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Section 2 Test Methodology and Assessment

A rapid colorimetric assay for mold spore germination using XTT tetrazolium salt

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A Rapid Colorimetric Assay for Mold Spore Germination Using XTT Tetrazolium Salt

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ABSTRACT

Current laboratory test methods to measure efficacy of new mold inhibitors are time consuming, some require specialized test equipment and ratings are subjective. Rapid, simple quantitative assays to measure the efficacy of mold inhibitors are needed. A quantitative, colorimetric microassay was developed using XTT tetrazolium salt to metabolically assess mold spore germination. Quantitative values for the inhibition of mold spore germination using the XTT assay were 30-fold lower for thiabendazole and Durazol than the calculated MIC_{90} based on subjective ratings using current standard methods. 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.

Keywords: XTT tetrazolium salt, antifungal, colorimetric assay, mold fungi, spore germination

1. INTRODUCTION

Research on the development of mold inhibitors for interior protection of cellulose-based building materials has increased proportionally with the rise in consumer complaints about air quality issues from mold growth. Current laboratory test methods to measure efficacy of new biocide formulations require four to eight weeks incubation depending on the test method selected and rely on subjective visual ratings of wood specimens (ASTM 2010; AWPA 2010). Rapid, simple quantitative methods to measure the efficacy of mold inhibitors are needed.

Colorimetric tetrazolium assays have been developed to test vegetative viability of clinically important yeasts such as *Candida* sp. and *Saccharomyces cerevisiae* (Kuhn, et al. 2003; Levitz and Diamond 1985). Most of the previously reported research has been on yeasts and *Aspergillus fumigatus* (Levitz and Diamond 1985), a human pathogen. These assays utilize 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 which, in the presence of an electron-coupling agent, 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.

A number of variations have been reported for XTT antifungal susceptibility of both yeasts and biofilms. Hawser et al. (1998) conducted susceptibility testing against numerous clinical isolates of *Candida* spp. using XTT and compared results with those from a standardized clinical test (NCCLS 1997). Their results showed that one hundred percent of the isolates assayed by the two methods demonstrated agreement within two (2-fold) dilutions of the MIC obtained for amphotericin B, itraconazole, ketoconazole, fluconazole and flucytosine. Pierce et al. (2008) developed a method based on fungal biofilm development. A protective extracellular matrix renders biofilms increasingly resistant to antifungal agents, such as azoles and polyenes. In their model, biofilms of medically-important molds and yeasts grown in a 96-well microtiter plate were subjected to antifungal drugs. After a pre-determined exposure time, the tetrazolium salt was added to measure the metabolic activities of cells within the biofilm. By this method, biofilm formation was shown to be reproducible and there were no statistical differences in the susceptibility tests when values were compared between pairs of rows on the plate with each row representing eight replicate results.

The objective of this study was to develop a rapid quantitative colorimetric assay for screening the efficacy of antifungal agents in preventing mold spore germination.

2. MATERIALS AND METHODS

2.1 Assay reagents

Growth medium 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.

2.2 Antifungal agents

Table 1. Biocides evaluated for XTT assay proof of concept

Biocide	**Source**	**Initial concentration**
Durazol[a]	USDA Forest Products Lab, Madison, WI, USA	20 mg/ml
Propiconazole	Janssen Pharmaceutical, Titusville, NJ, USA	1024 µg/ml
Thiabendazole	Sigma-Aldrich, St. Louis, MO, USA	1024 µg/ml
3-Iodo-2-propynyl butylcarbamate (IPBC)	Sigma-Aldrich, St. Louis, MO, USA	1024 µg/ml
Disodium octaborate tetrahydrate (DOT)	US Borax, Inc. Valencia, CA, USA	40 mg/ml
Boric acid	National Boraxx, Cleveland, OH, USA	40 mg/ml
Didecyldimethyl ammonium chloride (DDAC)	Lonza, Inc. Fair Lawn, NJ, USA	20 mg/ml

[a]*US patent # 7,858,125* (Clausen et al. 2010)

2.3 Spore inoculum preparation

Isolates of *Aspergillus niger, Penicillium chrysogenum* PH02, *Trichoderma viride* ATCC 20476 were grown and maintained on 2% malt extract agar (Difco, Detroit, MI) in Petri dishes. A spore suspension was prepared by flooding the surface of a two-week-old culture of the individual test fungi 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. They were then diluted in RPMI 1640 as needed for optimizing the inocula described in section 2.4.

