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Inhibition of wood decay and termite damage by calcium precipitation

by

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INHIBITION OF WOOD DECAY AND TERMITE DAMAGE BY CALCIUM PRECIPITATION

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ABSTRACT

Fungal decay of wood in service, especially brown-rot, results in billions of dollars (US) of losses annually. Recent environmental restrictions, both U.S. and international, are limiting or eliminating the use of broad spectrum biocides for wood preservation, primarily due to problems with disposal. In order to design new, environmentally benign methods for control of wood decay fungi, it is essential to understand the precise sequence of biochemical events as wood is colonized. The production of polygalacturonase (PG) and hydrolysis of bordered pit membranes during incipient brown-rot decay has recently been described by our laboratory. One key to pectin hydrolysis by plant pathogens 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, it was hypothesized that in situ precipitation of existing calcium ions in wood may prevent the cascade of biochemical events involved in colonization of wood by brown-rot fungi, especially hydrolysis of bordered pit membranes. Preliminary experiments in our laboratory have shown that brown-rot fungi, white-rot fungi, and termites are inhibited from effecting weight loss of wood following pretreatment of wood blocks with the selective water soluble calcium precipitating agent N,N-naphthaloylhydroxylamine (NHA).

INTRODUCTION

Brown-rot decay is the meet destructive and costly form of decay of wood in service. The decay mechanism can best be characterized by diffusion of low molecular weight (LMW) agents into the wood cell wall causing extensive oxidative depolymerization of polymeric polysaccharides accompanied by measurable strength loss of wood prior to weight loss (Green and Highley, 1995; Winandy and Morrell, 1993). Brown-rot fungi normally colonize softwoods via rays and axial resin canals and from there, develop via penetration of window pit membranes into axial tracheids (Daniel, 1994). The precise mechanism by which brown-rot fungi initiate and sustain this biochemical alteration remains a mystery.

Production of oxalic acid has been described as one key to the mechanism of brown-rot decay (Bech-Anderson, 1987; Green et al ., 1991), simply because it appears to be involved in many chemical processes simultaneously. First, oxalate is reported to be integrally involved, directly or indirectly, in formation of hydroxy radicals from H_2O_2 and iron. Oxalate acts as a reducing agent for conversion of Fe+++ to Fe++ required for the Fenton chemistry which depolymerizes polysaccharides (Backa et al ., 1992; Schmidt et al., 1981).

Second, oxalic acid is a moderately strong iron chelator and the only chelator found universally in brown-rot fungi (Hyde and Wood, 1995). Viikari and Ritschkoff (1992) prevented brown-rot decay with both an organic (EDTA) and inorganic (tripolyphosphate) iron chelator. Third, oxalic acid has also been implicated in pH reduction and direct acid catalyzed hydrolysis of the wood substrate especially hemicellulose (Espejo and Agosin, 1991; Green et al ., 1991 and Shimada et al ., 1994). Fourth, oxalic acid is involved in chelation of other cations, i.e. Ca++, especially from the calcium pectate in pit membranes, compound middle lamellae (CML), and ray cells (Beck Anderson, 1987, 1993; Evans et al ., 1994 and Green et al ., 1995a & b) and metal complexation of zinc (Wang et al ., 1992). Production of oxalate would therefore enable the fungi to weaken the wood structure, thus increasing the pore size to permit penetration by lignocellulolytic enzymes (Dutton et al ., 1993). Calcium oxalate frequently forms crystals which can be readily visualized by scanning electron microscopy in fungi, wood, and Boil which may serve to sequester and detoxify excess calcium (Dutton et al ., 1993; Graustein et al ., 1977; Hintikka et al ., 1979; Conolly and Jellison, 1995).

The least understood of the above observations is the role of oxalic acid in calcium chelation and the formation of calcium oxalate crystals. Brown-rot fungi may conserve the functions of oxalic acid shown to predominate among plant pathogenic fungi, i.e. depolymerization of pectin by (a) direct disintegration of pectic substances, (b) by synergistic action with polygalacturonase activity (Bateman and Beer, 1965; Punja and Jenkins, 1984; Tanaka and Nonaka, 1981) (c) by lowering pH and chelating calcium from calcium pectate (Magro et al ., 1984). Pectin has been shown to induce polygalacturonase and oxalic acid in brown-rot fungi grown in vitro (Green et al ., 1994; Green et al ., 1995). In addition to providing a calcium sink for wood derived calcium, calcium oxalate may form from soil translocated calcium *in* order to provide regulation of pH during decay (Bech-Anderson, 1987; Connolly and Jellison, 1994).

