Accelerated Colorimetric Micro-assay for Screening Mold Inhibitors

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ABSTRACT

Rapid quantitative laboratory test methods are needed to screen potential antifungal agents. Existing laboratory test methods are relatively time consuming, may require specialized test equipment and rely on subjective visual ratings. A quantitative, colorimetric micro-assay has been developed that uses XTT tetrazolium salt to metabolically assess mold spore germination. Using isothiazolinone as a model mold inhibitor, the minimum inhibitory concentration (MIC) for the XTT assay was compared with the 4 week ASTM plate method (D4445) to test fungicides for controlling mold and the 8 week AWPA environmental chamber method (E24) for evaluating wood product surfaces to mold growth. Quantitative values for the 2-day XTT assay estimated the same MIC for isothiazolinone (50 ppm) for all three test fungi. For the ASTM and AWPA methods, there was a sharp cutoff at a MIC of 50 ppm and 100 ppm isothiazolinone, respectively that was sustained for 12 weeks. While XTT and ASTM methods utilized the same test fungi, the AWPA method exposed treated specimens to a broad range of mold spores naturally present in unsterilized soil so a higher MIC might be expected. Because the XTT assay is conducted in a microplate, it saves resources and reagents in addition to time. The XTT microplate assay can be used to demonstrate the relative resistance of different mold species to individual antifungal agents, to compare the relative inhibition of a new antifungal agent to existing antifungal agents, or to screen new wood preservative formulations for their susceptibility to mold growth.

Key words: XTT tetrazolium salt, mold inhibitors, micro-assay, mold fungi, spore germination

INTRODUCTION

Wood preservative formulations have not traditionally been developed with mold inhibition in mind. Mold fungi often grow uninhibited and occasionally grow preferentially on wood treated with copper-based preservatives, so research on mold inhibition has become increasingly important to the wood preservation industry. Current laboratory test methods to screen efficacy of mold inhibitors require four to eight weeks for test results depending on the test method selected. The two standards method most frequently used rely on subjective visual ratings of wood specimens (ASTM 2010; AWPA 2010). Accelerated quantitative methods to measure the efficacy of mold inhibitors are needed.

Colorimetric assays using tetrazolium were originally developed to verify vegetative viability of clinically important yeasts, such as *Candida spp.* and *Saccharomyces cerevisiae*, and human pathogenic molds, such as *Aspergillus fumigatus* (Kuhn, et al. 2003; Levitz and Diamond 1985). These assays utilized yellow tetrazolium salts, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) or 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[9phenyl-amino)carbonyl]-2H-tetrazolium hydroxide (XTT). Both tetrazolium salts are cleaved by mitochondrial dehydrogenases of metabolically active yeasts to form a formazan derivative. In the presence of an electron-coupling agent, the formazan derivative absorbs light in a specific nanometer range that can be read in a microplate reader (Altman 1976; Hawser et al. 1998; Meletiadis et al. 2001). Colorimetric tetrazolium assays are reported to produce clear-cut endpoints for minimum inhibitory concentration (MIC) determination of antifungal agents (Hawser et al. 1998; Meletiadis et al. 2001).

Comparative studies conducted with the XTT assay and those from a standardized clinical test demonstrated agreement within two (2-fold) dilutions of the MIC obtained for amphotericin B, itraconazole, ketoconazole, fluconazole and flucytosine (Hawser et al. 1998; NCCLS 1997). Clausen and Yang (2011) reported the development of an XTT microassay for rapid colorimetric screening of mold inhibitors. They showed that the XTT assay can be used to demonstrate the relative resistance of different fungal species to antifungal agents, to compare the relative inhibition of a new antifungal agent to existing antifungal agents, or to screen new wood preservative formulations for their susceptibility to mold growth. The objective of this study was to compare the MIC of a known mold inhibitor using the XTT assay with the MIC using standardized ASTM and AWPA laboratory methods.