2.4 Assay parameters

2.4.1 Optimizing inoculum

To standardize inocula, wells in a 96-well microtiter plate were aseptically inoculated with 100 µl of 10^5, 10^6, or 10^7 spores/mL prepared in RPMI 1640. The plate was sealed and pre-incubated for 24 hr at 27°C. The inoculum level that repeatedly produced an absorbance reading at least 3 times greater than the RPMI background absorbance was selected as the standard inoculum for further testing. Standardized inoculum varied for each fungal species. Spore controls consisting of 80 µl spores and 80 µl RPMI were included for each fungus/antifungal combination during every assay.

2.4.2 XTT assay protocol

For each mold isolate, the pre-determined concentration of fungal spore inoculum was pre-incubated in RPMI at 27°C for 24 hours. In a sterile flat-bottom microplate, 80 µl of RPMI and 80 µl saline were added to each well in column A (n=8). One microplate was required to screen 10 concentrations of each antifungal agent. Pre-incubated spores were aseptically added to all wells in columns B through H. Serial 2-fold dilutions of individual antifungal agents were prepared in sterile RPMI beginning with the concentration listed in Table 1 and 80 µl of each dilution was added to wells in columns B through G (n=8). Eighty microliters of sterile RPMI was added to all wells in column H. The plate was sealed and incubated for 24 hours at 37°C. Following exposure to the antifungal agent, the assay with XTT-menadione reagent was prepared by combining 10 mL XTT stock solution with 1 µl menadione stock solution just before use. Eighty microliters of XTT-menadione reagent was added to all wells of the plate which was 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.

3. RESULTS

3.1 Assay parameters

Assay development typically requires the optimization of numerous parameters including reagent proportions, incubation time and temperature for individual assay steps, inocula, selection and pH of buffers, reagents and media that are conducive to the biological system being tested. Synthetic RPMI medium was selected as the growth medium for the XTT assay because of its suitability for antifungal susceptibility testing of yeasts in clinical standards (NCCLS 1997). Spore inoculum was optimized at 1×10^6 spores/mL by pre-testing 10-fold dilutions of spore preparations for individual test fungi. Assay volume was partially dictated by the microplate well capacity along with other considerations such as dilution of biocide and optimal concentration of tetrazolium salt substrate. Reagent proportions were optimized using the method of Pierce et al. (2008) as a general guide, but the final proportions were altered based on the inoculum volume that provided consistent and reproducible absorbance readings.

3.2 Assay profiles

Figures 1-3 show the absorbance profiles for individual mold fungi exposed separately to antifungal agents in the XTT assay. It is apparent from the profiles that individual fungi used in this study are more or less susceptible to the same antifungal agent. For example, in Figure 1, *Aspergillus niger* is inhibited by IPBC and both azoles at concentrations ranging from 0.002 and 0.016 mg/mL but is relatively resistant to boric acid and DOT (10 mg/mL). Figure 3 shows that *Trichoderma viride* is less susceptible to IPBC than either *Penicillium chrysogenum* or *Aspergillus niger*. *T. viride* has a reputation for biocide resistance which is not clear-cut from the absorbance profiles. On the other hand, the known susceptibility of *P. chrysogenum* to biocides is evident in the profiles (Figure 2). Indeed, profiles of the same fungus exposed to different antifungal agents are strikingly different. Most notably in Figure 2 *P. chrysogenum* was more susceptible (i.e. has a higher MIC) to propiconazole than to IPBC or thiabendazole. Metabolic activity for *P. chrysogenum* was completely inhibited when exposed to either boric acid or DOT at a concentration of 1.25 mg/mL or greater, but the inhibition rapidly diminished upon further dilution.

