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Inhibition of decay fungi using cotton cellulose hydrolysis as a model for wood decay

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Abstract

Environmental pressures to replace chromium and arsenic in fixed waterborne preservatives have been increasing. Potential inhibitors of brown-, white- and soft-rot fungi need to be evaluated as alternative preservatives by screening and testing in, in vitro. model systems. This paper reports the inhibition of cellulose depolymerization and weight loss of selected decay fungi by 11 chemical compounds. The ability to depolymerize carbohydrate polymers is analogous to strength loss of wood which can occur independently of utilization (weight loss). Cotton cellulose was pretreated with 1% solutions of compounds selected for their unique ability to stain wood components, dye cellulose or to act as a scavenger of active oxygen species. Cotton cellulose was exposed to three brown-rot fungi (*Gloeophyllum trabeum* MAD-617; *Postia placenta* MAD-698 and *Tyromyces palustris* TYP-6137) and the white-rot fungus *Trametes versicolor* MAD-697. After 12 weeks exposure to the fungi in modified soil-block tests. cotton samples were removed and tested for weight loss, reduction in degree of polymerization (DP) and elemental analysis by inductive coupled plasma (ICP) spectroscopy. Only two compounds tested (NHA and ruthenium red) inhibited weight loss for all fungi tested. The remaining compounds were selective in their ability to prevent weight loss or inhibit reduction in DP. In general. antioxidants were only effective against brown-rot fungi. Independent mechanisms of cellulose hydrolysis by brown- and white-rot fungi demonstrates one problem inherent in development of target specific wood preservatives not evident in preservatives containing broad-spectrum biocides, i.e. different mechanisms require different inhibitors. Published by Elsevier Science Ltd.

Keywords: Brown-rot; antioxidant; Cellulose hydrolysis; Wood decay; Exoglucanase

1. Introduction

Fungal decay of wood · in-service results in billions of dollars (US) of losses annually (Green III and Highley, 1997b; Highley and Illman, 1991). Of this, brown-rot decay is the most costly and destructive form of deterioration of wood in-service. The mechanism of brown-rot decay is thought to occur by diffusion of low molecular weight agents into the wood cell wall resulting in extensive oxidative depolymerization of polymeric polysaccharides, accompanied by strength loss of wood, prior to weight loss (Green III and Highley, 1997a; Winandy and Morrell, 1993; Green III et al., 1991). Precise confirmation of the mechanism(s) of brown-rot will likely be investigated well into the 21st century. There is a polite myth that all the brown-rot mechanisms are the same (Green III and Highley, 1997a). Most researchers believe that the primary mechanism of brown-rot is the production of hydroxy radicals by means of Fenton chemistry or one-electron oxidation (Flournoy, 1994; Highley and Flournoy. 1994; Enoki et al., 1997). Cellobiose dehydrogenase (CDH) has also been implicated in the autoxidation of Fe(II)-oxalate complexes to produce H_2O_2 in *Coniophora puteana* (Wood, 1994; Hyde and Wood. 1997). Recently, Kerem et al. (1998, 1999) and Paszczynski et al. (1999) have proposed a hydroquinone based mechanism of $Fe^{3+} \rightarrow Fe^{2+}$ reduction and H_2O_2 production for *Gloeophylum trabeum*. White-rot fungi like *Phanaerochate chrysosporium* have also been shown to generate hydroxy radicals during wood degradation (Tanaka et al., 1999). Hydroxy radicals (OH') arc

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widely implicated as a major damaging species in free radical pathology (Winterbourn, 1987). Thus, wood decay is an ideal system to test for antioxidant inhibition.