MATERIALS AND METHODS

Assay Reagents

Growth medium for spore germination consisted of RPMI 1640 medium without sodium bicarbonate supplemented with L-glutamine and buffered with 165 mM morpholinepropanesulfonic acid, pH 7.0 (Sigma-Aldrich, St. Louis, MO) (Pierce et al. 2008). The tetrazolium salt selected for this method was 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2*H*-tetrazolium hydroxide (hereafter called XTT) (Sigma-Aldrich). XTT (0.5 g/L) was prepared in phosphate buffered saline, (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) (Sigma-Aldrich, St. Louis, MO), filter-sterilized and stored in 10 mL aliquots protected from light at -70°C. A 10 mM stock of menadione (2-methyl-1,4 naphthoquinone) (Sigma-Aldrich), an electron-coupling agent, was prepared in 100% acetone and stored in 50 µl aliquots at -70°C. Isothiazolinone (SUPATIMBER® H.E. 14, supplied by Viance, Charlotte, NC) served as the model mold inhibitor in this study. Two-fold dilutions of isothiazolinone were prepared resulting in concentrations ranging 0 to 800 ppm. Isothiazolinone was diluted in RPMI for the XTT assay and in water for the standardized test methods.

Spore Inoculum

Isolates of *Aspergillus niger* (2.242)*, Penicillium chrysogenum* (PH02), and *Trichoderma viride* (ATCC 20476) were grown and maintained on 2% malt extract agar (Difco, Detroit, MI) in Petri dishes. Two spore suspensions were prepared as described below.

For the XTT assay, spores from individual test fungi were collected by flooding the surface of a two-week-old culture with 10 mL of sterile PBS plus 0.025% Tween-20 (Sigma-Aldrich). The resulting spore suspension was removed via aspiration to a sterile container, washed twice in sterile PBS and collected by centrifugation at 3,000 x*g* for 20 min each at 4°C. Spores suspended in RPMI 1640 were counted with a hemocytometer and further diluted in RPMI to yield 1 x 10^6spores/mL.

For standardized tests, spores from individual test fungi were collected in 10 mL of sterile deionized water (DI) according to ASTM standard D4445-10 (ASTM 2010). Spores were collected, counted and equal numbers of spores for each test organism were transferred to a spray bottle. The spore mixture was diluted with deionized (DI) water to yield approximately 3 x 10^7 spores mL. The spray bottle was adjusted to deliver 1 mL inoculum per spray and was shaken frequently during inoculation to ensure a homogeneous inoculum.

Specimen Preparation

Southern pine sapwood specimens that were either 7 x 20 mm cross section x 7 cm long or 75 x 100 mm x 12.5 mm thick were cut from kiln-dried lumber and conditioned at 27ºC and 70% relative humidity (RH). Ten random replicates of each size specimen and each concentration of test solution were vacuum-treated for 40 min at -172 kPa submerged in individual test solutions. Treated specimens were air-dried for 1 week prior to conditioning at 27ºC and 70% RH for 21 days.

XTT Assay Protocol

For each mold isolate, 1 x 10^6 spores were pre-incubated in RPMI at 27°C for 24 hours. In a sterile flat-bottom microplate, 80 µl of RPMI and 80 µl of saline were added to each well. Eighty microliters of a range of 2-fold dilutions of isothiazolinone were prepared in sterile RPMI and added to each well (n=10) with appropriate positive and negative control wells. The plate was sealed and incubated for 24 hours at 37°C. Following exposure to isothiazolinone, 80µl XTT-menadione reagent was added to all wells of the plate which was then wrapped in foil and incubated for 3 hr at 37°C. Absorbance was read with a PowerWave XS 2 microplate reader (BioTek Instruments, Inc., Winooski, VT) at 490nm.

ASTM D4445-10 Protocol

Treated specimens (n=10) were placed on top of a polyethylene mesh spacer in Petri dishes (150 x 25 mm) (B-D Falcon, Los Angeles, CA) containing 4 layers of blotting paper that was saturated with 30 mL DI water. Specimens were sprayed with 1 mL of mixed mold spore inoculums, sealed in polyethylene bags to retain 100% RH, and incubated at 27ºC and 70% RH. Individual specimens were visually evaluated for mold growth after 4, 8, and 12 weeks of incubation on a scale of 0 to 5 with 0 indicating no mold growth and 5 indicating heavy mold growth on all sides of the specimen.

AWPA E24 Protocol

Environmental chambers fabricated according to AWPA E24 specifications were used to suspend treated test specimens (n=10) over non-sterile soil pre-inoculated with spores from the three test fungi. The test chambers were placed in a conditioning room at 27ºC. Treated specimens were vertically suspended across the width of the chamber over inoculated soil and sprayed with spores of the test fungi. Individual specimens were rated for mold growth on a scale of 0-5 after 4, 8, and 12 weeks incubation.