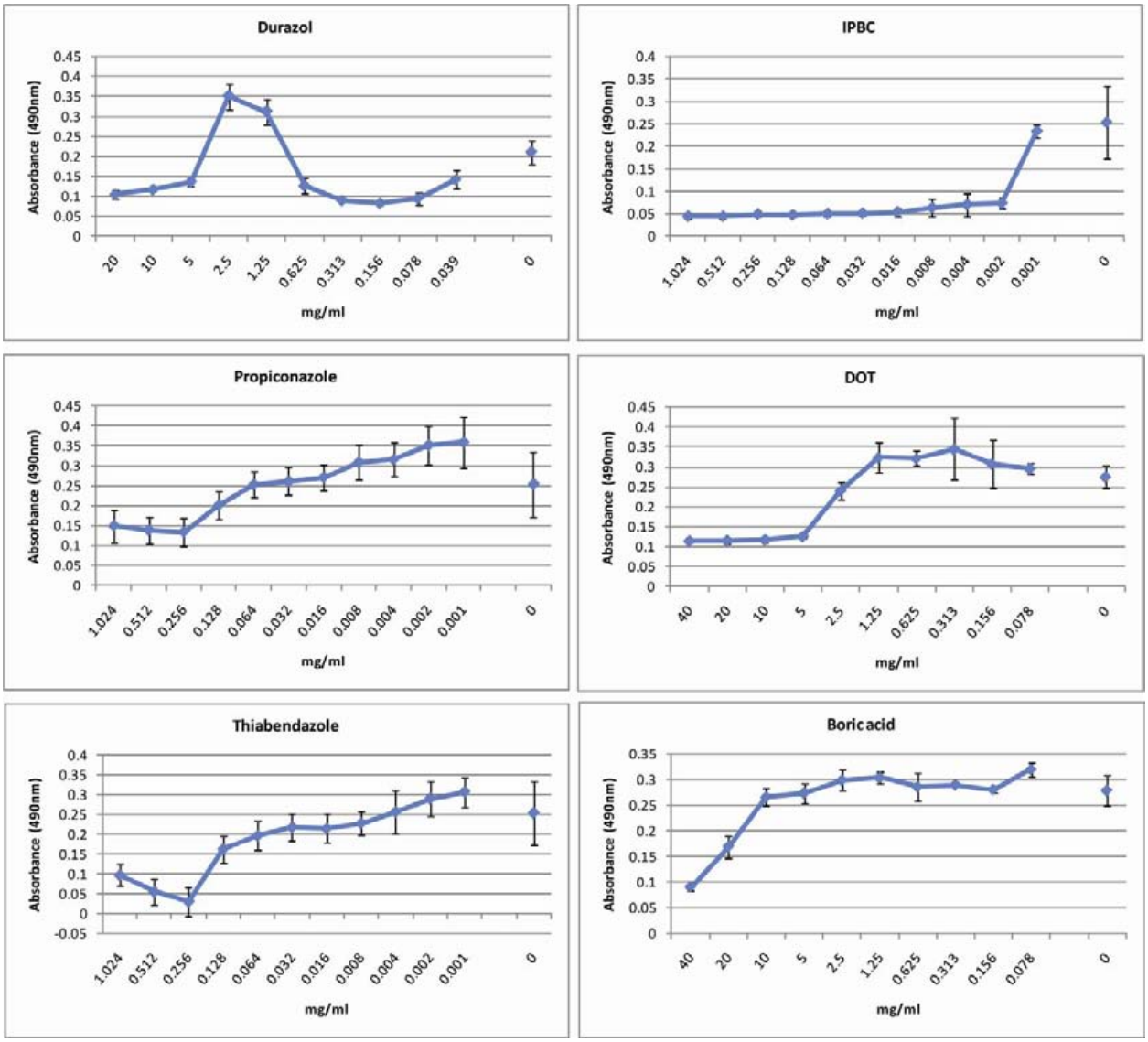

Figure 1. Metabolic activity for *Aspergillus niger* spores following exposure to Durazol, propiconazole, thiabendazole, 3-Iodo-2-propynyl butylcarbamate (IPBC), disodium octaborate tetrahydrate (DOT), or boric acid quantitatively measured using the XTT colorimetric assay.

