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Protection of Southern Pine from Fungal Decay and Termite Damage with *N,N*-Naphthaloylhydroxylamine

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The design of environmentally benign methods for preserving wood in service requires an understanding of the precise sequence of the biochemical events that occur as wood is colonized. We hypothesize that *in-situ* precipitation of existing calcium ions in association with pectin in wood may prevent the cascade of biochemical events involved in fungal colonization. Preliminary experiments showed that pretreatment of wood blocks with the selective water-soluble calcium-precipitating agent *N,N* -naphthaloylhydroxylamine (NHA) inhibited decay caused by brown-rot and white-rot fungi as well as damage caused by eastern subterranean termites. Published by Elsevier Science Limited

INTRODUCTION

Recent environmental restrictions, both US and international, are limiting the use of broad-spectrum biocides for wood preservation, primarily due to problems with disposal. There is an urgent need for new, sharply targeted, and benign wood-preserving methods. Brown-rot decay is the most destructive and costly form of decay of wood in service, resulting in the loss of billions of US dollars annually. Brown-rot fungi normally colonize softwoods via rays and resin canals, and from there enter tracheids via window pit membranes (Daniel *et al.,* 1996). The decay mechanism can be best characterized by diffusion of low molecular weight agents into the wood cell wall, which causes extensive oxidative depolymerization of polymeric polysaccharides and is accompanied by measurable strength loss of wood prior to weight loss (Green & Highley, 1995; Winandy & Morrell, 1993). The precise mechanism by which brown-rot fungi initiate and sustain this biochemical alteration remains a mystery.

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Production of oxalic acid has been described as one possible key to the mechanism of brown-rot decay (Bech-Andersen, 1987; Green *et al.,* 1991), simply because it appears to be involved in many chemical processes simultaneously. Oxalate is reported to be integrally involved, directly or indirectly, in the formation of hydroxy radicals from hydrogen peroxide (H_2O_2 and iron. Oxalate may act as a reducing agent for the conversion of Fe^{3+} to Fe^{2+} required for the Fenton chemistry that depolymerizes polysaccharides (Schmidt *et al.,* 1981; Backa *et al.,* 1992; Suttie *et al.,* 1996). Oxalic acid is a moderately strong iron chelator and the only chelator found universally in brown-rot fungi (Hyde & Wood, 1995). Viikari & Ritschkoff (1992) prevented brown-rot decay with both an organic (ethylene diamine tetraacetate) and inorganic (tripolyphosphate) iron chelator. Oxalic acid has also been implicated in pH reduction and direct acid catalyzed hydrolysis of the wood substrate, especially hemicellulose (Espejo & Agosin, 1991; Green *et al.,* 1991; Shimada *et al.,* 1994). Oxalic acid is also involved in chelation of other cations, i.e. Ca^{2+}, especially from the calcium pectate in pit membranes, compound middle lamellae, and ray cells (Beth-Andersen, 1987; Beth-Andersen *et al.,* 1993; Evans *et al.,* 1994; Green *et al.,* 1995a,b) and the metal complexation of zinc (Wang *et al.,* 1992).

The production of oxalate therefore would 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 in fungi, wood, and soil that can be readily visualized by scanning electron microscopy. The formation of these may serve to sequester and detoxify excess calcium (Graustein *et al.,* 1977; Hintikka *et al.,* 1979; Dutton *et al.,* 1993; Connolly & Jellison, 1995).

The least understood of these 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) synergistic action with polygalacturonase activity (Bateman & Beer, 1965; Tanaka & Nonaka, 1981; Punja & Jenkins, 1984; Amadioha, 1993) and (c) lowering of pH and chelation of 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, 1995). In addition to providing a calcium sink for wood-derived calcium, calcium oxalate may form from soil-translocated calcium to neutralize acidic conditions harmful to fungal hyphae (Bech-Andersen, 1987; Connolly & Jellison, 1994).

We have been investigating the applicability of using Ca^{2+} precipitating agents to inhibit fungal degradation of wood. One such water-soluble compound, *N,N*-naphthaloylhydroxy lamine (NHA), first used by Beck (1951) for determination of serum calcium, has been shown to form an extraordinarily insoluble precipitate with Ca^{2+} (Slocum & Wang, 1982; Zeichmeister, 1979). Here, we report on the capacity of NHA to prevent fungal growth on agar *in vitro,* weight loss of Southern Pine in ASTM (1993) soil-block tests for fungal inhibition, and termite damage in treated pine.