RESULTS AND DISCUSSION

XTT Assay

Depending on the mode of action of a mold inhibitor, the estimated MIC from the XTT assay has been reported to be more sensitive than the statistically calculated MIC of a mold inhibitor based on standardized test methods (Clausen and Yang 2011). In this study, complete inhibition of mold growth using two standardized methods was compared with complete inhibition of metabolic activity in the XTT assay using isothiazolinone as the model mold inhibitor. Figure 1 shows the absorbance readings for three test fungi exposed to two-fold dilutions of isothiazolinone. Absorbance at 490nm is indicative of metabolic activity (i.e. mold spore germination). For all three test fungi, complete inhibition of metabolic activity occurred at dilutions containing ≥50 ppm isothiazolinone. Lower dilutions of isothiazolinone demonstrated decreasing levels of inhibition.

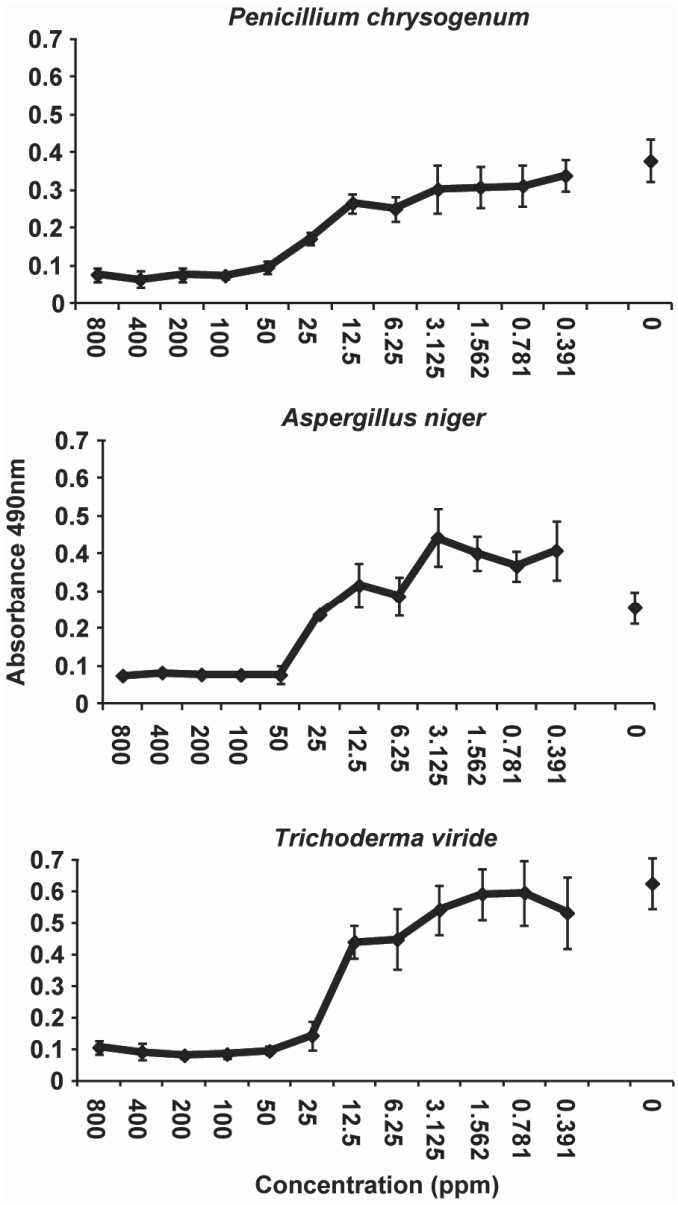

Figure 1: XTT assay absorbance readings for mold spores exposed to isothiazolinone.

Standard deviation in quantitative results for the XTT assay increased at concentrations of isothiazolinone that did not completely inhibit spore germination. This result would be anticipated due to the sensitivity of the assay to mitochondrial dehydrogenase activity and inherent metabolic variability in mold spores.

Standardized Test Results

Comparative ratings for two standardized laboratory mold tests using vaccum-treated southern pine specimens are summarized in Table 1. Even though ASTM D4445 is a 4 week test and AWPA E24 is an 8 week test, incubation of test specimens was extended to 12 weeks to evaluate sustained inhibition. Average ratings and standard deviation for the four-week duration of this test are highlighted in gray. Ratings based on visual evaluations of mold growth can have a high standard deviation highlighting the importance of testing 10 replicates. Specimens treated with ≥50 ppm isothiazolinone substaintially inhibited mold growth in the D4445 test over the 12-week duration of the test.