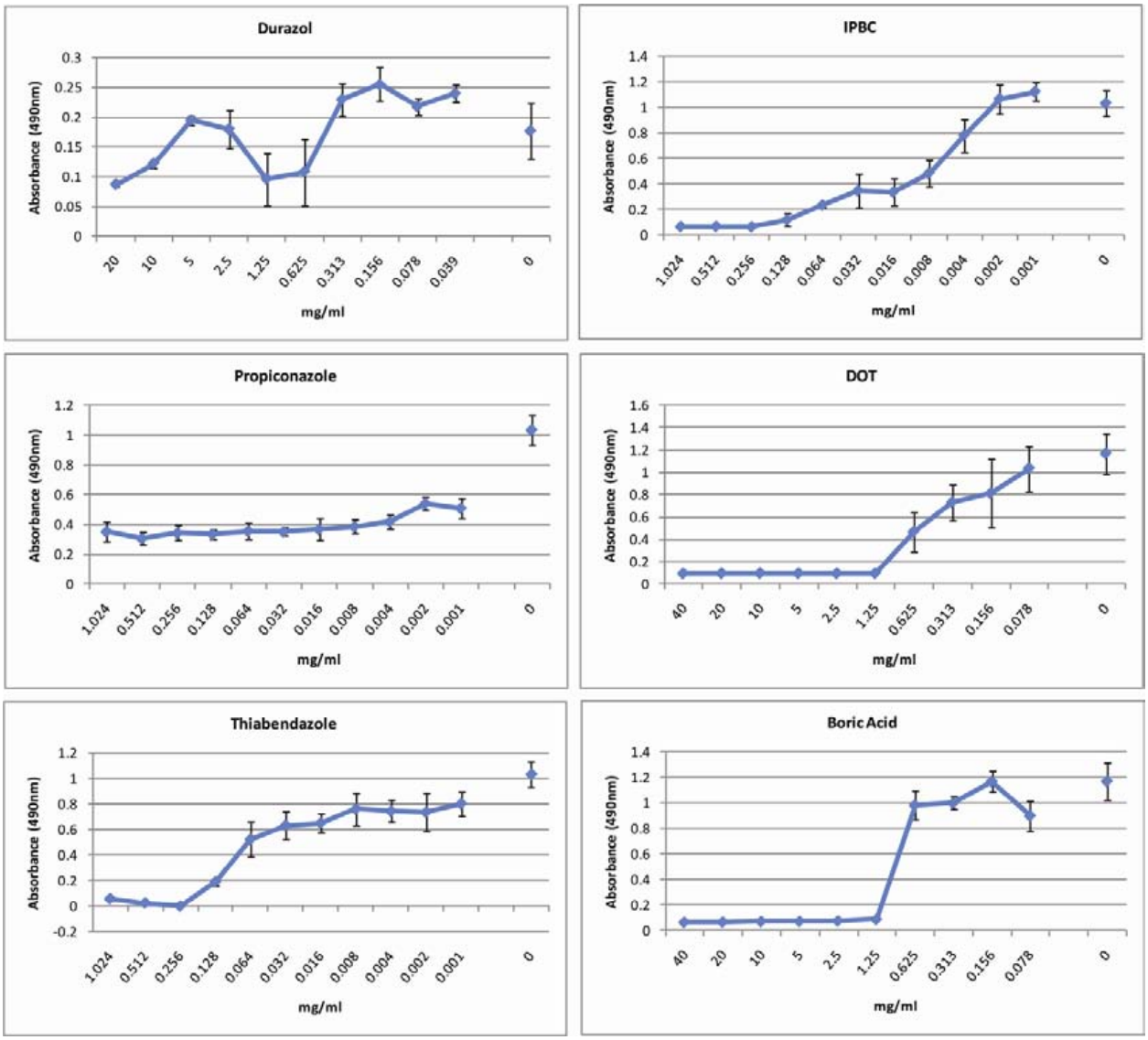

Figure 2. Metabolic activity for *Penicillium chrysogenum* spores following exposure to Durazol, propiconazole, thiabendazole, 3-Iodo-2-propynyl butylcarbamate (IPBC), disodium octaborate tetrahydrate (DOT), or boric acid quantitatively measured using the XTT colorimetric assay.

