958. Studies of the methodology of soil-block testing. Rept. 2114. USDA Forest Serv., Forest Prod. Lab., Madison, Wis. 126 pp.
16. Duwadi, R. and P.D. Hilbrich. 1996. National Conf. on Wood Transportation Structures. Gen. Tech. Rept. FPL-GTR-94. USDA Forest Serv., Forest Prod. Lab., Madison, Wis. 494 pp.
17. Flemming, C.A. and J.T. Trevors. 1989. Copper toxicity and chemistry in the envi-

ronment: A review. Water, Air, and Soil Pollution 44:143-158.

18. Gadd, G.M. 1993. Tansley Review No. 47. Interactions of fungi with toxic metals. New Phytol. 124:25-60.
19. _____ and C. White. 1985. Copper uptake by *Pencillium ochro-chloron*: Influence of pH on toxicity and demonstration of energy-dependent copper influx using protoplasts. J. General Microbiology 131: 1875-1879.
20. Gilbertson, R.L. 1981. North American wood-rotting fungi that cause brown rots. Mycotaxon 12:372-416.
21. Hallas, L.E. and J.J. Cooney. 1981. Tin and tin-resistant microorganisms in Chesapeake Bay. Appl. Environ. Microbiology 47:446-471.
22. Nordgren, A., E. Baath, and B. Soderstrom. 1985. Soil microfungi in an area polluted by heavy metals. Can. J. Bot. 63:448-455.
23. Richards, C.A. and R.M. Addoms. 1947. Laboratory methods for evaluating wood preservatives: Preliminary comparison of agar and soil culture techniques using impregnated wood blocks. *In*: Proc. American Wood-Preservers' Assoc. 43:41-58.
24. Schmidt, O. and U. Moreth. 1996. Biological characterization of *Poria* indoor brown-rot fungi. Holzforschung 50:105-110.
25. Starkey, R.L. 1973. Effect of pH on toxicity of copper to *Scytalidium* spp., a copper tolerant fungus and some other fungi. J. General Micrbiology 18:217-225.
26. Sutter, H.-P., E.B.G. Jones, and O. Walchli. 1983. The mechanism of copper tolerance in *Poria placenta* (Fr.) Cke. and *Poria vaillantii* (Pers.)Fr. Material und Organismen 18:241-262.
27. Thornton, J.D. and M.A. Tighe. 1987. Comparative laboratory testing of strains of the dry rot fungus *Serpula lacrymans* (Schum. ex Fr.) S. F. Gray III. The action of copper naphthenate in wood. Holzforschung 41: 195-197.
28. Tsunoda, K., K. Nagashima, and M. Takahashi. 1997. High tolerance of wood-destroying brown-rot fungi to copper-based fungicides. Material und Organismen: 31:31-45.
29. Wazny, J. and J.D. Thornton. 1986. Comparative laboratory testing of strains of the dry rot fungus *Serpula lacrymans* (Schum. ex Fr.) S. F. Gray. Holzforschung 40:383-388.
30. Williams, G.R. and R. Fox. 1994. The control of copper tolerant basidiomycete fungi in preservative treated wood in ground contact. *In*: Proc. American Wood Preservers' Assoc. 90:156-176.
31. Yamamoto, H., K. Tatsuyama, and T. Uchwiwa. 1985. Fungal flora of soil polluted with copper. Soil Biol. Biochem. 17:785- 790.
32. Young. G.A. 1961. Copper tolerance of some wood-rotting fungi. Rept. 2223. USDA Forest Serv., Forest Prod. Lab., Madison, Wis. 10 pp.
33. Zabel, R.A. 1954. Variations in preservative tolerance of wood-destroying fungi. J. Forest Prod. Res. Soc. IV(4):166-169.