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Correlation between oxalic acid production and tolerance of *Tyromyces paulstris* strain TYP-6137 to *N',N*-naphthaloylhydroxamine

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ABSTRACT

Eleven strains of *T. palustris* were evaluated for mass loss and production of phosphate buffer soluble oxalic acid on pine wood blocks treated with 0.5% *N'N*-naphthaloylhydroxamine (NHA) in a soil-block test. After 12 weeks higher percentage mass loss was observed in control groups for 10 strains, while TYP-6137 was shown to be tolerant with no difference between the untreated controls and 0.5% NHA-treated blocks. Strain TYP-6137 was evaluated at 6, 8, and 10 weeks for mass loss and production of oxalic acid in a colorimetric assay with increasing levels of NHA treatment (0.5 - 1%). *Tyromyces paulstris* TYP-6137 was shown to be successful in degrading NHA-treated wood in the 0.5-0.8% treatment range, but was inhibited in the 0.9-1% treated groups. Although there is no simple linear correlation between mass loss and production of oxalic acid, there was an inverse relationship at the 6-week time period. Thus, it was concluded that production of oxalic acid in *T. palustris* TYP-6137 at 6 weeks may facilitate mass loss at 8-10 weeks in the 0.5-0.8 NHA-treated blocks. In the 0.9-1% treatment groups this response was not seen. The broad variation seen in oxalic acid production among the 11 *T. palustris* strains suggests that high oxalic acid production alone is not he sole mechanism of NHA tolerance. Oxalic acid appears to be non-specifically produced in response to the preservative treatment, yet its association with mass loss could not be clearly defined.

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1. Introduction

Fungal tolerance to active chemical compounds is a critical consideration in development of new wood preservatives. Tolerance is defined as mass loss in treated wood bolcks equal to or greater than that of untreated control blocks. Tolerance of brown-rot fungi to copper has been linked to oxalic acid production (Clausen and Green, 2003; Green and Clausen, 2003, 2005). Wood-decay fungi exhibit tolerance to other preservative components, but the precise mechanisms are not well definde. Tolerance to common wood preservatives for type cultures is noted in the American Wood Preservers Association (AWPA) Standards (AWPA, 2003). *Gloeophyllum trabeum* MAD-617, 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 is known to be 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 (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, *T. palustris* TYP-6137 was found to produce high levels of oxalic acid (OA) on untreated southern yellow pine (SYP) and to cause 42% mass loss on copper-citrate-treated southern yellow pine (SYP) (Green and Clausen, 2003, 2005).

Oxalic acid ($C_2H_2O_4$) is produced in measurable amounts by most brown-rot fungi and is known to be a metabolic byproduct of the decay process (Bech-Anderson, 1987; Hastrup et al., 2006; Sierra-Alvarez, 2007.) Recently, Munir et al. (2001) concluded that *T. palustris* acquires biochemical energy for growth by oxidizing glucose to oxalate. Other studies have also implicated oxalic acid production as a key fungal metabolite in precipitation and neutralization of copper by brown-rot fungi (Munir et al., 2001). A paper by Green and Clausen (2005) examine copper tolerance to arsenic-free preservatives (alkaline copper quat (ACQ-D). *N',N*-naphthaloylhydroxamine (NHA), copper borate (CuBor)), by *T.* palustris strains TYP-6137 and TYP-L-15755, resulting in nearly 10% mass loss on 0.5% NHA-treated blocks but little or no mass loss in ACQ-D or CuBor-treated blocks. In previous studies on NHA, TYP-6137 caused significantly higher mass loss of NHA-treated SYP and

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balsa blocks than did *G. trabeum* or *Meruliporia incrassata* (Green and Highley, 1998). *Tyromyces palustris* TYP-6137 also showed capacity to cause similar mass loss of 0.5% NHA-treated blocks and SYP controls in an experiment by Hastrup et al. (2005). From these observations, it was concluded that the T palushis strains TYP-6137 and, to a lesser extent, TYP-L-15755, exhibit tolerance to NHA that has thus far not been observed in other common brown-rot fungi (Hastrup et al., 2005).