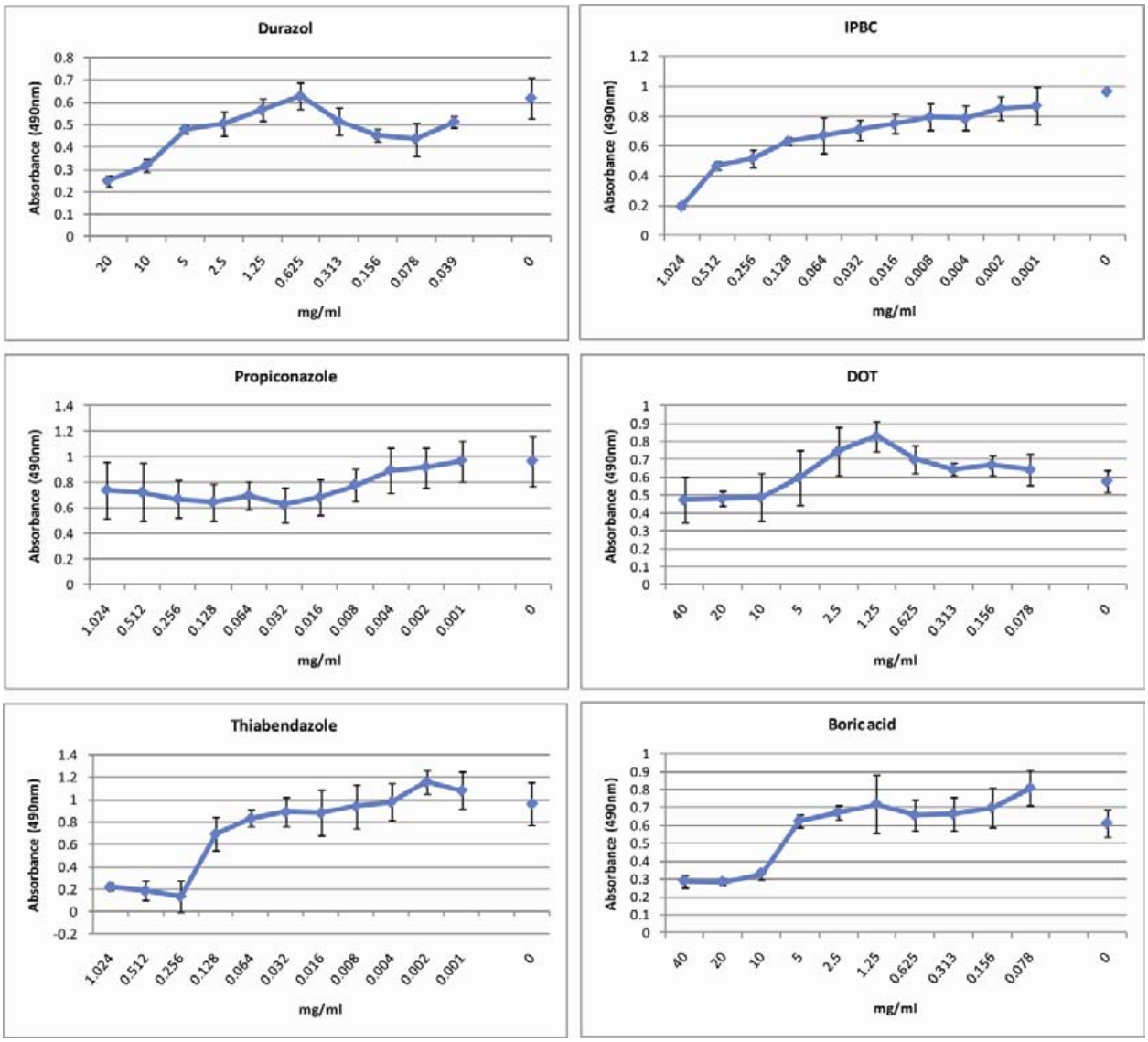

Figure 3. Metabolic activity for *Trichoderma viride* spores following exposure to Durazol, propiconazole, thiabendazole, 3-Iodo-2-propynyl butylcarbamate (IPBC), disodium octaborate tetrahydrate (DOT), or boric acid quantitatively measured using the XTT colorimetric assay.

