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The role of oxalic acid in tolerance to N'N-napthaloylhydroxylamine in *Tyromyces palustris*

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The role of oxalic acid in tolerance to N'N-naphthaloylhydroxylamine in *Tyromyces palustris*

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ABSTRACT

Certain wood decay fungi exhibit tolerance to one or more wood preservatives. Copper tolerance of brown-rot fungi has been studied in our laboratory for the past six years. We have observed some degree of tolerance to N'N-naphthaloylhydroxamine (NHA), a recently patented termite bait, by the brown-rot fungus *Tyromyces palustris* TYP-6137. In an effort to try and confirm this tolerance in other isolates of *T. palustris* and to dissect the mechanism of tolerance, an additional ten isolates of *T. palustris* were tested in soil-block bottles for 12 weeks against southern yellow pine wood blocks vacuum treated with 0.5 % NHA. Of the isolates tested, only TYP-6137 showed complete tolerance to NHA. The other 10 isolates showed varying degrees of tolerance, but at a lower NHA retention than TYP-6137. This shows that although preservative tolerance is directly related to the mechanisms of decay, caution should be used when making generalizations based on the metabolism of a single isolate (Clausen et al., 2000). Wood blocks exposed to *T. palustris* were tested for oxalic acid production at week 12. Two isolates produced more oxalic acid on NHA treated blocks than untreated SYP blocks, but we did not find any direct relationship between the amount of oxalic acid produced and the NHA tolerance of *T. palustris.* A separate test on malt agar plates with 0.1% NHA confirmed the notion of tolerance in two *T. palustris* strains, 6137 and TYP-L-15755, by solubilizing the NHA and migrating across the agar plate. Other fungi tested, *G. trabeum* 617, *Trametes versicolor* 697, *Postia placenta* 698, *Serpula lacrymans* 12C and *Antrodia vaillantii* TFFH, were not able to colonize malt agar supplemented with NHA. Thus, we conclude that NHA tolerance observed in *T. palustris* is apparently not mediated solely by overproduction of oxalic acid and that the apparent variation among strains in NHA tolerance is comparable to copper tolerance previously reported in *Wolfiporia coccos.*

Keywords: N'N-naphthaloylhydroxamine(NHA), copper, termite bait, preservative tolerance, *Tyromyces palustris*

1. INTRODUCTION

Fungal tolerance to wood preservatives has been recognized for many years, and is critically important to the development of new preservatives. The tolerance to common wood preservatives for type cultures is in fact noted in the AWPA Standards (AWPA, 2003). *Gloeophyllum trabeum* MAD-6 17, for example, is particularly tolerant to phenolic and arsenic compounds. One important group of decay fungi that has been studied extensively are those that are copper tolerant such as *Postia placenta* MAD-698, which has been shown to be particularly tolerant to copper and zinc compounds (AWPA, 2003). Recently, *Tyromyces palustris* and *Serpula lacrymans,* the European dry rot fungus, have also been shown to be copper tolerant (Green and Clausen, 2005; Hastrup et al 2005).

Replacement of CCA-treated wood in US residential markets with copper-based organic systems has renewed concerns about the decay capacity of copper-tolerant fungi as copper remains the primary component of the new generation of preservatives. In a series of papers on copper tolerance, *Tyromyces palustris* TYP 6137 produced high levels of oxalic acid on untreated southern yellow pine (SYP) and 42% mass loss on copper citrate-treated SYP (Green and Clausen 2005; Green and Clausen 2003). Oxalic acid (OA) production has previously been implicated as a key fungal metabolite in precipitation and neutralization of copper by brown-rot fungi (Clausen et al, 2000; Munir et al, 2001). In a study of copper tolerance to arsenic-free preservatives (ACQ-D, NHA, CuBor) *T. palustris* strains, TYP 6137 and TYP-L-15755, produced nearly 10% weight loss on 0.5% NHA treated blocks but little or no weight loss in ACQ-D or CuBor-treated blocks (Green and Clausen 2005). In previous studies on N'N-naphthaloylhydroxylamine (NHA) leachability, TYP-6137 caused significantly higher weight loss of NHA-treated SYP and balsa blocks than *Gloeophyllum trabeum* or *Meruliporia incrassata* (Green and Highley 1998). T. *palustris* TYP 6137 also showed equal capacity to cause weight loss of 0.5% NHA-treated blocks and SYP controls (44.3 +/- 4.8 vs 48.2 +/- 6.4)(Hastrup et a1 2005). We concluded from these observations that the T. *palustris* strains, TYP 6137 and TYP-L-15755, show partial tolerance to NHA that is not observed in other common brown-rot fungi (Hastrup et a1 2005).