The actual role of oxalic acid in tolerance seems to vary with treatment and fungal species. Green and Clausen (2003) studied oxalic acid production of 15 brown-rot species on copper-citrate-treated wood blocks in which the oxalic acid production was found to be 2–17 times greater in the Cu-treated blocks than in the untreated pine. Conversely, in a study of the role of oxalic acid in copper tolerance by the brown-rot fungus *Wolfporia cocos*. Clausen et al. (2000) found no direct statistical relationship between the amounts of oxalic acid produced and copper tolerance in the 19 isolates examined. The later study, however, only examined oxalic production at week 12.

N′,N-Naphthaloylhydroxylamine (Na-NHA:$C_{12}H_6NNaO_3$) is a naphthalene derivative, and a selective calcium precipitating compound (Green et al., 1997) that has been patented and recently licensed as a termite bait (Rojas et al., 2004). It has also been recognized as an experimental, heavy-metal-free wood preservative and has been shown to inhibit most decay fungi, possibly by means of precipitation of calcium ions (Green et al.. 1997, 2002; Palfreyman et al.. 1996).

Many recent studies of the mechanism of decay have surmised that oxalic acid is a major factor leading to tolerance of various copper-based wood preservatives. This study aims to determine if oxalic acid is, in part, contributing to the unique tolerance to NHA by *T. palustris* as well as the effect of preservative treatment on oxalic acid production. Variation of tolerance in a range of treatment concentrations will also be evaluated to determine the threshold concentration of NHA as well as the amount of soluble oxalic acid in the wood in various NHA concentrations at different time periods.

2. Materials and methods

2.1. Soil block decay test

Eleven strains of *T. palustris* (Berk. and Curt) Murr.: FP94152, FP105403-T, FP103951-sp, FP100004-T, P13a, FP105394-sp, MS-48, TYP-L-15755, TYP-6137, FP97400-T or 14757-RLG and one strain of *G. trabeum* (Pers.: Fr.) Murr. MAD-617 were examined in an ASTM (D1413) soil block test (ASTM, 1996). Southern yellow pine wood blocks (1 × 1 × 1 cm) were conditioned to 27°C and 70% relative humidiy (RH), weighed, and vacuum-treated with 0.5% aqueous NHA 100 mmHg for 30 min. Treated blocks were air-dried and conditioned to 27°C and 70% RH and re-weighed prior to autoclaving. Untreated SYP blocks served as controls. Soil bottles (5.5 × 5.5 × 13.5 cm; Owens-Brockway, Owens, OH) using SYP feeders were inoculated with the test fungal strain. Groups of six blocks were added to each soil bottle after the fungus had completely colonized each feeder. After 12 weeks blocks were removed, brushed free of mycelium, dried in an oven at 60°C overnight conditioned to 27°C and 70% RH, and reweighed.

2.2. Oxalic acid analysis

Accumulation of phosphate buffer soluble Oxalic acid war determined by a colorimetric microarray (Trinity Biotech Co., Wicklow, Ireland). Individual blocks ($n = 6$) were extracted in 3.0 ml of 0.1 M phosphate buffer (pH 7.0) for 2 h with agitation (Clausen and Green, 2003). Test results were determined using a 96-well Dynex microplate reader, in which 10 μl of test solution and Oxalic acid standards were inserted (Dynex Technologies, Chantilly, VA). Two hundred microliters of DMAB/oxalate oxidase reagent were added to each well followed by 20 μl of MBTH reagent. The plate war then incubated for 5 min at 37°C. Optical density was measured at 590 nm in a Dynatech MRXII microplate reader and oxalate (mmol) was determined from a standard curve.

2.3. Oxalic acid response and mass loss to varying concentrations of NHA

Southern yellow pine wood blocks (1 × 1 × 1 cm) were conditioned at 27°C and 70% RH weighed, and vacuum-treated with 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, or 1% NHA at 100 mmHg for 30 min. Wood blocks were air-dried, conditioned to 27°C and 70% RH, and reweighed before autoclaving. Untreated SYP blocks served as controls. Test blocks were then added to mil bottles that had been inoculated with TYP-6137. Average percentage mass loss at 6, 8, and 10 weeks was determined ($n = 6$), and oxalic acid assays were performed on individual test blocks using the colorimetric microarray described above (Trinity Biotech Co., Wicklow, Ireland).

