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Section 1   Biology

Mechanisms of Protection by NHA Against Fungal Decay

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Abstract

Treating wood with the water-borne sodium salt of N'-N-naphthaloylhydroxylamine (Na-NHA) protects wood against decay and termite damage. Initial testing indicated little or no inhibition of sapstain fungi, molds, or soft-rot fungi by Na-NHA, suggesting that the mechanism by which this compound protected wood was complex and not that of a broad-spectrum biocide. Previously, we (Green et al, 1997) suggested that the protective mechanism was due to Na-NHA complexing with calcium ions to disrupt fungal metabolism, and/or binding of Na-NHA to the calcium in pit membranes, which acts as a physical barrier to fungal colonization. More recent experiments suggest an alternate mechanism. Specifically, pKa measurements of Na-NHA show that the sodium salt will be essentially fully protonated when impregnated into SYP which is naturally acidic (pH at or below 5.5). Furthermore, the protonated form (H-NHA) was more fungicidal than Na-NHA against two white- and two brown-rot fungi, with the bioactivity approaching that of several commercial organic fungicides. The moderate fungicidal activity of H-NHA is not surprising; the compound is a polyaromatic hydrocarbon much like many of the compounds in creosote. By precipitating mainly at the pit membranes, H-NHA or Ca-NHA serves as a fungicidal barrier to inhibit fungal hyphae from spreading to adjacent cells through the pits. Studies of H-NHA combined with three commercial organic biocides showed that only one combination was synergistic, which further suggests that NHA protects wood by a biocidal mechanism rather than being simply a compound is extremely water insoluble and will thus not leach from wood in outdoor exposure. NHA may also have metal chelating and/or antioxidant properties which further assists in protecting wood. non-biocidal additive. Once H-NHA is formed this

Introduction

Recent public announcements of the phasing out of CCA for preserving wood for home use, especially decking, in the United States emphasizes the need to develop a new generation of environmentally friendly wood preservatives (USA Today January 6, 2002). Off the shelf replacements for CCA currently include a group of copper-organic mixtures, such as ammoniacal copper quat (ACQ) , copper azole, and copper boron combinations. Although copper is not as toxic as its metallic cousins chromium and arsenate, there is national and international concern for increased accumulations of copper into the environment, especially against aquatic environments (Green and Shultz 2002). Thus, the new "second generation" replacements for CCA just coming onto the market may have only a brief commercial use.

One key to the development of new environmentally benign biocides is a more thorough understanding of the precise mechanisms of fungal colonization and decay in order that key steps may be targeted for inhibition. One such chemical model is N'N-naphthaloylhydroxylamine (Na-NHA;$C_{12}H_6NNaO_3$). Originally evaluated as a selective calcium precipitating agent (Sobota et.al. 1988), Na-NHA was thought to disrupt normal calcium cycling in the fungal hyphae

rendering the agent either fungicidal or fungistatic. Calcium serves to i) balance pH; ii) transport calcium receptor proteins; iii) aid in the synthesis of RNA and DNA in mitochondrial energy supplies; and iv) support calcium levels for calcium pumps acrossed membranes or as a messengers for homeostatic signaling between cells or endoplasmic reticulum. (Symington et al 1999)

In early investigations on Na-NHA (Green et.al. 1997, Green et.al. 1996, Crawford and Green 1999), experiments were limited to inhibition by the water soluble sodium [or basic] form form of NHA. We also observed (Green et.al.1997) that Na-NHA precipitated onto the pit membranes of bordered pits in southern pine. We hypothesized that the Na-NHA was precipitating on the calcium intercalated between the polygalacturonic (pectin) acids, and preventing hydrolysis of pits. This in turn could physically block hyphae from bridging cells through the pit aperture and thus block fungal colonization.

More recent experiments (Green and Shultz 2002) have demonstrated the possibility that simple calcium precipitation is not the only explanation for fungal inhibition and decay prevention by Na-NHA. The acid or protonated form of H-NHA (hydroxynaphthalimide) will not react with Ca++ in the same way that the sodium form of NHA does after preservative treatment but is as effective in protecting wood against fungal attack. The objective of this paper is to examine and discuss alternative mechanisms for the preservative action of NHA in wood.

Materials and Methods

Fungi: *Gloeophyllum trabeum* (Madison 617; ATCC 11539); *Irpex lacteus* (Madison 517; ATCC 11245); *Postia placenta* (fr) Lars.et Lomb (MAD-698, ATCC 11538) ; and *Trametes versicolor* (L.:Fr.) Pil. (MAD-697; ATCC 12679)

Agar Plate tests: The agar-plate testing procedure was similar to earlier descriptions by Archer et al (1995) and Schultz et al (1991). Each biocide/fungus combination was replicated five times on 2% malt agar plates. Fungi were transferred to test plates in 5mm agar plugs. Plates are read when the radial growth of the control plates is within 1cm of the edge of the plate. IC_{50} values were calculated by regression of the relative growth versus the log of the concentration over the liner range of the semi-log plot. The biocides were added after the agar had been autoclaved and cooled to 45^0C.