N'N-naphthaloylhydroxylamine (Na-NHA: $C_{12}H_6NNaO_3$) is a naphthalene derivative, and a selective calcium precipitating compound (Green et a1 1997). NHA has recently been patented and licensed as a termite bait (Rojas et al 2004), and has been shown to inhibit decay fungi (Green et al., 1997, 2002). NHA tolerance is logically linked to the mechanism of NHA inhibition. Previous investigations into this mechanism have shown that the protinated form of NHA (H-NHA) is more inhibitory to decay fungi than the sodium form (e.g. Na-NHA)(Green et. al., 2002). Due to excess OA production during incipient decay by T. *palustris,* we assume that most or all of the soluble Na-NHA will rapidly be converted into the acid form (H-NHA), at least initially. What type of subsequent decay event could then render the H-NHA inactive? We have theorized at least five possible mechanisms: i) translocation of calcium, iron or manganese into the decay site which would precipitate the H-NHA and replace the hydrogen atom (e.g. Bech-Anderson noted that the dry rot fungus *S. lacrymans* required a calcium source of building materials to neutralize extracellular OA (Bech-Anderson 1987)); ii) cleavage by extracellular hydrolytic enzymes; iii) oxidative Fenton chemistry (one electron oxidation); iv) a barrier effect initiated by precipitation of NHA on or outside the hyphal sheath – e.g. calcium oxalate is observed to be precipitated near or onto the hyphal sheath (Green et a1 1996); and iv) binding of carbonyl groups of NHA to carbohydrate wood components at very low pH (~1.0) by acid-catalyzed aldol condensation (personal communication, 2005).

2. MATERIALS AND METHODS

Three main experiments were designed, the first of which includes 11 strains of the fungus T. *palustris* to see if NHA tolerance varied throughout the species or if it was only observed in TYP 6137 and TYP-L-15755. The second experiment was designed to determine if the NHA was actually "degraded" by T. *palustris* by secondarily testing TYP 6137 or TYP-L-15755 degraded wood blocks against non-NHA-tolerant wood decay fungi after the method described by Duncan and Deverall (1964). The third experiment was designed to estimate fungal inhibition or growth on 0.1% NHA malt agar plates.

2.1 Soil block decay test

In order to better understand strain variation in the fungus *T. palustris,* 11 different strains were examined along with one brown-rot fungus, *G. trabeum* MAD 617, to serve as comparison. A total of 432 SYP wood blocks (10 x 10 x 10mm) were divided into two groups: 216 were used as control blocks and 216 were vacuum treated at 100mm Hg for 30 minutes with a 0.5% NHA solution. All blocks were air-dried overnight and conditioned to 27°C/70% RH. A conditioned weight was determined for all blocks prior to being steam sterilized for 30 minutes.

Soil bottles [55 x 55 x 135mm; Owens-Brockway; Owens, OH] were filled with 120 grams of soil, 20 mL of distilled water, and a SYP feeder was placed on the soil surface. Bottles were autoclaved for 45 minutes. Groups of 6 were then inoculated with one of the 11 strains of T. *palustris*: FP94152, FP105403-T, FP103951-sp, FP10004-T, P13a, FP105394-sp, MS-48, L15755, 6137, FP97400-T, 14757-RLG, or with *G. trabeum* MAD-617. After the fungus had completely covered the SYP feeder strip, 18 treated blocks were added to 3 of the bottles for each fungus while 18 untreated blocks were added to 3 bottles for controls. At 6, 9, and 12 weeks, two blocks were removed from each bottle, brushed free of mycelium, dried in an oven at 60°C overnight, conditioned for 1 week at 27°C/70% RH, and weighed. Average percent mass loss of each group was then calculated from the weights before and after decay testing. At week 12, oxalic acid production was determined by microassay (Sigma, St. Louis, MO).

2.2 Two-step decay test

A total of 30 SYP feeders [3 x 27 x 40mm] were vacuum treated at 100mm Hg for 30 minutes with a 0.5% NHA solution. After treatment, blocks were dried overnight in a biological hood and then conditioned to 27°C/70% RH. Conditioned weights were recorded and blocks were steam sterilized for 30 minutes. Three sets of 3 glass bottles [55 x 55 x 135mm; Owens-Brockway; Owens, OH] were filled with 1 inch of 2% malt extract agar (Difco Laboratories, Detroit, MI), laid on their side to harden, and inoculated with either T. *palustris* 6137, *T. palustris* L15755, or *G. trabeum* 617. After the agar was covered with fungal growth, 10 treated feeders were placed in the bottles and inoculated for 10 weeks.

Standard soil block bottles were prepared for secondary exposure of the treated feeders to fungi along with 10 SYP untreated controls. Bottles were inoculated with *G. trabeum* 617 or *P. placenta* MAD 698.

