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Targeted inhibition of wood decay (Using everything but the kitchen sink)

by

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SUMMARY

Low molecular weight oxidative decay agents have been implicated in the degradation of wood by brown-rot decay as evidenced by chemical analysis of brown-rotted wood and detection of oxalic acid and hydroxy radicals. Fenton chemistry (H_2O_2 / Fe++) is often proposed as the mechanism for generating hydroxy radicals. Previous authors have shown iron to enhance the brown-rot hydrolysis of wood, while others have shown suppression of brown-rot by organic and inorganic metal chelators. We have attempted to inhibit brown-rot and white-rot decay of southern pine and maple wood blocks in a series of soil block decay tests using a variety of chemicals targeted specifically at key components of proposed brown-rot mechanisms. Included in these tests were inorganic and organic chelators, calcium coordinating compounds, wood binding dyes, microbial siderophores and common antioxidants--some previously tested. All chemicals were screened at 1% aqueous (w/v). Only 2 of 28 compounds were effective in significantly reducing wood weight loss by all fungi tested in 12 weeks: napthaloylhydroxylamine (NHA)--a calcium precipitating agent; and ruthenium red (RR)--a pectin stain. Both compounds bind preferentially to pit tori and ray parenchyma cells as observed by light microscopy. Targetting the woody substrate for inhibition of decay looks more promising than targetting fungal physiology.

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 decay 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 debated well into the 21st century. There is polite myth that all brown-rot mechanisms are the same (Green and Highley, 1997).

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 this wood is taken out of service. In order to develope 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. 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 iron promotes brown-rot decay and that iron chelators (ie EDTA) inhibit decay (Paajanen and Ritschkoff, 1993; Viikari. and Ritschkoff, 1992; Suttie et al ., 1996). 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 promotors in our ASTM soil block tests.

The primary objective of this paper is to try and build upon what is already known or surmised 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 decay of wood by brown- and white-rot fungi. The targeted mechanisms include scavengers of Fenton hydroxy radicals by antioxidants like butylated hydroxytoluene(BTH) and ferrozine, calcium precipitation by napthaloylhydroxylamine (NHA), chelation of iron by 1,10 phenanthroline, deferroxamine mesylate and EDTA.

MATERIALS AND METHODS

Fungi

Five brown-rot fungi were used: Postia placenta (Fr.) M. Lars et Lomb. (MAD-698) Gloeophyllum trabeum (Pers:Fr.) Murr. (MAD-617), Meruliporia incrassata (Berk. and Curt.) Murr. (MAD-563), Fomitopsis palustris (Berk. and Curt.) Gibn. and Ryv.) (L-15755) and F . palustris (Typ-6137); and one white fungus was included, Trametes versicolor (L:Fr.) Quel (MAD-497). All fungal isolates were maintained on 2% malt-extract agar tubes at 4°C for the duration of the study.

Wood

We used southern pine (pinus sp.) and maple (Acer . sp.) sapwood blocks (3/4 in square, 1.91 cm square). Southern pine blocks were exposed to the brown-rot fungi and maple to the white-rot fungus.

Treatment of blocks

Blocks were steam sterilized (lOO°C) for 30 minutes and impregnated with sterile 1 percent solutions of the test chemicals (Table 1). Ruthenium red and NHA were also tested at lower concentrations of chemical. Four replicate blocks were treated.

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, test blocks were removed from bottles, oven dried at 80°C, and weighed. Weight loss was used as an estimate of decay susceptibility.

Inhibition of fungal growth by RR and NHA on agar

Various concentrations of RR and NHA were added to 2% malt agar cooled to 50°C and then dispersed into petri dishes to produce a solid medium for inoculation with the decay fungi. Cores (5 mm diameter) removed from the margins of actively growing cultures were inoculated in the centers of plates that were then incubated in the dark at 27°C. Three replicate plates were set up for each test. Inhibition of growth of the decay fungi was recorded as the difference in mean radial growth of the decay fungi in the presence or absence (control) of the chemicals when the decay fungi in controls reached the edge of plates. These values were then used to calculate the inhibition of hyphal extension as a percentage of hyphal extension in the absence of the chemicals.

RESULTS

The calcium binding agent, NHA, and the pectin binding dye, RR, were the only compounds that substantially reduced decay by all the fungi tested at the one percent level (Tables 2-4). The iron chelator, 1,10 phenanthroline, was also-effective but it was tested for only six weeks and against only 3 test fungi. (Twelve week results will likely lead to increased decay.)

NHA and RR were tested for fungitoxicity in malt/agar medium (Table 5). Both compounds completely inhibited fungal growth at 0.1 percent concentration and killed the fungi. NHA was more inhibitory than RR at the lower concentrations.