2.4. Statistical analysis

Percentage mass loss and oxalic acid production after 12 weeks exposure of *T. palustris* strains were compared with the Wilcoxon signed-rank test for paired data using S-PLUS® Version 7.0 (Insightful Corp., 2005).

Trellis boxplots were used as a statistical tool to explore the distribution of oxalic acid and percentage mass loss of individual soil blocks over NHA treatments (0.5–1%) and exposure periods (6, 8, and 10 weeks). Results show a summary of data with vertical bars on the plots encoding the medians, the distance between the ends of the boxer representing the interquartile ranges, while outside values are graphed individually and are represented by open circles (Insightful Corp., 2005).

The second experiment was conducted as a split-plot with whole plot factor NHA treatment (0.5–1%) and subplot factor exposure period (6, 8, and 10 weeks) with several blocks inmultiple bottler. However, identifying bottle information used within a particular treatment combination was not retained. An alternative to analyzing there data, without assuming independence ofindividual mil blocks, is to take means (or means of an appropriate transformation) for each treatment combination and use the unreplicated experiment methods of Milliken and Johnson (1989). A response surface (i.e., regression with quantitative factors) approach was used for two reasons: (1) to be able to evaluate multiple sources of variation due to the split-plot structure of the experiment, and (2) to help determine quantitatively the minimal percentage NHA that effectively treated the blocks by reducing mass loss and minimizing Oxalic production. The mean percentage mass loss and mean logarithmic oxalic acid were calculated and used as the response variable for each time by treatment combination. Mean percentage mass loss and median Oxalic acid (estimated by the antilogarithm of the mean logarithmic oxalic acid) are marked in the boxplots as closed circles (Fig. 3). Models were fit in SAS® Version 9.1 (SAS Institute, 2004).

3. Results and discussion

3.1. Soil block decay test

The decay capacity and the accumulation of soluble oxalic acid of the 11 strains of T palushis and one strain of *G. trabeum* MAD-617 after 12 weeks decay are shown in Table 1 as accumulated μmol oxalic acid as well as μmol oxalic acid/g. The Wilcoxon signed-rank test for the equivalence of percentage mass loss between the paired untreated and treated T palushis groups indicated a significant difference between the untreated and treated wood ($p = 0.0049$. $n = 11$). Significantly higher percentage mass loss (~5–30%)was observed in control groups for 10 of the *T. palustris* strains. Only

Table 1
Mass loss and production of soluble oxalic acid/oxalic acid per gram of wood weight in 11 strains of *T. palustris* and 1 strain of *G. trabeum* MAD-617 after 12 weeks decay[a]

Fungal strain	Control group			0.5%NHA-treated group		
	Mass loss (%)	OA (μmol)	OA (μmol/g)	Mass loss (%)	OA (μmol)	OA (μmol/g)
FP94152	62.5 (2.4)[b]	315 (265)	1486.4	42.2 (9.9)	653 (399)	1924.5
FP105403-T	53.2 (2.9)	739 (407)	2647.0	38.0 (4.7)	345 (271)	904.3
FP103951-sp	60.5 (7.0)	363 (105)	1530.7	30.0 (10.9)	1558 (391)	4066.7
FP100004-T[c]	46.8 (2.4)	4463 (209)	13415.3	29.6 (6.8)	1313 (298)	3277.8
P13a	66.8 (3.6)	516 (164)	2447.1	55.7 (4.5)	242 (111)	997.8
FP105394-sp	45.3 (9.1)	635 (363)	2055.1	39.8 (11.6)	908 (592)	2538.7
MS-48	60.0 (2.1)	192 (38)	812.2	53.6 (4.6)	588 (416)	2123.5
TYP-L-15755	60.9 (4.3)	206 (70)	886.9	39.4 (12.0)	979 (259)	2662.2
TYP-6137	39.5 (11.1)	321 (99)	865.2	49.6 (5.6)	263 (181)	908.1
FP97400-T	64.7 (3.8)	785 (367)	3869.8	52.7 (24.2)	158 (55)	443.1
14757-RLG	61.8 (5.3)	711 (369)	3032.6	31.7 (11.1)	310 (389)	766.5
G. trabeum 617	64.1 (2.1)	187 (23)	845.6	30.4 (12.1)	253 (69)	603.5
Means of TYP	56.5 (4.9)	478 (225)		42.0 (9.6)	601 (306)	
Medians of TYP	60.5	516		39.8	588	

[a] Modified in part from Arango et al. (2006).
[b] Standard deviation in parentheses, $n_i = 6$.
[c] *T. palustris* strain FP100004-T OA data not included in the means.