We have been investigating the applicability of using Ca++ precipitating agents to inhibit fungal degradation of wood. One such water soluble compound, N,N-naphthaloylhydroxylamine (NHA), first used by Zeichmeister (1979) for ultrastructural localization of Ca++, has been shown to form an extraordinarily insoluble precipitate with Ca++ (Slocum and Wang, 1982). The present paper reports on the capacity of NHA to prevent weight loss of Southern pine in ASTM (1991) soil block tests for fungal inhibition.

MATERIALS AND METHODS

Test organisms. All fungal isolates were maintained on 2% malt-extract agar tubes at 4°C for the duration of the study. Four brown-rot fungi, postia placenta (Fr.) Lars. et Lomb. (MAD-698/ME20), Gloeophyllum trabeum (Pers.: Fr.) Murr. (MAD-617), Meruliporia incrassata (Berk. & Curt.) Murr. (MAD-563) and Fomitopsis palustris (Berk. and Curt.; Gilbn. and Ryv,) (L-15755), and one white-rot fungi, Trametes versicolor (L.: Fr.) Pil. (MAD-697). The eastern subterranean termite, Reticulitermes flavipes (Kollar), was harvested from Janesville, WI.

Calcium binding compound. The following water soluble calcium binding compound N,N-naphthaloylhydroxylamine (NHA), was examined for its ability to inhibit wood-decay fungi in vitro and in situ (Sigma, St. Louis), at 0.5%, 0.1%, and 1.0%.

Funual decay and termite teats. The soil-block decay test and the subterranean termite test were conducted, with some modifications in accordance with ASTM Standards D1413-76 and D3345-74(2). Test blocks (1.9 cm cubes) were cut from Southern yellow pine (Pinus spp.) sapwood. Four replicates (blocks) per test board were used in each test. Feeder strips of Southern pine were used in the decay chambers. Supplementary food source (paper pulp) were included with wood blocks in termite chambers. In leachability soil-block tests standard AWPA E11-87 methods were followed.

ICP spectroscopy. Total nitrogen and total inorganic constituents were estimated by inductive coupled plasma spectroscopy at the UWEX Soil and Plant Analysis Lab, Madison, WI.

RESULTS

Calcium is the most abundant element in sound blocks of southern pine (Table 1) and black spruce (Bailey and Reeve, 1994). During decay by the brown-rot fungus Postia placenta (MAD 698) calcium content goes up 2-3 fold in 4 weeks.

Table 1. Elemental Analysis of Sound and Brown-rotted Southern Pine

SAMPLE*	% Total N	PPM P	K	CA	MG	S	FE	MN
CONTROL	.03/.03	18/20	276/245	548/583	133/137	27/33	1/1	120/129
MAD-698	.17/.13	65/97	178/180	1860/1612	573/514	128/136	106/62	276/257
ME-20	.11/.08	17/22	80/62	1076/1162	411/383	84/80	1/1	180/181

* - Duplicate wood samples

Growth inhibition of decay, stain and mold fungi on 2% agar plates was tested with three concentrations of NHA.

Table 2. Percent reduction in radial growth of fungi on NHA/malt agar

FUNGUS	NHA CONCENTRATION: 0.001%	0.01%	0.1%
P. placenta MAD698	72%	100%	100%
M. incrassata MAD563	74%	100%	100%
G. trabeum MAD617	18%	66%	100%
T. versicolor MAD-697	6%	59%	100%
C. minor C188	70%	80%	100%
Trichoderma sp. P71H	0%	6%	100%

These results suggest that NHA is more inhibitory to brown-rot fungi, previously shown to be oxalic acid accumulators (MAD-698 & MAD-563), and less inhibitory to molds like Trichoderma except at the highest concentration (0.1%) where all fungi were uniformly inhibited.

Growth inhibition of sapstain fungi and mold fungi on wood (Southern pine and maple) pretreated with three concentrations of NHA. No inhibition was detected on wood except for c . minor at 0.5 and 1.0% NHA. These results appear to exclude broad spectrum toxic inhibition of fungal growth (Table 3).

