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Targeted Inhibition of Wood Decay Fungi: Degradation of Cotton Cellulose

by

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Targeted Inhibition of Wood Decay Fungi: Degradation of Cotton Cellulose

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Brown-rot decay is responsible for 80% of the damage and replacement of wood in service. For nearly thirty years, researchers have postulated a one-electron oxidase system combining F^{+2} oxalic acid, and H_2O_2 in the production of hydroxy radicals which ultimately oxidize cellulose *in situ.* Recently, researchers have been investigating antioxidants, in combination with biocides (Schuhz, *et al.*, 1998), as targeted inhibitors of the decay process and potential wood preservatives. In order to target the cellulolytic mechanisms of brown-rot and white-rot decay fungi, cotton cellulose was treated with free radical scavengers, antioxidants, dyes and hydroxy-radical detectors 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in order to assess their ability to retard cellulose hydrolysis. Weight loss of cotton cellulose is the most reliable and accurate measure of exoglucanase activity. Cotton cellulose was exposed to *G. trabeum* MAD 617, *T. palustris* 6137 and *T. versicolor* MAD 697 in soil-block tests. After 12 weeks exposure, cotton was evaluated for weight loss, change in DP, and elemental analysis by ICP spectroscopy. Only 2 out of 11 compounds tested (NHA and ruthenium red) showed less than 1% weight loss for all three fungi tested. All other compounds were selective, underscoring difficulties in sharply targeting decay mechanisms. Weight loss and DP of cellulose are analogous to degradation and strength loss of wood caused by decay fungi.

Key words: mechanism of brown-rot, antioxidant, cellulose hydrolysis, wood decay, exoglucanase

Introduction

Fungal decay of wood in service results in billions of dollars (US) of losses annually. 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 best characterized 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 and Highley 1997, Winandy and Morrell 1993, Green et al., 1991). Precise confirmation of the mechanism(s) of brown-rot will likely be investigated well into the 21st century. There is the polite myth that all brown-rot mechanisms are the same (Green and Highley, 1997a). Production of hydroxy radicals by means of Fenton chemistry or one-electron oxidation holds the majority view. (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* (Hyde & Wood, 1997). Recently, Kerem et al., (1998, 1999) have proposed a hydroquinone based mechanism of Fe^{+3} --> Fe^{+2} reduction and H_2O_2 production for *G. trabeum.* Hydroxy radicals (OH·) are widely implicated as a major damaging species in free radical pathology (Winterbourne, 1987). Thus, brown-rot 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, primarity due to increased disposal problems as treated wood is taken out of service. In order to develop new, environmentally 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 more environmentally friendly or benign wood preservatives. To date, no commercially available wood preservatives have been developed to 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, where known, have always been identified after, not before, fungicide discovery (Brent, 1995).

Previously published IRG results have reported that free radical scavengers (antioxidants) in concert with didecyldimethylammonium chloride (DDAC) are synergistic in their antifungal action (Schultz et al., 1998). Similarly, a series of IRG 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 to try and build upon what is already known about the mechanism of wood decay using strong inference (Platt, 1971) and to test targeted chemicals for their ability to interfere, interrupt or inhibit the *in situ* hydrolysis of cellulose by brown- and white-rot fungi. In a previous IRG document, we examined inhibition of fungal decay on southern yellow pine (Green et al., 1997). The targeted mechanisms in this study include scavengers of Fenton hydroxy radicals by antioxidants like butylated hydroxytoluene (BTH), butylated hydroxy anisole (BHA) and lapachol, calcium precipitation by napthaloylhydroxylamine (NHA) and chromogenic substrates (ABTS) for peroxidase and hydroxy radicals.

Materials and Methods

Fungi

Three brown-rot fungi were used: *Postia placenta* (Fr.) M. Lars et Lomb. (MAD-698) *Gloeophyllum trabeum* (Pers. :Fr.) Murr. (MAD-617), and *Tyromyces palustris* (Typ-6137); and one white fungus was included, *Trametes versicolor* (L. :Fr.) Quel (MAD-697). All fungal isolates were maintained on 2% malt-extract agar tubes at 4°C for the duration of the study.

Cotton cellulose test method

Two half-gram samples of cotton cellulose were tested per soil block bottle (n=4). 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 (D2017).

Treatment of cotton

Cotton cellulose was steam sterilized (100°C) for 30 minutes and impregnated with sterile 1% solutions of the test chemicals (aqueous or 95% Etoh; Table 1). Four replicate cotton samples were treated. Aqueous solutions were dried in a lyophilizer.

Soil-block test

The standard ASTM D2017 soil-block method (ASTM, 1993) was used to test the ability of the chemicals to prevent degradation by the brown- and white-rot fungi. Following incubation, cotton samples were removed from bottles, oven dried at 40°C, and weighed. Weight loss was used as an estimate of decay susceptibility.

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)

Degree of polymerization

A viscometric assay using 0.5M cupriethylenediamine (GFS Chemicals, Columbus, OH) was used to determine the cellulose DP of cotton after exposure to decay fungi.