Recent environmental restrictions, both US and international, are limiting the use of broad spectrum biocides for wood preservation, primarily due to increased disposal problems as treated wood is taken out of service. In order to develop new, environment friendly (benign) methods for the control of wood decay fungi, it is essential to take an "educated guess" approach to control, i.e. start using what we already know to stop decay rather than wait until the mechanism is completely understood. There is little evidence to support the hypothesis that advances in understanding the precise mechanisms of brown-rot decay have contributed to the development of more environment friendly or benign wood preservatives. To date. no commercially available wood preservatives have been developed that interfere with any specific wood decay mechanism. In fact, just the opposite is true. These efforts to identify targets are generally unsuccessful and effective target sites, when known, have always been identified after, not before. fungicide discovery (Brent, 1995).

Previously published results have reported that free radical scavengers (antioxidants) in concert with didecyldimethylammonium chloride (DDAC) are synergistic in their antifungal action (Schultz and Nicholas, 1994; Schultz et al., 1998). Similarly, a series of papers by Beth-Anderson (1987, 1993) suggested that calcium is a requirement for dry-rot decay by *Serpula lacrymans.* We have included these inhibitors in our ASTM soil-block tests on cotton cellulose.

The primary objectives of this paper are: (i) to try and build upon what is already known about the mechanism of wood decay using strong inference (Platt, l964), and (ii) test targeted chemicals for their ability to interfere, interrupt or inhibit the in situ hydrolysis of cellulose by brown- and white-rot fungi. In previous publications, we. examined inhibition of fungal decay on southern yellow pine (Green III et al., 1997a, 1997b). The targeted mechanisms, in this study, include scavengers of Fenton hydroxy radicals by antioxidants like butylated hydroxytoluene (BTH) and butylated hydroxy anisole (BHA) natural wood products like lapachol (Jin and Laks, 1995) calcium precipitation by napthaloylhydroxylamine (NHA) and chromogenic substrates (ABTS) for detection of peroxidase and hydroxy radicals.

2. Materials and methods

2.1. Fungi

Three brown-rot fungi were used: *Postia placenta* (Fr.) M. Lars et Lomb. (MAD-698), *G. trabeum* (Pers.:Fr.) Murr. (MAD-617), and *Tyromyces palustris* (Berk. & Curt.) Gilb. & Ryv. (TYP-6137), and one white fungus was included, *Trametes versicolor* (L.:Fr.) Quel (MAD-697). All fungal isolates were stored on 2% malt-extract agar tubes at 4°C prior to their use in this study.

2.2. Cotton cellulose test method

Two 0.5 g samples of cotton cellulose were tested per modified soil-block bottle (n = 4) (see Fig. I). Southern pine feeders were exposed to the brown-rot fungi and maple feeders were exposed to the white-rot fungus per modified ASTM soil-block method (i.e. cotton cellulose is substituted for test wood blocks) (ASTM, 1993).

2.3. Treatment of cotton

Cotton cellulose was steam sterilized (100°C) for 30 min and impregnated with sterile 1% solutions of the test chemicals (aqueous or 95% EtOH; see Table 1). Four 0.5 g replicate cotton samples were weighed and dip treated. Treated samples were dried in a lyophilizer. Cotton samples were not reweighed after chemical treatment.

2.4. Modified soil-block test

The standard ASTM D2017 soil-block method (ASTM, 1993) was modified to test the ability of the chemicals to prevent degradation by the brown- and white-rot fungi (i.e. cotton cellulose was substituted for wood blocks). Following incubation. cotton samples were removed from bottles, oven dried at 40°C, and

Fig. 1. Soil-block bottles showing 7-day cotton cellulose testing of *P. placenta* MAD-698. (Cellulose treatments left to right: ABTS; Control; $CeCl_3$; RR.)

weighed gravimetrically. Weight loss was used to estimate decay.

2.5. Elemental analysis

Inductive coupled plasma (ICP) spectroscopy was performed at the University of Wisconsin Extension Soil and Plant Analysis Lab in Madison, WI on duplicate samples: P, K, Ca, Mg, S, Za, B, Mn, Fe, Cu, Al and Na contents were estimated in ppm.