After 10 weeks in the agar bottles treated blocks, were removed, dried in an oven at 60°C overnight, conditioned for 1 week at 27°C/70% RH, weighed, steam-sterilized, and placed in the second set of soil bottles with *G. trabeum* 617 or *P. placenta* MAD 698. Following a 10-week incubation in the soil bottles, blocks were removed, brushed free of mycelium, and dried in the oven. Blocks were then re-conditioned for 1 week and final weights were taken.

2.3 Agar toxicity test

In order to compare growth inhibition of T. *palustris* vs. other decay fungi in the presence of NHA, 2% MEA plates treated with 0.1% NHA were inoculated with 6 fungal strains: *P. placenta* 698, *T. versicolor* 697, TFFH *Serpula Incrassata, S. lacrymans* 12-C, *T. palustris* TYP-6137, and *T. palustris* TYP-L-15755 and incubated at 27°C/70% RH. After 16 days, color change to the plate as well as fungal growth was noted.

3. RESULTS AND DISCUSSION

3.1 Soil block test results

The decay capacity and OA production of the eleven isolates of T. *palustris* and *G. trabeum* MAD 617 after 12 weeks decay are shown in Table. 1. Higher percent mass loss (5 - 30%) was observed on controls for ten of the *T. palustris* isolates. Only TYP-6137 showed no apparent difference between 0.5% NHA and the untreated controls. The mean mass loss for control blocks was 57%, and 42% for NHA. Higher average OA was seen in the NHA-treated blocks of (600 µmoles) vs. 478 µm in the untreated blocks. This is similar to copper tolerance in which the oxalic acid was 2-17 times greater in the Cu-treated blocks than in the untreated pine. This fact alone suggests a potential role for OA in the observed NHA tolerance. A greater correlation between OA production and NHA tolerance may have been observed at an earlier time point during the test as OA has been shown to peak at 6-10 weeks in copper tolerant by brown-rot fungi (Green and Clausen, 2003).

Table 1: Mass Loss and Oxalic Acid Production of 11 strains of *Tyromyces palustris* and 1 strain of *Gloeophyllum trabeum* MAD-611

	Control Group		NHA Treated Group	
***T. palustris* isolate**	**Mass Loss [%] [a]**	**OA Production [µmol]**	**Mass Loss [%]**	**OA Production [µmol]**
FP94152	62.5 (2.4)	315 (265)	42.2 (9.9)	653 (399)
FP105403-T	53.2 (2.9)	739 (407)	38.0 (4.7)	345 (271)
FP103951-sp	60.5 (7.0)	363 (105)	30.0 (10.9)	1558 (391)
FP10004-T[b]	46.8 (2.4)	4463 (209)	29.6 (6.8)	1313 (298)
P13a	66.8 (3.6)	516 (164)	55.7 (4.5)	242 (111)
FP105394-sp	45.3 (9.1)	635 (363)	39.8 (11.6)	908 (592)
MS-48	60.0 (2.1)	192 (38)	53.6 (4.6)	588 (416)
L15755	60.9 (4.3)	206 (70)	39.4 (12.0)	979 (259)
6137	39.5 (11.1)	321 (99)	49.6 (5.6)	263 (181)
FP97400-T	64.7 (3.8)	785 (367)	52.7 (24.2)	158 (55)
14757-RLG	61.8 (5.3)	711 (369)	31.7 (11.1)	310 (389)
***G. trabeum* MAD 617**	64.1 (2.1)	187 (23)	30.4 (12.1)	253 (69)
Means *T. palustris*	56.5 (4.9)	478 (225)	42.0 (9.6)	601(306)

[a] Standard deviation in ( )
[b] *T. palustris* isolate FP 100004-T OA data not included in the means

3.2 Two-step decay test results

In the two-step test, NHA- treated pine was exposed progressively to T. *palustris* TYP-6137, L-15755, *or G. trabeum* MAD-617 for up to 10 weeks in agar bottles followed by decay in soil-

block test by NHA-susceptible brown-rot species. The results are shown in Figure 1. No weight loss occurred in NHA treated blocks in MEA bottles. The inability of MAD-6 17 or Mad-698 to decay the blocks in the soil-block test underscores the inability of *G. trabeum* to chemically alter or degrade the NHA into a less potent form. This suggests that in the absence of OA production the NHA remains antifungal. This apparent change in mass loss following colonization by TYP-6137 (16%) and L-15755 (28%) indicates a change in the NHA inhibition and may be due to solubiliztion, degradation, or alteration of calcium NHA complexes.