NHA and RR were also tested in soil-block tests at concentrations lower than one percent (Table 6). NHA was effective at 0.5 percent; RR was not tested at this concentration. Neither compound was effective in stopping decay at 0.1 percent.

We felt that treating wood with calcium compounds might stop brown-rot decay by precipitation of oxalic acid before the oxalate can have the required effect (Table 4). However, this was not the case as more of the compounds stopped decay although only $CaOH_2$ significanlty reduced it. Iron has been reported to enhance brown-rot decay but addition of iron dextran to wood did not enhance weight loss.

DISCUSSION

Transition metals e.g. iron have been hypothesized to play a key role in the formation of highly reactive radicals such as OH which initiate the breakdown of wood cell polymers. Therefore, materials that interfere with peroxide/radical levels such as metal chelators or antioxidants would be expected to retard wood decay. However, Highley (1982) and Highley and Murmanis (1985) found that decay by both white-rot and brown-rot fungi in most cases was not reduced in wood treated with a number of chelators and radical peroxide quenching agents in soil-block tests. This was confirmed in the present study even when higher concentrations of compounds were used to treat blocks. An exception was 1,10 phenanthroline which reduced brown-rot decay and completely inhibited the white-rot fungus in the six week test. In a previous study (Highley, 1982) it did not inhibit decay when used at a lower concentration.

However, Viikari and Ritschkoff (1992) and Suttie et al ., (1996) report inhibition of brown-rot decay by inorganic and organic chelators. Suttie et al ., (1996) found that deferoxamine and ferrozine at 1mM stopped decay by Coniophora puteana in 6-week tests but EDTA was not particularly effective. Low weight loss was obtained in control blocks in their study and weight losses were also quite variable. Viikari and Ritschkoff (1992) used higher concentrations of material (50mM) than Suttie et al ., (1996) or those used in our study and obtained good control of decay with EDTA and tripolyphosphate chelators. Better control of decay by the chelators in these studies than we observed might be explained by the different decay tests used. Both Suttie et al ., (1996) and Viikari and Ritschkoff (1992) exposed test blocks over a malt-agar medium whereas we exposed blocks over a soil medium. Soil contains more metals such as iron than malt-agar and thus may provide sufficient amounts of metals to overcome the effect of the chelators.

Iron has been reported to promote brown-rot decay (Morris, 1992; Paajanen, 1993). Paajanen (1993) found that iron added to malt-agar medium increased decay of pine by Serpula lacrymans . We did not obtain an increase in decay when iron was added as iron dextran to blocks and exposed over soil but sufficient iron may have already been present in the soil.

Thompson (1964) looked at five metal-binding compounds as wood preservatives--thioglycolic acid, potassium xanthogenate, 8-hydroxyquinoline, dimethylglyoxime, and 1-nitroso-2-naphthol. His idea was to render micronutrients required for fungal growth unavailable. The treated wood was exposed to T . versicolor and P . placenta in soil-block tests. He found that the two fungi varied widely in their susceptibility to the chelating compounds. Only dimethylglyoxime was effective against both fungi at relatively low retention levels.

There is an increasing body of information to suggest that one of the mechanisms in decomposition of wood by brown-rot fungi involves iron-catalyzed oxygen radical-induced cell wall decomposition. Our results with the chelators and antioxidants do not support this theory but the fact that they had little effect on the ability of the decay fungi to decompose wood doesn’t rule out Fenton

chemistry as part of the degradation system. The fungi need to survive in a harsh environment and therefore may produce compounds such as siderophores, or acids that out-compete and/or inactivate these treatments. Unfavorable, ion pH conditions, for example, may affect the ability of chelators to form stable chelates. Some work beet at high PH. Wood, especially partially decayed, can be very acid (Green et al ., 1991) which would reduce the effect of these chelators.

The production of pectin degrading enzymes and hydrolysis of bordered pit membranes during incipient brown-rot decay has recently been described by Green et al ., (1995). One key to pectin hydrolysis has been shown to be fungal production of oxalic acid which lowers the pH of the substrate and chelates calcium ions. Production of oxalic acid may serve a similar role during incipient wood decay as calcium oxalate has been visualized by scanning electron microscopy during both brown-rot and white-rot decay. Therefore, we hypothesized that in situ precipitation of calcium ions or pectin binding dyes may prevent the cascade of biochemical events involved in colonization of wood by decay fungi, especially hydrolysis of bordered pit membranes.