TYP-6137 showed a negative difference between the untreated controls and 0.5% NHA. with a higher mass loss in the treated wood compared to the untreated SYP. Variation of inhibition and tolerance seen in the different strains is similar to what has been noted in other studies on copper tolerance (Clausen et al., 2000). The mean and median mass loss from *Tyromyces* strains was 57% and 61% for control blocks, and 42% and 40% for NHA-treated blocks. Elevated oxalic acid values (1.5–5×) were observed in selected strains in the NHA-treated blocks and the means for strains (Table 1) were 600 µmol compared with 478 µmol in the untreated blocks. However, the medians of 588 µmol and 516 µmol are not significantly different based on a Wilcoxon signed-rank test ($p = 0.8984$. $n = 11$). Although not all copper-tolerant fungi are NHA-tolerant, the role of oxalic acid in both processes may give an indication as to the role of oxalic acid in preservative tolerance. Brown-rot fungi generally show higher tolerance toward copper than do white-rot fungi and were also shown to accumulate considerable quantities of oxalic acid (up to 44.3 mM) in liquid medium (Sierra-Alvarez, 2007). This increase may be a non-specific response of the fungus to the preservative and/or a failure to initiate degradation. Although the highest oxalic acid producer per gram in the control group and the second highest in NHA-treated blocks (µmol OA/g). FP100004 appears to be inhibited by low concentrations of NHA (Table 1). In copper-treated SYP, we have shown precipitation of copper oxalate, which abrogates the toxicity of copper ions from the treated wood (Clausen et al., 2000). The production of oxalic acid in NHA-tolerant TYP-6137 may therefore be promoted by the presence of a preservative rather than the cause of tolerance. This is supported by the fact that strain FP100004-T was the highest producer of oxalic acid, but was inhibited by NHA treatment.

3.2. Oxalic acid response and mass loss to varying concentrations of NHA

It has been shown that oxalic acid peaks between 6 and 10 weeks in copper-tolerant brown-rot fungi (Green and Clausen. 2003). Additional oxalic acid readings at time periods earlier than 12 weeks were performed. A linear range of NHA treatment (0.5–1%) was chosen since previous experiments had shown this to be the working range of the preservative. At 0.5% NHA and below, *T. palushis* TYP-6137 exhibits tolerance, and above 1% the fungus is inhibited; therefore, by calculating mass loss between these percentages we can determine the minimum concentration necessary for fungal inhibition.

Net production of soluble oxalic acid by TYP-6137 documented at three time periods showed a peak at 6 weeks in 0.5–0.8% NHA-treated blocks, followed by a decrease in oxalic acid production from week 8 to week 10 in treated blocks. This coincides with the hypothesis that oxalic acid production is one of the early steps in the decay process (Green et al., 1991). Conversely, in the control blocks, the fungus typically produced less oxalic acid at this early stage. It is possible, therefore, that the preservative treatment either induced early production of oxalic acid or that the fungus produces the acid in response to the presence of preservative treatment. Because oxalic acid production is indicative of fungal metabolism, NHA sensitivity was demonstrated at 0.9 and 1%. At these higher concentrations mass loss and oxalic acid production were reduced, especially at later exposures, signifying a decreased ability of the fungus to degrade the wood. Therefore, it can be concluded that the concentration needed to inhibit TYP-6137 is approximately 0.9% NHA. below which TYP-6137 appears to be tolerant (Figs. 1 and 2). It has been observed that leached 1% NHA blocks will fall below the inhibitory concentration of the preservative treatment, thereby making them susceptible to decay by *T. palushis* TYP-6137, though this needs further study (unpublished data).

Some authors have differentiated water-soluble oxalic acid from acid-soluble or total oxalic acid (Shilling and Jellison. 2005). Total oxalic acid assumes the formation of calcium oxalate crystals during the drying process as often visualized by scanning electron microscopy (Green et al., 1996). There is, however, no way of determining if acid-soluble oxalic acid is an artifact of drying and not an essential part of the decay process. Eased upon the recent paper of Clausen et al. (2008). it is not clear just what fraction of oxalic acid is estimated by the phosphate buffer of the colorimetric test, although they saw no statistically different values for acid-soluble or phosphate-buffer-soluble oxalic acid using either the colorimetric test or HPLC. Our own observations indicate that the colorimetric test can estimate water-soluble and acid-soluble oxalic acid in the working range of pH 1–3.