All absorbance profiles except those for Durazol indicated a gradual increase in metabolic activity as the antifungal agent became more dilute until they eventually equaled the absorbance reading for the control. The bi-phasic nature of Durazol's absorbance profile was consistent, but not surprising since Durazol was the only multi-component anti-fungal included in this study. Thiabendazole, comprising 0.1% of the Durazol formulation, is the key mold inhibitory component in Durazol although dicocomethyl amine (DCMA) also contributes to mold inhibition. In an attempt to analyze the Durazol absorbance profile, thiabendazole and DCMA were assayed separately. However, the opacity of DCMA interfered with the XTT assay and therefore, the affect of DCMA on the Durazol profile could not be assessed. The concentration of Durazol that inhibited the metabolic activity of *T. viride* and *P. chrysogenum* spores by this method was estimated at 0.625 mg/mL, but the bi-phasic profile made it difficult to estimate an end point for *A. niger* spores. Two percent Durazol (20 mg/mL) completely inhibits these test fungi in the ATSM D4445 method (Clausen and Yang 2005).

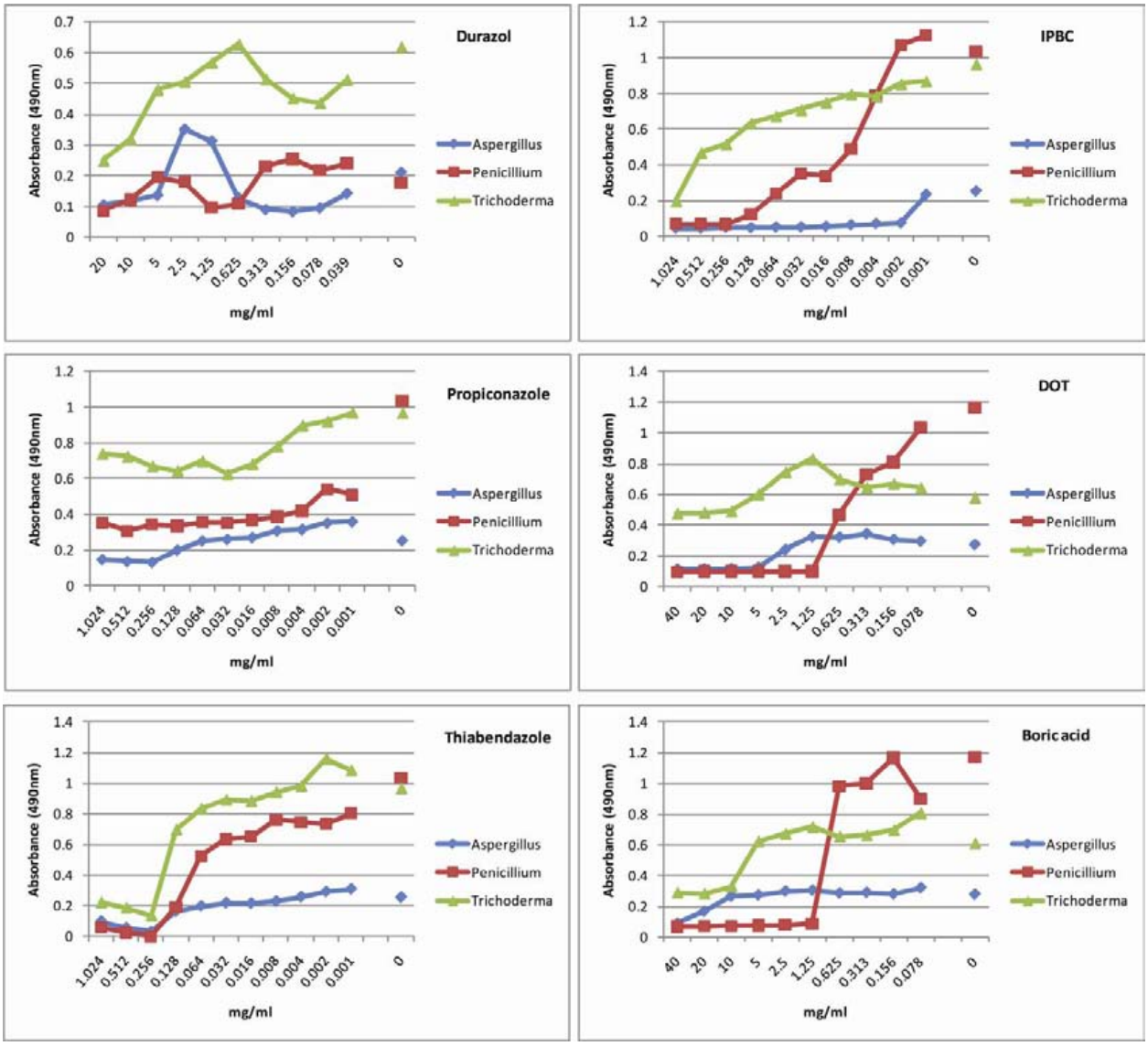

Figure 4. Combined absorbance profiles (i.e. metabolic activity following exposure to antifungal test chemicals) show comparative variation in susceptibility of the three test fungi to Durazol, propiconazole, thiabendazole, 3-Iodo-2-propynyl butylcarbamate (IPBC), disodium octaborate tetrahydrate (DOT), or boric acid.

Figure 4 is a compilation of absorbance profiles for spores from the three test fungi exposed to each antifungal agent. Note the different scales on the X-axis. The starting concentration for each antifungal agent was selected from statistically calculated MIC's based on ratings from ASTM D4445-10. Using a standardized inoculum (1 x 10^6 spores/mL) the rate of metabolic activity was different for each test fungus and becomes apparent in the compilation figure. *Trichoderma viride* and *Penicillium chrysogenum* showing consistently higher control absorbance readings in the XTT assay under the conditions of this study.

Thiabendazole provided metabolic inhibition of *Penicillium* spores at 0.001 mg/mL and both *Aspergillus* and *Trichoderma* spores at 0.008 mg/mL. The MIC_{90} for thiabendazole was previously calculated to be 0.016% with data from the ASTM D4445 test method (Clausen and Yang 2005). IPBC inhibited all test fungi at very low concentrations, e.g.0.002 to 0.004 mg/mL.