Table 3. Growth of fungi on NHA treated wood blocks

ORGANISM	UNTREATED		0.1%		0.5%		1.0%	
	SYP	MAPLE	SYP	MAPLE	SYP	MAPLE	SYP	MAPLE
C-188 Ceratocystis minor	4[a]	4	4	4	4	3	4	3
RWD-9472B Ceratocystis pilifera	4	4	4	4	4	4	4	4
MDX-18 Aureobasidium pullulans	4	4	4	4	4	4	4	4
P71H Trichoderma sp.	4	4	4	4	4	4	4	4
Aspergillus niger	4	4	4	4	4	4	4	4
Penicillium sp.	4	4	4	4	4	4	4	4

[a]Growth Scale:

1. Clean (<5%) 2. Light (5-20%) 3. Moderate (20-40%) 4. Heavy (>40%)

Decay resistance was demonstrated in a six week ASTM D 1413-76 soil block exposure test of Southern pine wood blocks (48 test/16 control blocks) to three brown-rot fungi and one white-rot fungus (1 June-13 July 1995). Wood weight loss from wood decay fungi was less than or equal to 1.6% when treated with 0.5% NHA, while the mean weight loss in the untreated controls was 49%. Tenth percent aqueous NHA is ineffective, indicating the minimal inhibitory concentration (MIC) is between 0.1 and 0.5% NHA (see Table 4).

Table 4. Weight loss of Southern pine (AWPA Soil Block Test)

NHA LEVEL	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%	54.6 ± 2.1	49.1 ± 3.1	32.2 ± 14.0	33.2 ± 4.9
0.1%	37.2 ± 7.4	46.8 ± 6.4	18.6 ± 17.2	10.9 ± 11.2
0.5%	zero	1.6 ± 1.4	zero	zero

[1] Mean and standard deviation of four replicates

Similar wood blocks were tested to determine the leachability of NHA using the standard method (AWPA E1l-87) (Table 5). This comparison of leached samples should be helpful in differentiating the indirect effects of calcium precipitation in wood from direct toxic effects of residual, unbound chemicals on the fungi.

Table 5. Mean percentage weight loss of NHA treated pine wood (leached and unleached) blocks in soil block tests.

NHA LEVEL	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	53.6 ± 0.9	57.8 ± 4.5	49.0 ± 1.8	45.6 ± 5.1
0.5%	1.5 ± 0.2	23.2 ± 1.8	15.9 ± 7.0	15.4 ± 2.5
0.5% leached	8.7 ± 9.8	20.4 ± 6.6	14.6 ± 8.6	15.5 ± 4.3
1.0%	1.4 ± 0.1	10.0 ± 4.8	3.6 ± 1.3	2.1 ± 0.4
1.0% leached	3.3 ± 2.6	5.4 ± 2.7	1.4 ± 0.1	1.9 ± 0.9
2.0%	1.5 ± 0.2	2.7 ± 0.6	1.6 ± 0.1	1.8 ± 0.2
2.0% leached	1.1 ± 0.2	3.0 ± 0.6	1.2 ± 0.3	0.8 ± 0.1

[1] Mean and standard deviation of four replicates

These results indicate greater weight loss at 0.5% than table 4. With the exception of MAD-563 (at 0.5%), threr is virtually no difference in weight losses between leached and unleached wood blocks suggesting either fixation or precipitation of NHA to wood.

Table 6. Mean percentage weight loss and termite mortality for Reticulitermes falvipes in pine sapwood blocks vacuum treated with NHA or CCA.

TREATMENT	Percent weight loss of wood block[1]	Attack rating of wood block	Percent survival of termites[1]
Untreated	18.4 ± 0.6	heavy	100.6 ± 2.2
0.05% NHA	14.0 ± 1.7	heavy	100.4 ± 3.6
0.1% NHA	1.8 ± 0.0	light	60.9 ± 19.6
0.5% NHA	1.6 ± 0.3	none	0.0 ± 0.0
1.0% NHA	1.5 ± 0.0	none	21.4 ± 17.8
0.4 pcf CCA	1.3 ± 0.3	light	78.2 ± 6.3

[1] Mean and standard deviation of four replicates

Decay resistance of Southern pine sticks (15 cm X 2.0 cm X 0.3 cm) treated with 1% aqeuous N'N-naphthaloylhydroxylamine (NHA) was compared to untreated sticks in a modified ASTM soilblock test. Results are shown in Figure 1.