Table 1: Ratings for two standardized mold tests on southern pine vacuum-treated with isothiazolinone

Treatment concentration	**Average mold ratings**		
(ppm)	4 wk	8 wk	12 wk
ASTM D4445			
800	0	0	0
400	0	0	0
200	0.1 (.03)	0.1 (0.3)	0.4 (0.4)
100	0.4 (0.5)	0.2 (0.4)	0.3 (0.5)
50	0.1 (0.5)	0.2 (0.4)	0.4 (0.7)
25	1.0 (1.0)	0.4 (0.7)	0.6 (1.0)
0	3.2 (1.6)	3.3 (1.4)	3.7 (1.1)
AWPA E24			
800	0	0	1.4 (1.0)
400	0	0	1.3 (1.1)
200	0	0	1.6 (0.7)
100	0.1 (0.3)	0.3 (0.7)	1.1 (1.1)
50	1.4 (1.0)	4.6 (0.7)	5
25	2.0 (1.4)	4.6 (0.7)	5
0	2.3 (1.8)	4.9	5

Shaded ratings demonstrate nearly complete mold inhibition for the normal duration of the test. Standard deviation is in parentheses.

In the AWPA E24 test, specimens treated with ≥100 ppm isothiazolinone substantially inhibited mold growth for the 8-week duration of the test (highlighted in gray), but not for 12 weeks. Standard deviation for each treatment group was lower than that of the D4445 test method. As with the ASTM method, the visual rating system is subjective and a high number of replicate specimens per treatment group is important for precision and reproducibility. In many commercial practices, the use of mold inhibitor(s) is in combination with various wood preservative systems. The required concentration of mold inhibitor(s) in a wood preservative system can vary considerably due to the chemical properties of the components in the particular preservative formulation (Jin et al. 2012).

Despite micro- to macro- differences in the three test methods compared in this study, a critical factor for a screening method is the estimated minimal inhibitory concentration (MIC) for the chemical being evaluated. The estimated MIC for isothiazolinone for the XTT assay and D4445 test are in agreement (≥50 ppm), and are within one (two-fold) dilution from the estimated MIC for isothiazolinone in the E24 method (≥100 ppm). A higher MIC in the E24 test might be expected due to specimen exposure to a greater variety of air and soil microbes in a non-sterile test apparatus compared to the monocultures utilized in the other two methods.

For screening mold inhibitors, the XTT assay offers several advantages over the other standardized methods (Table 2). Quantitative results are obtained in just 2 days without the need to treat wood specimens. The XTT assay is susceptible to incompatibilities of assay reagents or the polystyrene test plate with certain antifungal agents. When ethanol is used as a diluent at greater than approximately 12% of the sample volume, the ethanol itself inhibits spore germination and appropriate

controls must be added to correct for that influence. Additionally, mold inhibitors that are comprised of more than one antifungal component have demonstrated a multi-phasic response in the XTT assay, so it is advised to screen individual components.

Table 2: Comparative criteria for the XTT assay, ASTM D4445 and AWPA E24 test methods

Criteria	**Test Method**		
	XTT	D4445	E24
Test type	Quantitative	Qualitative	Qualitative
Culture type	mono-culture	mixed or mono-culture	poly-culture
Equipment	Microplate Reader	Petri Dishes	Environmental Chamber
Substrate	RPMI	Wood	Wood
Substrate variability	No	Yes	Yes
Time	2 days	4 weeks	8 weeks
Interferences	Opacity, Ethanol, Bi-phasic	None	Volatiles
Biological variability	Yes	Yes	Yes

The redeeming feature of the D4445 test is the low cost for disposable Petri dishes and a roll of gutter guard. It is also a relatively simple test to conduct and can test either mono-cultures or defined mixed cultures. Fabricating environmental chambers for the E24 test is initially costly and chambers are cumbersome to clean between tests. Specimens in the E24 environmental chamber are susceptible to possible influence of volatiles from adjacent samples and multiple chambers are needed to randomize specimens and/or isolate volatile treatments.

CONCLUSIONS

The rapid, quantitative XTT micro-assay designed to screen mold inhibitors was compared to two standardized methods using isothiazolinone as a model mold inhibitor. The estimated MIC for the micro-assay was in agreement with the ASTM D4445 method and within one (two-fold) dilution of the AWPA E24 test. The XTT assay can be used to 1) demonstrate the relative resistance of different fungal species to a given mold inhibitor, 2) compare the relative inhibition of a new biocide to existing mold inhibitors, and 3) screen new wood preservative formulations for their susceptibility to mold growth.

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