Oxalic acid has been considered by some authors to be the key element of brown-rot decay (Bech-Andersen, 1987). Oxalate production may be required to decrease the pH of the woody substrate below pH 2.0 where hemicelluloses fall apart and initiate decay (Green and Highley. 1997a,b). The active mechanisms of decay including acid production, enzymes. or oxidative systems (e.g., Fenton chemistry) may damage or inactivate the NHA molecules. Overproduction of oxalic acid may also result in direct damage to the fungal hyphae (Bech-Andersen, 1987). Thus NHA

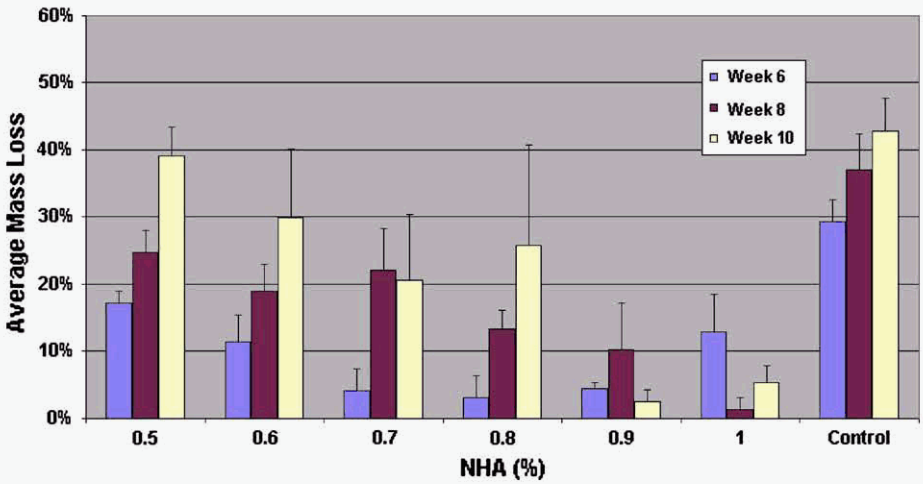

Fig. 1. Mass loss of 0.5-1% NHA-treated blocks against decay by *Tyromyces palustris* TYP-6137.

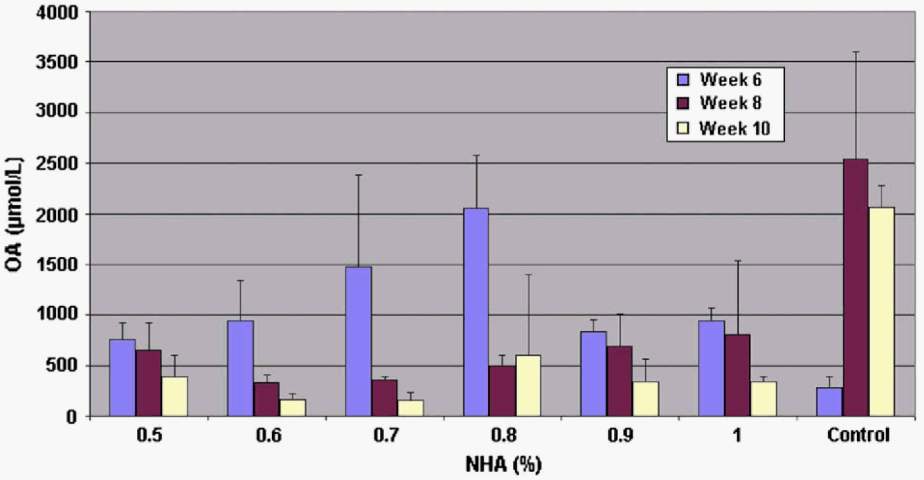

Fig. 2. Oxalic acid production on 0.5–1% NHA-treated and control blocks by the fungus *Tyromyces palustris* TYP-6137.

may be capable of out-competing oxalate in TYP-6137 for calcium by forming a precipitate. Disruption of calcium regulation by precipitation with NHA may be reversed by additional oxalate production followed by calcium translocation.