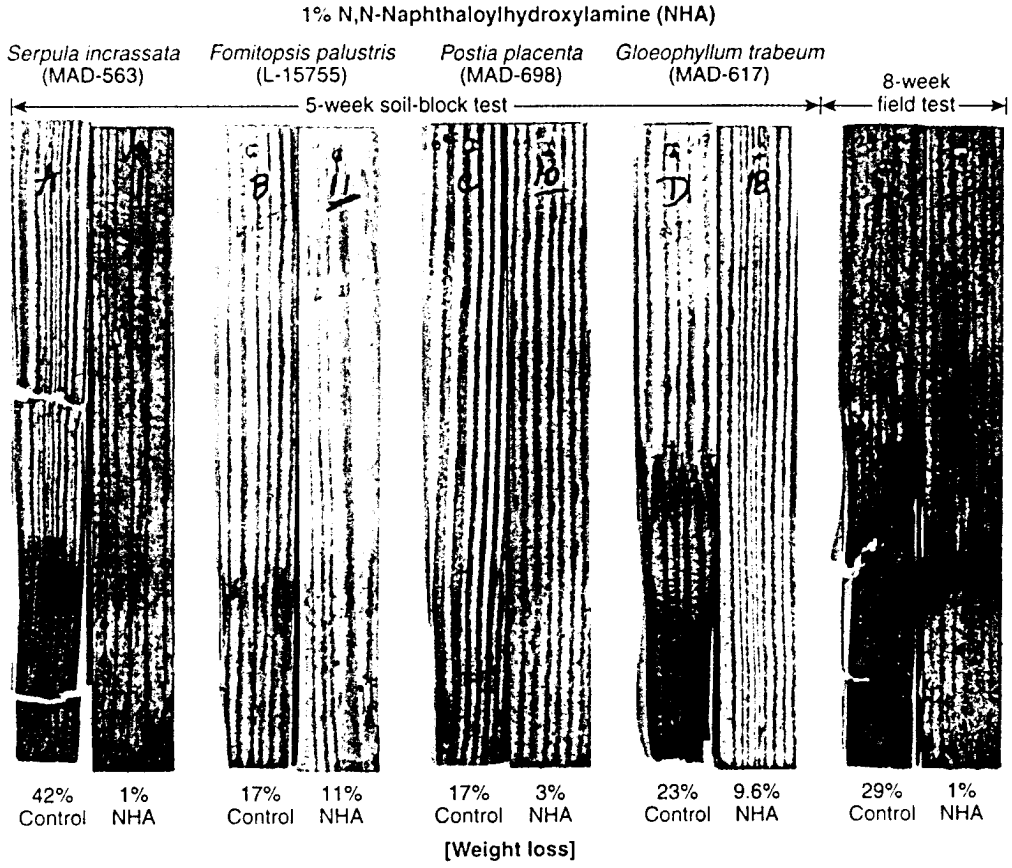

Figure 1. Comparison of brown-rot decay of Southern pine sticks treated with and without 1% NHA.

Discussion

The primary location of calcium in wood is associated with pectin which is found in the tori of pit membranes, the middle lamella, cell corners and the ray cell parenchyma (Militz, 1993 a & b; Murmanis and Chudnoff, 1979; Bailey and Reeve, 1994; and Tschernitz and Sachs, 1973). The effects of treating conifer wood with commercial pectinases to improve penetration of preservatives has been thoroughly studied. Tschernitz (1973) demonstrated that the penetration of sapwood of Douglas fir increased following commercial pectinase treatment, as long as treatment was combined with either low pH or a calcium chelator, such as ammonium oxalate or sodium hexametaphosphate. The use of chelating agents to remove tissue calcium and thereby increase the volubility of pectic materials has long been practiced; ammonium oxalate was one of the first chelators and in later years EDTA, CDTA, and sodium hexametaphosphate were used (Van Buren, 1990).

Calcium is considered an important cross-linking agent in regulating plant cell wall hydrolysis (Rihouey et al ., 1995). The calcium ions within pectin are intercalated between the polygalacturoic chains in an "egg-box" system, binding to carboxyl groups between opposing chains (Liners et al ., 1989). Pectin is a good chelator of Ca++ and acts as a selective binder for Ca++ ions in undignified tissues (Bailey and Reeve, 1994). In parenchymatous plant tissue, the middle lamella is thought to consist principally of the calcium salts of pectic substances. Cell wall separation can be effected with calcium-chelating agents or pectolytic enzymes. Extraction with chelating agents [ammonium oxalate, sodium hexametaphosphate, ethylene diamine tetraacetate (EDTA), cyclohexane diamine tetraacetate (CDTA)] generally yields pectins with a relative high degree of methylation. Removal of Ca++ ions is critical for hydrolysis of pectic acid by polygalacturonase, since these enzymes split glycosidic linkages adjacent to free carboxyl groups (Voragen et al., 1995).

Whewellite and weddellite crystals, the mono- and dihydrate, respectively of calcium oxalate, may have a large effect on the biological and biochemical processes of wood decay because (i) the crystals are a reactive reservoir of calcium (ii) even small amounts of oxalate in solution increase the volubility of iron and aluminum; (iii) oxalate effects the pH of a solution by being both the anion of a weak acid and chelator of iron and aluminum (Graustein et al., 1977).