Boric acid and DOT have well-defined end points for complete inhibition of *Penicillium chrysogenum* (1.25 mg/mL), but provided only slight inhibition of the other two test fungi at higher concentrations. The calculated MIC_{90} for boric acid based on results from the ASTM D4445 test was 3.7% (Clausen and Yang 2005).

3.3 Assay interference

The XTT assay was not suitable for evaluating certain antifungal agents. Opacity in chlorothalonil masked the XTT reaction. DDAC became cloudy in the presence of the XTT-menadione reagent creating a high background absorbance. The interfering background absorbance could not be subtracted from the reactive absorbance, so a profile could not be obtained for DDAC by this method. Ethanol was required as the diluent or part of the formulation for some antifungal agents such as thiabendazole, Durazol and IPBC. It was apparent from the absorbance readings that ethanol inhibited spore germination until it was diluted to approximately 12% of the preservative concentration. Ethanol inhibition of spore germination is well known and has previously interfered during method development by these authors. In instances where ethanol was needed for biocide solubility, additional controls comprised of ethanol and spores were used to correct for the effect of ethanol on the reactive absorbance in wells containing antifungal agents with >12% ethanol. Multi-component biocide formulations (e.g. Durazol) may also be difficult to evaluate with this assay if they contain multiple mold inhibitors or components that interfere with the assay reagents.

4. CONCULUSIONS

A rapid quantitative assay for screening antifungal agents for inhibition of spore germination has been developed. The XTT assay can be used to demonstrate the relative resistance of different fungal species to a given antifungal agent and to compare the relative inhibition of a new biocide to existing antifungal agents. Quantitative values for mold inhibition using the XTT assay were 30-fold lower for thiabendazole and Durazol than the calculated MIC_{90} based on subjective ratings using current methods. The XTT assay could also be useful in screening new wood preservative formulations for their susceptibility to mold growth. If mold growth was enhanced in the presence of the preservative, the formulation could be altered prior to marketing to include a mold inhibitor.

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