2.6. Degree of polymerization

A viscometric assay using 0.5 M cupriethylenediamine (GFS Chemicals, Columbus, OH) was used to determine the degree of polymerization (DP) of the cotton cellulose after exposure to decay fungi (Cowling, 1960). Untreated cotton was used for a control.

3. Results

3.1. Weight loss or gain of cellulose in modified soil-block test

Cotton cellulose weight changes are shown in Table 2. Only ruthenium red (RR) and NHA prevented cellulosic weight loss (< 1%) by all the four fungi. Cerium chloride, uric acid and ABTS, a substrate oxidized to a green color by H_2O_2, and horseradish peroxidase (HRP), were only effective against the three brown-rot fungi (Table *3). T. versicolor* turned ABTS-treated cotton green in 48 h due to the production of peroxidase, lactase and H_2O_2 but, did not inhibit weight loss or DP (Table 4). Weight gains can be attributed to chemical and fungal mass and translocated cations with little subsequent decay.

Lapachol and indigo inhibited only *T. versicolor* (Tables 2 and 3). Two of the radical scavengers, mannitol and indigo, enhanced weight loss (i.e MAD-617 and TYP-6137) compared to the control.

3.2. ICP spectroscopy

Elemental analysis by ICP spectroscopy indicated that: (i) brown-rot fungi increase the ppm of Ca^{2+}, Fe^{3+}, Mn^{2+}, $A1^{3+}$; and (ii) white-rot increases that of Ca^{2+}, Mn^{2+}. Calcium remains a common target available for chemical inhibition. In control samples, brown-rot fungi translocated nearly every element except Na^{+}, preferentially Fe^{3+} (400-fold). The white-rot fungus. *T. versicolor*, preferentially translocated Mn^{2+} (> 1000-fold). In ABTS-treated cotton samples, *T. palustris* translocated Al^{3+}, Ca^{2+} and Fe^{3+}, probably by oxalic acid production (Green III and Clausen, 1999). Oddly, *T. palustris* exported sodium from sodium urate and did not translocate Fe^{3+} to the degree observed in untreated controls. Sodium was 50-80 times higher in the samples treated with sodium salts of NHA and urate.

3.3. DP of degraded cellulose

DP are shown in Table 4. Brown-rot fungi reduced the control cotton to the limit of degree of polymerization (LODP). Uric acid and NHA were most effective in preventing hydrolysis of cotton by brown-rot fungi, and indigo prevented reduction in DP by *T. versicolor.*

4. Discussion and conclusions

Targeted inhibition of wood decay has been previously examined (Schultz et al., 1998; Green III et al.. 1997b). Schultz et al. (1998) observed a synergistic effect of BHT and DDAC against *T. versicolor*. In

Table 1
Chemicals tested (1%) against wood decay fungi

Compound	Category
1. Ruthenium red (III) chloride oxide, (RR) (pH 5.9)[a]	Pectin stain
2. *N,N*-naphaloylhydroxylamine (NHA) (pH 8.6)	Calcium precipitator
3. Butylated hydroxy anisole (BHA)[b]	Antioxidant
4. Butylated hydroxy toluene (BHT)[b]	Antioxidant
5. Lapachol[b]	Antioxidant
6. Cerium chloride ($CeCl_3$) (pH 3.64)	Peroxide precipitator
7. Indigo (pH 10.00)	Textile dye
8. Mannitol (pH 4.17)	Antioxidant[c]
9. Uric acid (Na salt) (pH 7.58)	Antioxidant[c]
10. Benzoic acid	Antioxidant[c]
11. 2,2´-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (pH 5.43)	Chromogenic substrate

[a] All compounds were solubilized in water.
[b] Compounds were solubilized in 95% ethanol.
[c] Hydroxy radical scavengers; Winterbourn (1987)

Table 2
Percent weight loss (mean ± S.D.) of treated cotton cellulose in soil-block tests after 12 weeks[a]