Results

Cotton cellulose weight losses are shown in Table 2. Briefly, only ruthenium red (RR) and NHA prevented cellulosic weight loss (<1%) from all 3 fungi. Cerium chloride, uric acid and ABTS, a substrate oxidized to green color by H_2O_2 and horseradish peroxidase (HRP), was only effective against the two brown-rot fungi. *T. versicolor* turned ABTS cotton green in 48 hours due to the production of peroxidase, laccase and H_2O_2 however, this did not inhibit weight loss or DP (Table 4).

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

Elemental analysis by inductive coupled plasma (ICP) spectroscopy indicated that i) brown rot fungi increase Ca, Fe, Mn, Al and ii) white rot increases Ca^{++}, Mn^{++}. Calcium remains a common target available for chemical inhibition. In control cotton, brown-rot fungi translocated nearly every element except Na, preferentially Fe^{+++} (400 fold) white rot preferentially Mn (data not shown) and in ABTS-treated cotton samples, MAD 6137 translocated $A1^{+3}$, Ca^{+2} and Fe^{+3} likely by oxalic acid production (Green, Clausen, in press). Oddly, MAD 6137 exported sodium from sodium urate and did not translocate Fe to the degree observed in untreated controls. Sodium was 50 to 80 times high in sodium salts (NHA and urate).

Degree of polymerization are shown in Table 4. Both brown-rot fungi reduced the control cotton to limit of degree of polymerization (LODP). Uric acid was most effective in preventing hydrolysis of cotton by brown-rot fungi and indigo from *T. versicolor.*

Discussion

Targeted inhibition of wood decay has been previously examined (Schultz et al., 1998; Green et al., 1997). Schultz et al., (1998) observed a synergistic effect of BHT and DDAC against *T. versicolor.* In the latter paper, 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 (Highley, 1980, 1982, 1990, 1992, 1997; Green et al., 1994, 1993) In this study, NHA also inhibited the cellulolytic mechanism of brown-rot. This demonstrates that NHA inhibits by a mechanism unrelated to Ca^{++} precipitation in pectin containing wood structures (i.e. pit torus) as previously hypothesized (Green et al., 1997).

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, ruthenium red stains ray parenchyma cells, resin ducts and pits an intense 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^{++}-calmodulin in myosin and to inhibit Ca^{++} release from sarcoplasmic reticulum. Thus, RR may interfere with pectin degradation directly or indirectly by inhibiting Fenton chemistry (Meinicke et al., 1996).

Cerium chloride was only effective in the inhibition of both 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 which are visible in TEM (Briggs et al., 1975, Czaninsky et al., 1993) and oxidase enzymes (Kausch, 1985). Cerium chloride was not expected to inhibit enzymatic hydrolysis and utilization of cellulose by *T. versicolor.* Thus only brown-rot (OH·) and not white-rot (enzymatic mechanisms of cellulose hydrolysis) appear blocked:

Peroxide scavenger inhibition $CeCl_3 + H_2O_2$ --> Ce(OH) 2OOH

ABTS (the diazonium salt of 2,2'-azino-bis-3-ethyl-benzothiazoline-6-sulfonic acid) can trap H2O2 with the formation of a blue-green color or act as a substrate for laccase 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 in brown-rot fungi (without inhibiting depolymerization) but not the white-rot fungus.

The enzymatic depolymerization of cellulose by *T. versicolor* was inhibited by the textile dye indigo and the tropical heartwood extractive, lapachol. Because the indigo dye binds to the

cellulose, substrate modification is assumed to prevent enzymatic recognition. Although some heartwood extractives are also radical scavengers (Hageizman 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 et al., 1997).

In summary, antioxidants did not inhibit cellulose hydrolysis by *T. versicolor* and indigo did not inhibit brown-rot fungi (Table 3). 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. Different mechanisms for the inhibition of brown-rot and white-rot fungi underscores the problems inherent in target specific inhibition of decay, and the potential requirement for multiple compounds in sharply targeted preservatives.

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

Compound	Category
1. Ruthenium red (III) chloride oxide, (RR) (pH 5.9)	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[a]
9. Uric Acid (Na Salt) (pH 7.58)	Antioxidant[a]
10. Benzoic Acid	Antioxidant[a]
11. 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-sulfonic acid) (pH 5.43)	Chromogenic substrate

a. Hydroxy radical scavengers; Winterbourn (1987)

b. 95% ethanol

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

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

(+ sign indicates weight gained)

[a] - approximately 75% mycelial mass: 25% cellulose

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 decrease in degree of polymerization (DP) of cotton after 12 week soil block test.[b]

Treatment	MAD-617	TYP-6137	MAD-697
Control	150/196[a]	150/196[a]	952/1190
NHA	998/1576	936/1044	1088/1576
Ruthenium Red	734/844	828/906	734/766
Uric Acid	1446/1618	1272/1472	ND
Cerium chloride	652/844	1030/1230	968/1072
ABTS	484/750	734/684	814/1174
Indigo	ND	ND	1420 1524
Lapachol	ND	ND	968/1460

[a] Limit of degree of depolymerization (LODP)

[b] Untreated cotton DP is 1576/1896/2122.