We showed that both brown-rot and white-rot fungi are inhibited from causing wood decay following pretreatment of wood blocks with either the water soluble calcium precipitator N,N-napthaloylhydroxylamine (NHA) or the pectin dye ruthenium red (RR). (Note: Astral blue did not inhibit decay). NHA is a water soluble heterocyclic calcium capture agent (M.W. 235) used initially for quantitative determination of calcium content in serum (Beck, 1951) before being used to demonstrate cellular calcium in electron microscopy (Zechmeister, 1979). NHA forms a very stable and very selective complex with Ca++ which is insoluble in 100mM EGTA (Sobota et al ., 1988). Sobota et al ., (1987) reported retention of 93% cellular calcium using NHA. Leaching NHA does not appreciably change fungal inhibition (Green et al ., 1996).

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 vitro and in vivo 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).

A chemical aimed at a target mechanism should interfere with an early event in the decay process e.g. colonization, to be an effective preservative because decay fungi reduce the strength of wood very early. Targeting inhibition of pit hydrolysis by decay fungi with agents such as NHA or RR appears to accomplish this objective by limiting fungal access to non-lignified carbohydrates and preventing colonization. Such compounds must also have low mammalian toxicity (Table 7). In summary, these results suggest that hypothetical decay mechanisms are not easily inhibited and that rendering specific wood substrates undigestible to the fungi may be better targets for fungal control than fungal metabolism.

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Table 1. Chemicals tested against wood decay fungi.

Compound	Catagory
1. Ruthenium red (III) cloride oxide, (pH 5.9)	Pectin stain
2. Phthalimide, potassium salt	NHA analog
3. Deferoxamine mesylate	Siderophore, chelator
4. Ethylenediamine tetraacetic acid (EDTA)	Chelator
5. Iron Dextran (FeOH)	Fe Promotor
6. Iminodiacetic acid ($C_4H_7NO_4$)	Complexes Mg, Ca
7. Calcium chloride	Ca Accelerator
8. Calcium hydroxide	Ca Accelerator
9. Calcium carbonate	Ca Accelerator
10. Calcium pantothenate	Ca Accelerator
11 N,N Naphaloylhydroxylamine (NHA) (pH 8.6)	Calcium precipitator
12. Ammonium oxalate (pH 6.3)	Fenton Chelator
13. Ethylene glycol tetraacetic acid (EGTA)	Chelator
14. Potassium Pyroantimonate (pH 10.3)	Calcium binding
15. Murexide (pH 8.3)	Calcium binding
16. Sodium hexametaphosphate (pH 6.5)	Chelator
17. Butylated hydroxy anisole (BHA)	Antioxidant
18. Butylated hydroxy toluene (BHT)	Antioxidant
19. Vitamin E Acetate	Antioxidant
20. N-Propyl gallate	Antioxidant
21. 1,10,-Phenanthroline	Metal ion chelator
22. Nordihydroguaiaretic acid	Antioxidant
23. Morin	Antioxidant
24. Rutin	Antioxidant
25. Lapachol	Antioxidant
26. Ferrozine; PDT disulfonate	Chelator
27. Chrome azurol S	Chromophore
28. Astral Blue 6G11	Pectin dye

Table 2. Effect of chelators, calcium binding agents and dyes on weight loss by decay fungi in soil-block tests.

Treatment (1% conc)	Percent weight loss due to fungal decay[a]					
	M. incrassata (MAD-563)	*G. trabeum* (MAD-617)	*P. placenta* (MAD-698)	*F. palustris* (L-15755)	*F. palustris* (TYP-6137)	*T. versicolor* (MAD-697)
			-12 weeks-			
Ruthenium red	2.0	2.7	1.0	2.8	5.3	1.2
NHA	-0-	6.4	1.8	ND	ND	3.5
Ammonium oxalate	59.3	53.6	56.9	ND	ND	55.2
Deferoxamine mesylate	37.2	65.8	2.6	11.5	21.1	54.3
EDTA	45.2	65.2	30.0	22.8	23.5	55.3
EGTA	57.2	63.5	52.0	ND	ND	49.1
Sodium hexametaphosphate	52.8	52.2	52.5	ND	ND	47.8
Imino-diacetic acid	26.2	63.1	21.7	13.7	20.0	50.2
Phthalimide	40.2	65.5	37.5	19.7	20.1	51.7
Potassium pyroantimonate	42.9	63.6	48.6	ND	ND	16.5
Murexide	56.9	57.3	53.3	ND	ND	53.0
Untreated control	52.6	63.3	49.2	31.0	26.8	50.0
			-6 weeks-			
Chrome azurol S	ND	36.8	30.1	ND	ND	25.6
Astra blue 6 GLL	ND	28.7	18.7	ND	ND	23.6
Ferrozine	ND	27.8	23.3	ND	ND	25.6
1,10 Phenanthroline	ND	5.3	6.5	ND	ND	-0-
Untreated control	ND	26.8	28.1	ND	ND	27.5