Treatment	*P. placenta* MAD-698	*G. trabeum* MAD-617	*T. palustris* TYP-6137	*T. versicolor* MAD-697
Control	15.3 ± 7.4	18.9 ± 3.6	35.8 ± 5.0	26.4 ± 26.4
ABTS	+4.7 ± 4.5	+ 5.2 ± 0.8	+ 4.8 ± 1.7	22.7 ± 11.2
Lapachol	ND[b]	10.1 ± 6.3	17.9 ± 1.6	0.5 ± 0.3
RR	+ 6.8 ± 0.8	1.1 ± 1.9	+ 2.9 ± 1.7	+ 1.1 ± 1.2
Indigo	ND	7.5 ± 8.3	51.2 ± 6.1	1.1 ± 1.1
$CeCl_3$	+ 2.4 ± 1.0	1.6 ± 3.2	+ 2.8 ± 5.6	1.9 ± 0.8[c]
NHA	+ 11.4 ± 1.6	+ 11.0 ± 4.6	+ 10.0 ± 2.5	+ 6.9 ± 3.6
BHA	ND	2.5 ± 0.7	11.1 ± 3.1	11.7 ± 4.5
BHT	ND	5.1 ± 0.6	11.6 ± 6.5	40.9 ± 10.7
Mannitol	ND	25.1 ± 3.5	36.6 ± 3.8	12.4 ± 19.9
Uric acid	+ 2.0 ± 0.5	+ 10.4 ± 6.0	+ 1.1 ± 0.6	32.8 ± 19.6
Benzoic acid	ND	2.0 ± 4.8	17.2 ± 27.4	6.4 ± 6.8

[a] + sign indicates weight gained.
[b] Not determined.
[c] Visually 75% mycelial mass: 25% cellulose

Green III et al. (1997b), NHA was shown to be effective in inhibiting weight loss of southern yellow pine during soil-block tests. Weight loss of cotton cellulose and reduction in DP have previously been shown to experimentally represent the functional decay mechanisms of wood decay fungi (Green III et al., 1992, 1993; Highley, 1977, 1980, 1982, 1990, 1997; Kirk et al., 1991). In this study, NHA also inhibited the cellulolytic mechanism of brown-rot. This demonstrates that NHA inhibits by a mechanism unrelated to Ca^{2+} precipitation in pectin-containing wood structures (i.e., pit torus) as previously hypothesized (Green III et al., 1996, 1997a).

Ruthenium red (RR), an ammoniated form of ruthenium oxychloride, is an inorganic. synthetically prepared. intensely colored, crystalline compound. It has long been used as a standard stain for pectins in plant tissue for light microscopy. In normal pine, RR stains ray parenchyma cells, resin ducts and pits an intensely red colour (Highley and Lutz, 1970). In addition. RR has been widely used in the micromolar range as a strong and specific inhibitor of in vivo and in vitro Ca-mediated biochemical processes. RR has been shown to inhibit binding of Ca^{2+} -calmodulin in myosin and to inhibit Ca^{2+} release from sarcoplasmic reticulum (Meinicke et al., 1996). Thus. RR may interfere with cellulose degradation directly or indirectly by inhibiting Fenton chemistry. In this study, RR inhibited both brown- and white-rot fungi (Table 3).

Cerium chloride was only effective in the inhibition of the three brown-rot fungi. This is likely because cerium (Ce) acts as a trapping agent for all endogenous sources of peroxide, precipitating two forms of cerium perhydroxide that are visible in TEM (Briggs et al., 1975; Czaninski et al., 1993) and in localization of NADH oxidase enzymes (Kausch, 1987) as shown below:

Peroxide scavenger inhibition:

$$CeCl_3 + H_2O_2 \rightarrow \quad Ce(OH)2OOH$$

Cerium chloride was not expected to inhibit enzymatic hydrolysis and utilization of cellulose by *T. versicolor*. Only brown-rot ($OH^{\cdot}$) and not white-rot enzymatic mechanisms of cellulose hydrolysis appear blocked,