MATERIALS AND METHODS

Test organisms

The test organisms were four brown-rot fungi, *Postia placenta* (Fr.) Lars. et Lomb. (MAD-698 and ME-20), *Gloeophyllum trabeum* (Pers.: Fr.) Murr. (MAD-617), *Meruliporia incrassata* (formerly *Serpula incrassata)* (Berk. and Curt.) Murr. (MAD-563), and *Fomitopsis palustris* (Berk. and Curt.; Gilbn. and Ryv) (L-15755); a white-rot fungus, *Trametes versicolor* (L.: Fr.) Pil. (MAD-697); the sapstain fungi *Ophistoma minus* (Hedge) Syd. and P. Syd. (C-188) and *O. piliferum* (Fr:Fr.) Syd. and P. Syd. (RWD-9472B); four representative mold fungi, *Trichoderma* Pers:Fr. sp. (P71H), *Aurebasidium pullulans* (de Bary) G. Arnaud (MDX-18), *Aspergillus niger* Tiegh., and *Penicillium* Link:Fr. sp.; and the eastern subterranean termite *Reticulitermes flavipes* (Kollar). All fungi were maintained on 2% malt-extract agar. The termites were harvested from a natural forest habitat in Janesville, Wisconsin.

Toxicity tests

N,N-naphthaloylhydroxylamine (Sigma, St. Louis)[2] was examined for its ability to inhibit growth of mold, stain, and wood-decay fungi by inclusion in agar plates *in vitro* (0.001%, 0.01%, and 0.1%, w/v) and in Southern Pine *(Pinus* spp.) and maple *(Acer saccharinum)* wood blocks *in situ* (0.1%, 0.5%, and 1.0%, w/v). The pH of a 1% aqueous solution of NHA is 8.3.

Decay and termite resistance tests

Test blocks (1.9-cm cubes) were cut from Southern Pine sapwood. Four replicate blocks per treatment were used in each test.

Soil-block decay tests were conducted in accordance with ASTM Standard D1413-76 (ASTM, 1993), with some modifications. Southern Pine feeder strips were used in the decay chambers. Southern Pine sticks (15 × 2.0 × 0.3 cm) were used to test the efficacy of 1% aqueous NHA in a modified ASTM soil-block test; the sticks were pushed into the soil to a depth of 51–76 mm in a standard soil-block bottle for 5 weeks. One pair of sticks was exposed in the ground outdoors for 8 weeks. For the leachability soil-block tests, AWPA Standard E11-87 (AWPA, 1987) methods were followed.

The subterranean termite test was conducted in accordance with AWPA Standard El-82 (AWPA, 1982), with some modifications. Wood blocks treated with aqueous NHA (0.05%, 0.1%, 0.5%, and 1.0%) were compared to those treated with

[2]The use of trade or firm names in this publication is for reader information and does not imply endorsement by the US Department of Agriculture of any product or service.

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

Sample[b]		Element (ppm)						
	N (%)	P	K	Ca	Mg	S	Fe	Mn
Control	0.03/0.03	18/20	176/245	548/583	133/137	27/33	1/1	120/129
MAD-698	0.17/0.13	65/97	178/180	1860/1612	573/514	128/136	106/62	276/257
ME-20	0.11/0.08	17/22	80/62	1076/1162	411/383	84/80	1/1	180/181

[a]Duplicate samples.
[b]MAD-698 and M-20 are strains of *P. placenta.*

chromated copper arsenate (CCA) at 6.4 kg m^{-3}. A supplementary food source (paper pulp) was included with the wood blocks in the termite chambers.

ICP spectroscopy

Total nitrogen and total inorganic constituents were estimated in duplicate by inductive coupled plasma (ICP) spectroscopy at the Soil and Plant Analysis Laboratory of the University of Wisconsin-Extension (Madison, WI) to determine changes and the suitability of calcium as a target element during brown-rot decay by *P. placenta.*

Electron microscopy

For scanning electron microscopy (SEM), 1.3-cm cores of Southern Pine were soaked in a 1% solution of NHA overnight (under vacuum), then rinsed in distilled water and air-dried. The treated blocks and control blocks of southern pine were split and mounted on to aluminum stubs, gold-coated using a Denton Desk-1 sputter coater, and examined in a JEOL 840 SEM at 15 kV.