Results and Discussion

Na-NHA is highly water-soluble at the 2% level or higher forming a red colored salt solution. The pH of a 1% solution in distilled water is 8.2. We measured the pKa of NHA to be at 7.05 +/- 0.01, very close to the previously reported value of 6.8 (Slocum and Wang 1982). At pHs below this pKa the red color disappears indicating formation of H-NHA. When pine or Douglas fir is pressure treated with Na-NHA the centers of the woodblocks are white. This is caused by the natural acidity of the wood (i.e. 5.0-5.5) changing the Na-NHA into the colorless acid form [H-NHA]. Early on this phenomenon was incorrectly interpreted as "shell-treatment" of wood blocks; possibly due to blocking pit penetration of Na-NHA into the interior of the wood block by Ca-NHA. The outer sides of the woodblocks remain reddish orange indicating a higher concentration of Na-NHA in these sites overcoming the woods buffering capacity and remaining in the sodium form. [See figure 1 below]

Na-NHA (Basic)

H+   NaOH   Ca (2+)

NHA (Free Acid) ← Ca-NHA (Calcified)
H+
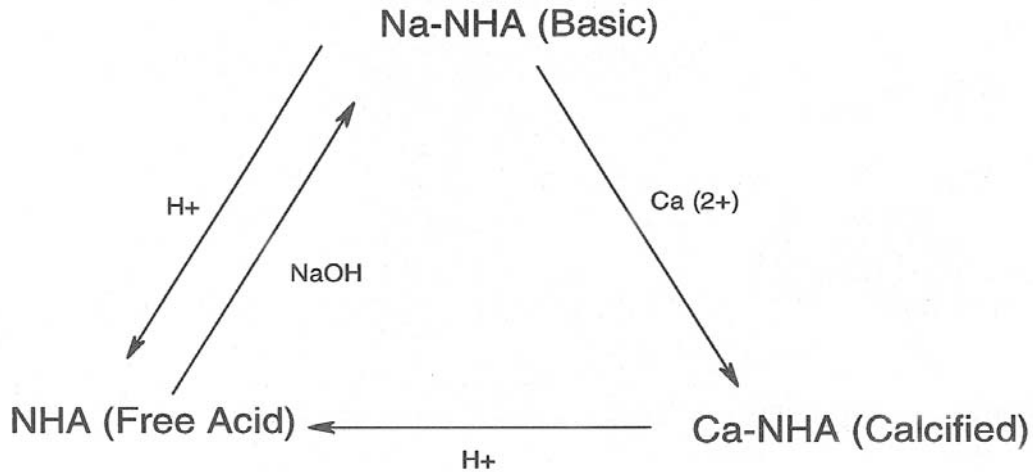
In order to determine the difference in fungicidal activities between Na-NHA and H-NHA both were examined using the agar plate test to determine the IC_{50} (inhibitory concentration required for 50% inhibition of radial growth)(see Table 1). In addition, fungicidal data for two commercial organic biocides and two extractives naturally present in decay-resistant heartwood, are also shown.

Table 1. Agar plate tests—IC_{50} [ppm]

Compound	I. lacteus	T. versicolor	G.trabeum	P.placenta
Na-NHA	>200ppm	58	>200ppm	20
H-NHA	79	33	50	5
DDAC	7	4	2	4
IPBC	4	1	6	1
Reservatol	ND	226	197	ND
DHM & DHK (2:1)	ND	533	197	ND

[No growth of *I .lacteus*, *T. versicolor*, or *P.placenta* was observed at 100ppm H-NHA]

These results indicate that the H-NHA is more biocidal than Na-NHA and comes close to the activity for the two commercial biocides, DDAC and IPBC, for the brown-rot fungus *Postia placenta*. H-NHA is also far more inhibitory than either of two natural heartwood extractives (Reservatol and DHM/DHK).

The key element in the mechanism of NHA is its three forms: basic or water soluble sodium Na-NHA; water insoluble calcium precipitated form Ca-NHA; and the barely water-soluble acid or protonated form H-NHA. These forms are interchangeable based upon the pH of the solution and the presence of calcium ions.

We propose that when wood is treated with Na-NHA, some of it immediately precipitates out as Ca-NHA in pectin rich areas like the compound middle lamellae (CML) and bordered pit membranes. In acidic interior areas, also the pectin rich bordered pits, where the pKa of the solution falls below 6.8-7.0, Na-NHA rapidly becomes protonated and precipitates out as the acidic H-NHA. In surface areas of the wood blocks, heavily penetrated by the treating solution, where the buffering capacity and naturally present wood calcium is overwhelmed, Na-NHA may remain as Na-NHA—but only under laboratory soil-block conditions. In field test conditions, any acid naturally present in the soil and free water, and soil calcium will rapidly convert all remaining Na-NHA to either the calcium precipitated form [Ca-NHA] or the protonated form [H-NHA] where ever rain or groundwater is acidic. Thus, during decay of wood stakes in ground-contact outdoor exposure, invading fungi will likely encounter either the Ca-NHA or H-NHA or both, especially in the bordered pit regions of tracheids of softwoods like southern pine. Acid production by decay fungi, especially oxalic acid production by brown-rot fungi (Clausen et al 2000; and Green and Clausen 2001) may convert the Ca-NHA to H-NHA, thus forming additional biocidal H-NHA. Since only the Na-NHA is the calcium precipitating form of this compound, calcium precipitation or chelation cannot play any significant role in inhibition of fungal growth.

In a recent publication (Green and Schultz, 2002) we also showed that when H-NHA was combined at low concentrations with three commercial biocides (DDAC, IPBC, and propiconizole) and tested by soil-block and agar-block tests against white and brown-rot fungi, synergism was only observed with one of the three: IPBC. We reasoned that if H-NHA was acting as a non-biocidal component (e.g.: antioxidant or chelator; Goodell et.al. 1997), such as chelating calcium, iron or manganese, then we would expect synergism to be observed with all three biocides. Since this was not observed, this also supports our current hypothesis that H-NHA is protecting the wood by simply acting fungicidally. Nevertheless, this data does not exclude the possibility of iron or manganese binding, or radical termination and we will be estimating metal binding constants in the near future. In summary, the fungicidal activity of H-NHA is not surprising, as the compound may simply mimic other polyaromatic hydrocarbon, similar to the compounds present in coal tar or creosote.

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