The most popular calcium-trapping agents used in ultrahistochemistry are oxalate and pyroantimonate anions (Sobota et al ., 1987). However oxalate has a high selectivity but low sensitivity for calcium (Caswell, 1979). Pyroantimonate also forms insoluble electron-dense products with the sodium and magnesium ions (Sobota et al ., 1987). For these reasons, both oxalate and pyroantimonate have been compared to N,N-naphthhaloylhydroxylamine (NHA). NHA is a water soluble heterocyclic calcium capture agent (M.W. 235) used initially for quantitative determination of the calcium content in serum (Beck, 1951) before being used to demonstrate cellular calcium in electron microcopy (Zeichmeister, 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. The NHA procedure for immobilization and visualization of cellular calcium is much simpler, possesses high sensitivity and selectivity, and is much simpler than

conventionally used pyroantimonate technique (Sobota et al ., 1988; Slocum and Wang, 1982).

The discovery of endo-polygalacturonase in brown-rot fungi and the likely synergistic role played by oxalic acid in hydrolyzing the membranes of bordered pits raised the issue of whether brown-rot fungi increased the permeability of wood during incipient decay (Green et al ., 1995a; Clausen and Green, submitted). Preliminary experiments demonstrated that the permeability of Douglas fir cores increased to maximum during colonization and decay by Postia placenta 698 (Green et al ., 1995a). However, P . placenta ME20, a nondecay isolate unable to accumulate oxalate or effect weight loss of wood was also unable to hydrolyze the pit membranes underscoring the important role of oxalic acid in calcium chelation and penetration of pit membranes during incipient decay. Logically, this suggested the possibility that precipitation of calcium in situ might prevent decay by i) preventing chelation of calcium from pectins by oxalate, ii) preventing hydrolysis of membranes of bordered pits, iii) preventing colonization of wood blocks by means of pit apertures, and iv) preventing brown-rot wood decay and weight loss of wood. Preliminary experiments with NHA have supported this theory, but further experiments are warranted to confirm the mechanism of inhibition since some direct toxicity of NHA is detectable in agar media against decay fungi.

Results of the NHA screening test for termite resistance showed nearly complete resistance to attack and evidence for termiticidal activity. Termite survival at 0.5% NHA was zero at 3 weeks. Higher survival at 1% NHA may be due to reduced feeding, and indirect death from starvation. Visual block rating showed that NHA was slightly superior to CCA (6.4 kg/m^3). Due to significant chewing and tunnelling of CCA blocks, termiticidal activity appeared to be related to "feeding deterrence", even for the adjunct cellulosic bait included with each test, possibly due to normal flora disruption at high pH (9.5) of NHA. Further testing of NHA in leached blocks and in field tests may shed further light on the mechanism of protection.

Many physiological processes in eukaryotic organisms are under the control of calcium, such as enzyme secretion, metabolic regulation, and cytoplasmic transport (Highley, 1990). Often calcium mediates cellular processes through the binding to specific proteins that serve as receptors for calcium. Of the calcium binding proteins, calmodulin is the most widely distributed. Calmodulin antagonists bind with high affinity to calmodulin and thereby inhibit calmodulin-dependent enzymes. T.W. Hill and R.G. Waggener (1984) reported that the calmodulin antagonists, chloropromazine and trifluoperazine, blocked secretion of B-1,4-endoglucanase by Trichoderma reesei, indicating that calmodulin may function as a regulatory agent in some critical stage in enzyme eecretion.

The future goal of this research will be to determine the mechanism by which the calcium trapping agent NHA inhibits fungal decay in wood. This will determine whether the inhibitory effects are directly toxic on the fungi or if indirect effects of calcium precipitation in wood prevent depolymerization and subsequent decay. Also, F . palustris and G . trabeum are able to decolonize NHA and partially circumvent this inhibition in modified soil block tests (Fig. 1). In preliminary tests, NHA did not inhibit mold and sapstain fungi on wood,

suggesting that direct toxic effects do not apply to all types of fungi, but may interfere only with the brown-rot/white-rot decay mechanism via calcium binding and interference with calcium cycling. NHA has been observed binding to pit tori by scanning EM (unpublished results). Our long term goal is to develop new and more specific approaches to preventing and controlling decay by brown-rot fungi. Because present wood preservatives pose a threat to the environment during treatment and disposal, there is an urgent need for new, sharply targeted, and benign wood preserving methods. In concert, these experiments may develop important information on new substrate targets in wood for potential inhibition of wood decay and how chemicals precipitate out and resist leeching. Treating wood with agents targeted specifically to the mechanism of degradation should provide environmentally friendly protection of wood.

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