RESULTS

Elemental analysis of sound and brown-rotted Southern Pine is shown in Table 1. During decay by the brown-rot fungus *P. placenta* MAD-698, the content of calcium and nearly all elements increased approximately two- to threefold in 4 weeks. *Postia placenta* MAD ME-20, a low decay senescent isolate, did not accumulate iron, whereas strain MAD-698 caused a 100-fold increase in iron.

Growth inhibition of decay, stain, and mold fungi on 2% agar plates was tested by incorporating three concentrations of NHA (Table 2). The results suggest that at the 0.001% concentration, NHA is more inhibitory to certain brown-rot fungi, especially oxalic acid accumulators *(P. placenta* MAD-698, *M. incrassata)* and blue-stain fungi *(O. minus)* and less inhibitory to white-rot fungi *(T. versicolor)* or molds like *Trichoderma.* At the 0.01% concentration, NHA exerted the same influence on the brown-rot fungus *G. trabeum* and the white-rot fungus *T. versicolor.* At the highest concentration (0.1%), NHA inhibited the growth of all fungi uniformly.

Table 2. Growth of Fungi on NHA/Malt Agar

Fungus	Reduction in radial growth (%)		
	0.001% NHA	0.01% NHA	0.1% NHA
P. placenta MAD-698	72	100	100
M. incrassata MAD-563	74	100	100
G. trabeum MAD-617	18	66	100
T. versicolor MAD-697	6	59	100
Ophiostoma minus C-188	70	80	100
Trichoderma sp. P71H	0	6	100

Growth inhibition of sapstain and mold fungi on wood (Southern Pine and maple) was estimated by pretreatment of the wood with three concentrations of NHA (Table 3). NHA inhibition was observed only for *O. minus* C-188 on maple treated with 0.5% and 1.0% NHA. These results appear to exclude broad-spectrum toxic inhibition of fungal growth.

Decay resistance was demonstrated in a 12-week ASTM D 1413-76 soil-block test of Southern Pine wood blocks (96 test/16 control blocks) using three brown-rot fungi and one white-rot fungus (Table 4). Wood weight loss from *G. trabeum* was $\leqslant$ 2.7% for wood treated with 2.0% NHA compared to 50% mean weight loss for the controls. The 1.0% NHA concentration was relatively ineffective for *G. trabeum,* indicating that the minimal inhibitory concentration (MIC) for decay fungi overall is between 1.0% and 2.0% NHA.

Table 3. Growth of Sapstain and Mold Fungi on NHA-treated Wood Blocks[a]

Fungus	Untreated		0.1% NHA		0.5% NHA		1.0% NHA	
	SP	Maple	SP	Maple	SP	Maple	SP	Maple
O. minus C-188	4	4	4	4	4	3	4	3
O. piliferum RWD-9472B	4	4	4	4	4	4	4	4
Aureobasidium pullulans[b]	4	4	4	4	4	4	4	4
Trichoderma sp. P71H	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) none (< 5%), (2) light (5–20%), (3) moderate (20–40%), (4) heavy (> 40%). SP, Southern Pine.
[b]MDX-18 strain.

Table 4. Effects of NHA on Leached and Unleached Pine in Soil-block Tests

Treatment	Weight loss caused by fungal attack (%)[a]			
	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% NHA	1.5±0.2	23.2±1.8	15.9±7.0	15.4±2.5
0.5% NHA leached	8.7±9.8	20.4±6.6	14.6±8.6	15.5±4.3
1.0% NHA	1.4±0.1	10.0±4.8	3.6±1.3	2.1±0.4
1.0% NHA leached	3.3±2.6	5.4±2.7	1.4±0.1	1.9±0.9
2.0% NHA	1.5±0.2	2.7±0.6	1.6±0.1	1.8±0.2
2.0% NHA leached	1.1±0.2	3.0±0.6	1.2±0.3	0.8±0.1

[a]Mean and standard deviation of four replicates.