[a]Southern pine used for brown-rot and maple for white-rot
ND = Not Determined

Table 3. Effect of antioxidants on weight loss by decay fungi in soil-block tests

Compound	G. trabeum (MAD-617)	P. placenta (MAD-698)	T. versicolor (MAD-697)
BHA	2.5	5.0	24.7
BHT	7.4	16.1	25.6
Vitamin E acetate	28.1	24.9	27.8
n-Propyl gallate	22.8	25.2	24.0
Nordihydroguairetic acid	28.6	19.6	21.7
Rutin	ND	26.1	25.5
Morin	ND	25.6	26.2
Lapachol	ND	26.2	27.1
Ferrozine	27.8	23.3	25.6
Control	26.8	26.8	27.5

[a]Weight loss in six weeks.
Southern pine used for brown-rotters and maple for the white-rotters.

Table 4. Effect of calcium and iron on brown-rot decay

Fungus	Percent weight loss[a] due to decay fungi in wood treated with:					
	Control (pH 6.3)	$CaCl_2$ (pH 5.5)	Ca pantothenate (pH 7.0)	$Ca(OH)_2$ (pH 1.3)	$CaCo_3$ (pH 1.4)	Iron dextran (6.3)
P. placenta	52.7	44.6	57.8	5.7	20.7	20.3
G. trabeum	40.8	23.9	40.7	9.7	22.9	40.5

[a]Weight loss in 10 weeks. Four replications. All treatments one percent.

Table 5. Percent reduction in radial growth of fungi on RR- or NHA-malt agar

FUNGUS	RR CONCENTRATION 0.01%	0.05%	0.1%	NHA CONCENTRATION 0.001%	0.01%	0.1%
P. placenta MAD698	27%	100%	100%	72%	100%	100%
G. trabeum MAD617	58%	100%	100%	18%	66%	100%
T. versicolor MAD 697	0%	84%	100%	6%	59%	100%

Table 6. Effect of RR on NHA concentration on decay in soil-block tests.

Treatment (CONC %)	Percent weight loss due to attack by the decay fungi[1] S. incrassata (Mad-563)	G. trabeum (Mad-617)	P. placenta (Mad-698)	T. versicolor (Mad-697)
Untreated	55.5 ± 5.6	55.1 ± 3.0	52.2 ± 2.9	33.8 ± 9.9
0.05 NHA	54.6 ± 2.1	49.1 ± 3.1	32.2 ± 14.0	33.2 ± 4.9
0.1 NHA	37.2 ± 7.4	46.8 ± 6.4	18.6 ± 17.2	10.9 ± 11.2
0.5 NHA	-0-	1.6 ± 1.4	-0-	-0-
Untreated	ND	13.8 ± 1.0	21.8 ± 3.2	43.2 ± 1.6
0.01 RR	ND	15.1 ± 0.9	26.6 ± 3.7	45.5 ± 0.5
0.05 RR	ND	15.7 ± 3.0	16.7 ± 2.7	28.4 ± 2.4
0.1 RR	ND	13.7 ± 0.9	10.1 ± 0.5	20.1 ± 2.4

[1] Mean and standard deviation of four replicates

NHA treated blocks exposed to decay fungi for 12 weeks and RR treated blocks exposed for 10 weeks. Southern pine used for brown-rotters and maple for the white-rotters.

Table 7. Acute mammalian toxicity of various wood preserving chemicals administered orally to mice.

	Oral LD_{50}
N'N-napthaloylhydroxylamine (NHA)	>3200 mg/kg[a]
Copper 8-Quinolinolate (DDAC)	450 mg/kg[b]
NP-1 (IPBC + DDAC)	400 mg/kg[c]
Copper Napthenate	110 mg/kg[d]
Pentachlorophenol	36-92 mg/kg[e]
AsO_3 (arsenic component of CCA)	3.15 ug/kg[e]

[a]Experimentally determined at the Forest Products Laboratory, 1996 (Reed and Muench, 1938).

[b]Lumber Protection Today, Forintek Canada Corp., 1991

[c]Koppers Company, Inc., 1985

[d]A preliminary toxicological evaluation of eight chemicals used as wood preservative, U.S. Army Medical Research, Fort Ditrick, MD, May 1984

[e]Registry of Toxic Effects of Chemical Substances (RTECS) Feb. 1996. In addition to acute toxic effects there are also adverse oncogenic, fetotoxic, carcenogenic and mutagenic effects. (very toxic)