Table 3
Relative efficacy of cotton weight reduction pattern of chemicals tested

Inhibits brown-rot	Inhibits white-rot	Inhibits both	Inhibits neither
ABTS	Lapachol	NHA	BHA
Uric acid	Indigo	RR	BHT
Cerium chloride			Mannitol
			Benzoic acid

Table 4
Effect of targeted chemicals on the decrease in degree of polymerization (DP) of cotton after 12-week soil-block test[a]

Treatment	MAD-698	MAD-617	TYP-6137	MAD-697
Control	196/196[a]	150/196[a]	150/196[b]	952/1190
NHA	920/1590	998/1576	936/1044	1088/1576
Ruthenium red	378/432	734/844	828/906	734/766
Uric acid	450/500	1446/1618	1272/1472	ND
Cerium chloride	304/340	652/844	1030/1230	968/1072
ABTS	466/484	484/750	734/684	814/1174
Indigo	ND	ND	ND	1420/1524
Lapachol	ND	ND	ND	968/1460

[a] Limit of degree of depolymerization (LODP) (N = 2).
[b] Untreated cotton DP is 1576/1896/2122 (N = 3).

confirming that hydroxy radicals are not required for cellulose degration by *T. versicolor.*

ABTS (the diazonium salt of 2,2´-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) can trap H_2O_2 with the formation of a blue-green color or act as a substrate for lactase or lignin peroxidase (LiP). ABTS has been used to detect hydrogen peroxide production by decay fungi (Highley and Flournoy, 1994). ABTS only inhibited weight reduction of cotton cellulose exposed to brown-rot fungi (without inhibiting depolymerization) but not cotton exposed to *T. verisicolor.* Many white-rot fungi have the capacity to oxidize lignin directly with lactase-ABTS complex (Muheim et al., 1992).

One alternative hypothesis to explain the inhibition of fungal decay is that 1% NHA (pH 8.3) and other inhibitors simply raise the pH of the wood blocks to an alkaline condition. However, Highley (1973) demonstrated that alkaline-treated wood was readily attacked by brown-rot fungi when exposed to a high-decay hazard via the ASTM soil-block test. Based upon agar testing and a minimum inhibitory concentration (MIC) for NHA of 0.01–0.05% in agar, NHA appears to inhibit by direct cytotoxicity, possibly by disrupting calcium cycling.

The enzymatic depolymerization of cellulose by *T. versicolor* was inhibited by the textile dye indigo and the tropical heartwood extractive lapachol. Due to the vat dye indigo intercallates into cellulose, substrate modification is assumed to prevent enzymatic recognition. Although some heartwood extractives are also radical scavengers (Hagerman et al., 1998), lapachol did not inhibit brown-rot in these experiments. Lapachol has been shown to have antifungal activity against dermatophytes and decay fungi (Ali et al., 1998). Lapachol (1%) did not inhibit *T. versicolor* in southern yellow pine (Green III et al., 1997a).

In summary, antioxidants did not inhibit cellulose hydrolysis by *T. versicolor* and indigo did not inhibit brown-rot fungi (Table 2). Although inhibition of weight loss and depolymerization of cotton directly address the brown-rot mechanism, H_2O_2 and hydroxy radicals (OH') can only be detected and inhibited by "non-specific reactions". Inhibition of cellulose hydrolysis by NHA and RR is not pectin dependent and both appear directly toxic to decay fungi (Green III et al., 1996, 1997b). The different mechanisms of cellulose hydrolysis by white-rot fungi and among different brown-rot fungi demonstrate an inherent weakness in working with highly target-specific wood preservatives. Such specificity was never a problem with the previous generation of highly toxic wood preservatives that contained broad-spectrum biocides. Multiple target-specific preservatives may need to be combined to provide full protection against decay fungi without endangering other biological systems.

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