Simultaneously, wood blocks were tested to determine the leachability of NHA in H_2O using standard method AWPA E11-87 (AWPA, 1987) (Table 4). This comparison of leached and unleached samples should be helpful in differentiating the indirect effects of calcium precipitation in wood from any toxic effects of residual, unbound chemicals. With the exception of wood exposed to *P. placenta* MAD-563 (at 0.5% NHA), there was little difference in weight loss between leached and unleached wood blocks, which suggests either fixation or precipitation of excess NHA into the wood.

The results of a modified soil-block stick test are shown in Fig. 1. Although all NHA-treated sticks showed less weight loss than did matched controls, the severity of this test is shown by the decay weight loss by *F. palustris* and *G. trabeum* specimens.

The 0.1%, 0.5% and 1.0% concentrations of NHA were effective at inhibiting termite damage (Table 5). For 0.5% NHA, termite mortality was 100% at 3 weeks, even with a favored supplementary food source. It should be noted that the food supplement came into contact with NHA from the wood blocks through both diffusion and termite tracking.

SEM of treated Southern Pine cores revealed the precipitation of cuboidal deposits of NHA on the torus of bordered pits (Fig. 2).

DISCUSSION

Calcium is the most abundant element in sound blocks of Southern Pine (Table 1) and black spruce *(Picea mariana* (Mill.) B. S. P.) (Bailey & Reeve, 1994). The primary location of calcium in Southern Pine is associated with pectin, which is found in pit tori, the middle lamella, cell corners, ray cell parenchyma, and resin canal parenchyma cells (Tschernitz & Sachs, 1973; Murmanis & Chudnoff, 1979; Militz, 1993a, b; Bailey & Reeve, 1994; Daniel *et al.*, 1996). The effects of treating conifer wood with commercial pectinases to improve penetration of preservatives has been thoroughly studied. Tschernitz (1973) demonstrated that commercial pectinase treatment improved preservative penetration of Douglas-fir sapwood by opening pit apertures, 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 practised. Ammonium oxalate was one of the first

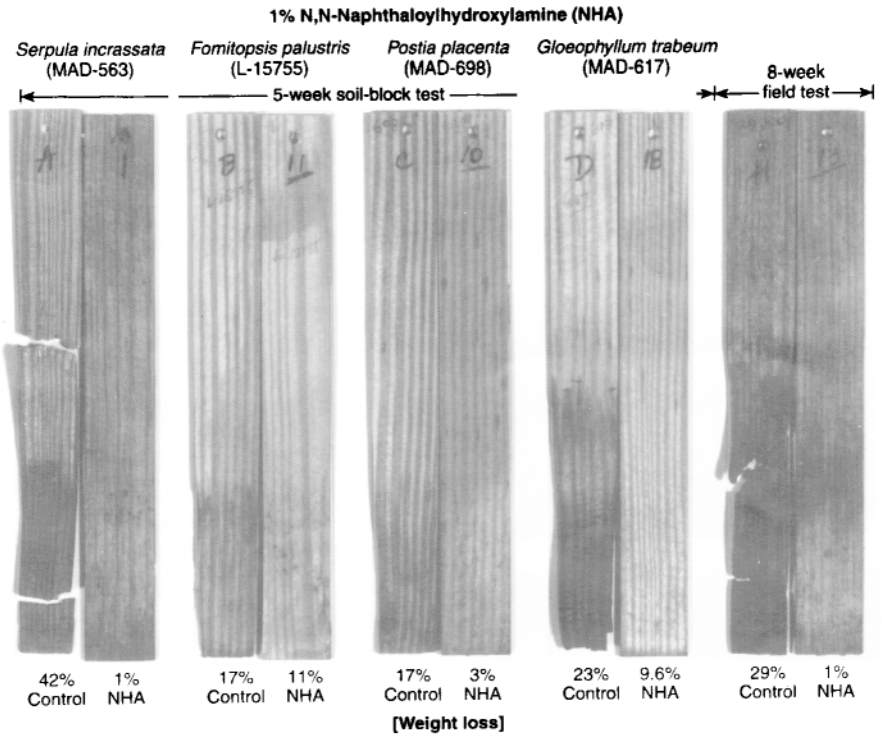

Fig. 1. Comparison of brown-rot decay of untreated and NHA-treated Southern Pine sticks.