Figure 1: Mass loss of 0.5% NHA feeders decayed first with 4137, L15755, or 417 (MEA) and secondarily by 617 or 498 (soil)

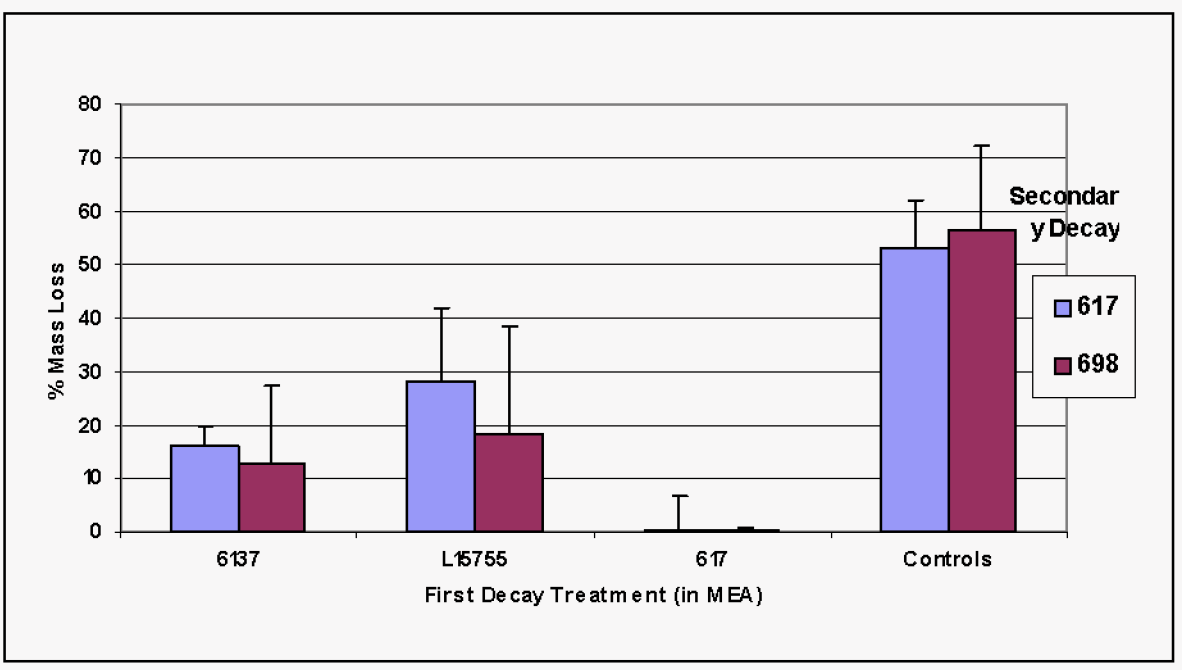

Figure 2: Growth of 4 Fungal Strains on 0.1% NHA treated MEA Plates
Top row: MAD 497, TYP 4137, TYP-L-15755
Bottom row: MAD 498, *S. lacrymans* 12C, TFFH *S. lacrymans*

3.3 Agar toxicity test results

The results of growth on the MEA plates treated with 0.1% NHA are shown in Figure 2. Of the 6 strains of fungi inoculated, only the *Tyromyces* strains were able to produce significant growth, covering almost half the plate. The apparent sequence of events with regard to the MEA plates begins with overproduction of OA by T. *palustris* TYP-6137 and TYP-L-15755 as well as by the other fungi, excluding the white-rot fungus, MAD 697. The OA converts the Na-NHA to the protonated H-NHA form, which is colorless. Additional OA lowers the microprobe pH of the agar to the pKa of OA (~1.24). Precipitated NHA crystals are solubilized at this low pH permitting the fungus to proceed forward with hyphal growth in the presence of the H-NHA. We theorize that this may be due to immobilization of NHA onto the hyphal sheath as seen in liquid cultures (unpublished result).

4. CONCLUSION

Preservative tolerance is a window into the mechanism of decay. *Serpula lacrymans* needs a woody substrate and a source of divalent cations to survive (Hastrup et. al. 2005, Palfreyman et. al. 1996). Oxalic acid is a potent metal chelator able to solubilize and translocate additional Ca/Fe/Mn into decay sites. *T. palustris* shows decay of NHA-treated blocks in soil-block tests, but not in MEA agar-bottles, likely due to lack of sufficient cations. We conclude that TYP-6137 and TYP-TYP-L-15755 are tolerant to higher levels of NHA, e.g. 1% leached, compared to other brown-rot fungi by some heretofore undescribed "barrier" or "blocking" mechanism, possibly mediated by the hyphal sheath, and solubilization of calcium oxalate complexes. Experiments to date have yet to confirm the precise mechanism of NHA tolerance.

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