Table 5. Results of Termite Tests on Pine Sapwood Vacuum-treated with NHA or CCA[a]

Treatment	Wood weight loss (%)	Attack rating[b]	Termite survival (%)
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
6.4 kg m^{-3} CCA	1.3±0.3	Light	78.2±6.3

[a]Mean and standard deviation of four replicates.
[b]Heavy, tunneling throughout springwood, holes; medium, 50% tunneling, no holes; light, surface nibbling; none, no feeding.

chelators studied, followed by ethylene diamine tetraacetate (EDTA), cyclohexane diamine tetraacetate (CDTA), and sodium hexametaphosphate (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 polygalacturonic chains in an 'egg-box' system, binding to carboxyl groups between opposing chains (Liners *et al.*, 1989). Pectin is a good chelator of Ca^{2+} and acts as a selective binder for Ca^{2+} ions in undignified tissues (Bailey & 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, such as ammonium oxalate, sodium hexametaphosphate, EDTA, and CDTA, generally yields pectins with a relative high degree of methylation. Removal of Ca^{2+} 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 respective mono- and dihydrate forms of calcium oxalate, may have a great effect on the biological and biochemical processes of wood decay for three reasons: (1) the crystals are a reactive reservoir of calcium (Arnott & Webb, 1983); (2) even small amounts of oxalate in solution increase the solubility of iron and aluminum; and (3) oxalate affects the pH of a solution by being both the anion of a weak acid and a chelator of iron and aluminum (Graustein *et al.*, 1977). Both forms of calcium oxalate crystals have been observed in brown-rotted wood (Green *et al.*, 1997).

The most popular calcium-trapping agents used in ultrahistochemistry are oxalate and pyroantimonate anions (Sobota *et al.*, 1987). Oxalate has high selectivity but low sensitivity for calcium (Caswell, 1979). Pyroantimonate also forms insoluble electron-dense products with sodium and

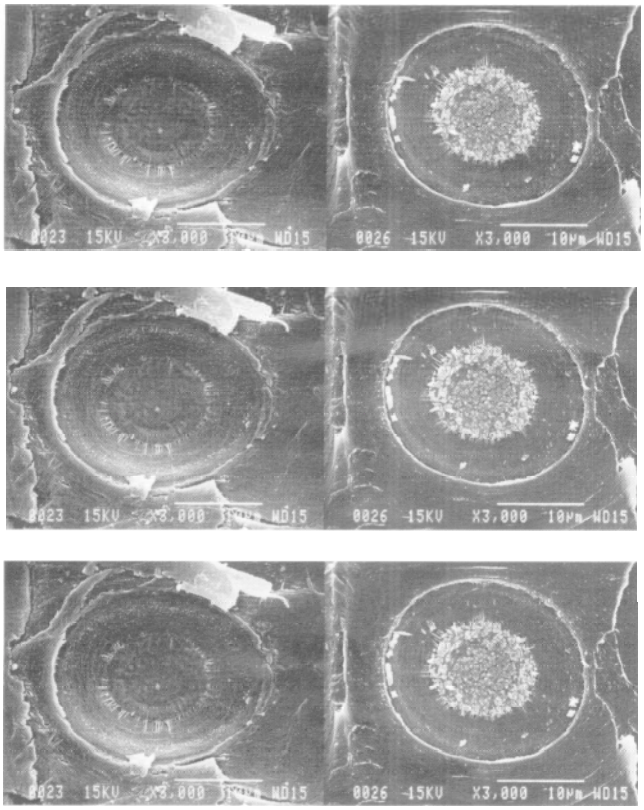

Fig. 2. Scanning electron micrographs of bordered pits of untreated (left) and pretreated (1% NHA, right) Southern Pine.

magnesium ions (Sobota *et al.,* 1987). For these reasons, both oxalate and pyroantimonate have been compared to NHA, a water-soluble heterocyclic calcium-trapping agent with a molecular weight of 235. Initially, NHA was used for quantitative determination of the calcium content in serum (Beck, 1951). It was then used by Voight (1957) to demonstrate calcium for light microscopy and by Zeichmeister (1979) to demonstrate cellular calcium in electron microscopy. NHA forms a very stable and very selective complex with Ca^{2+}, which is insoluble in 100-mM ethylene glycol tetracetic acid (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 possesses high sensitivity and selectivity and is much simpler than the conventionally used pyroantimonate technique (Slocum & Wang, 1982; Sobota *et al.,* 1988).

In our study, the precipitation of NHA on to the torus of pit membranes (Fig. 2) partially confirms the mechanism of NHA action to be calcium precipitation on to pectin-rich wood structures. Calcium oxalate crystals were also observed to plug pit apertures when wood blocks were exposed to 1% ammonium oxalate (Militz & Homan, 1993). Thus, NHA may prevent pectin hydrolysis by blocking access of fungal endopolygalacturonase.

The discovery of endopolygalacturonase in brown-rot fungi and the likely synergistic role played by oxalic acid in hydrolyzing the pectin in membranes of bordered pits raised the issue of whether brown-rot fungi increase the permeability of wood during incipient decay (Green *et al.,* 1995b; Clausen & Green, 1996). Preliminary experiments demonstrated that the permeability of Douglas-fir cores increased to maximum during colonization and decay by *P. placenta* MAD-698 (Green *et al.,* 1995b). However, *P. placenta* ME-20, a nondecay isolate unable to accumulate oxalate or effect weight loss of wood, was unable to hydrolyze the pit membranes, thus underscoring the important role of oxalic acid in calcium chelation and penetration of pit membranes during incipient decay. This suggested the possibility that precipitation of calcium *in situ* might prevent decay by preventing (a) chelation of calcium from pectins by oxalate, (b) hydrolysis of membranes of bordered and simple pits, (c) colonization of wood blocks by means of ray parenchyma pit apertures, and (d) brown-rot wood decay and weight loss of wood (Bailey & Reeve, 1994; Saka & Mimori, 1994). 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.

One alternative hypothesis to explain the inhibition of fungal decay is that 1% NHA (pH 8.3) simply raises 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.

Results of the screening test for termite resistance showed nearly complete resistance of NHA-treated Southern Pine to termite attack, providing evidence for termiticidal activity (Table 5). For 0.5% NHA, termite survival was zero at 3 weeks. The higher survival for 1% NHA may be due to reduced feeding and indirect death from starvation. Visual rating of blocks showed that NHA was slightly superior to CCA (6.4 kg m^{-3} retention). The significant chewing of and tunneling through CCA-treated blocks indicated that termiticidal

activity was apparently related to 'feeding deterrence,' even for the adjunct cellulosic bait included with each test, possibly due to normal hindgut flora disruption at the high pH (8.3) of NHA. All members of lower termites are provided with complex gut flora that consist of bacteria and protozoa (Yoshimura, 1995). 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, including enzyme secretion, metabolic regulation, and cytoplasmic transport (Highley, 1990). Often, calcium mediates cellular processes through binding to specific proteins that serve as receptors. 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. Hill & Waggener (1984) reported that the calmodulin antagonists chlorpromazine and trifluroperazine blocked secretion of β–1,4–endoglucanase by *Trichoderma reesei,* indicating that calmodulin may function as a regulatory agent in some critical stage in enzyme secretion. Highley (1990) showed that several calmodulin antagonists decreased production of extracellular carbohydrate-degrading enzymes in both brown- and white-rot fungi. Calcium precipitation *in situ* by NHA may also inhibit fungal metabolism.

The future goal of this research will be to determine the mechanism by which NHA inhibits fungal decay and termite damage in wood. This will determine whether the inhibitory effects are directly toxic to fungal metabolism or whether indirect effects of calcium precipitation in wood prevent depolymerization and subsequent decay. Also, *F. palustris* and *G. trabeum* were able to decolonize NHA and partially circumvent NHA inhibition in modified soil stick tests (Fig. 1), perhaps by lowering the pH of the wood. Clearly, direct soil contact of treated sticks reduced NHA inhibition. 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.

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 on the mechanism by which chemicals precipitate and resist leeching. Treating wood with agents targeted specifically to the mechanism of degradation should provide more environmentally friendly protection of wood.

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