3.3. Statistical analysis

Boxplot comparisons suggest possible trends of the role of oxalic acid in the mechanism of NHA tolerance. In several NHA-treated groups, production of soluble oxalic acid appears to occur at an earlier period (6 weeks) than in the control groups. Mass loss in control blocks at week 6, however, is significantly greater than in all other blocks except for the 0.5% NHA group at that time period. At week 8, control blocks peaked in their production of soluble oxalic acid, causing a significantly higher accumulation of oxalic acid than all of the NHA treatment groups. At week 10, oxalic acid production completely tapers off in treated groups compared to the controls (Fig. 3).

A linear response surface model adequately described percentage mass loss as a function of weeks' exposure and percentage NHA ($R^2 = 0.79$. adj-$R^2 = 0.76$, RMSE = 6.22. $p < 0.0001$), whereas oxalic acid production exhibited a more complicated relationship over the factors and the logarithmic oxalic acid was better described by a quadratic model ($R^2 = 0.69$, adj-$R^2 = 0.58$, RMSE = 0.50, $p = 0.0020$). Much of the complication for oxalic acid can be attributed to the inclusion of the 6-week exposure control in the model; a model without the control at 6 weeks results in a quadratic model with $R^2 = 0.82$, adj-$R^2 = 0.78$.

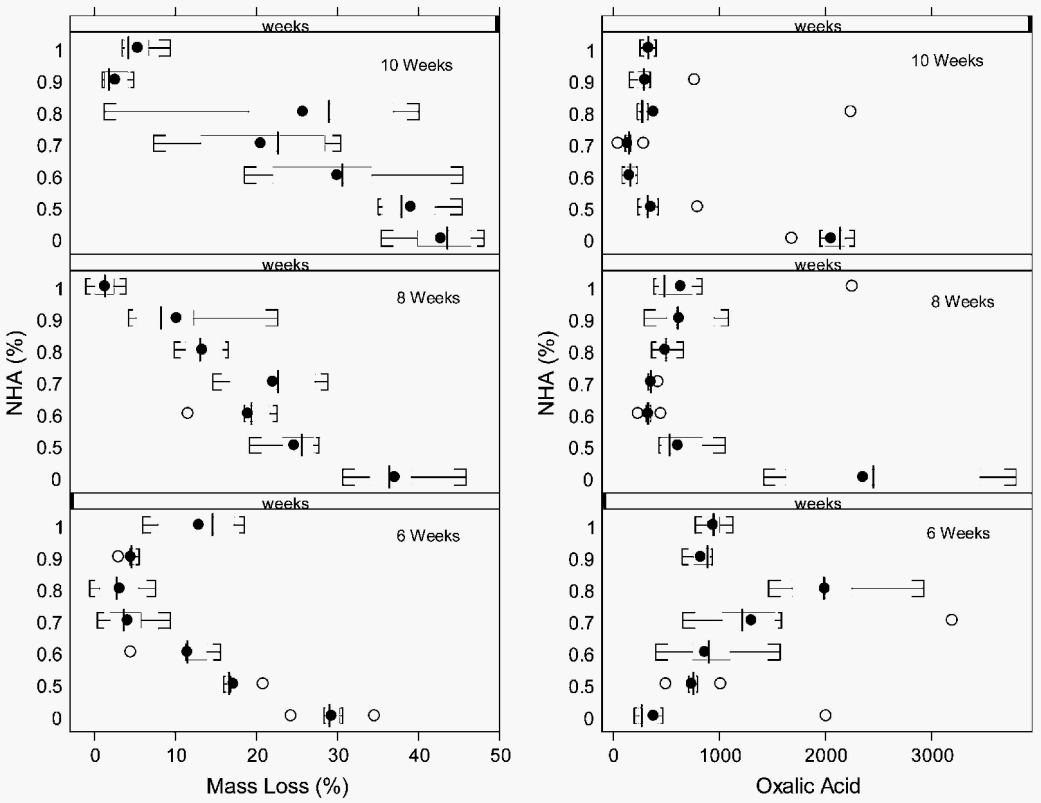

Fig. 3. Boxplot comparison of oxalic acid and percentage mass loss over NHA treatments and exposure period in TYP-6137.

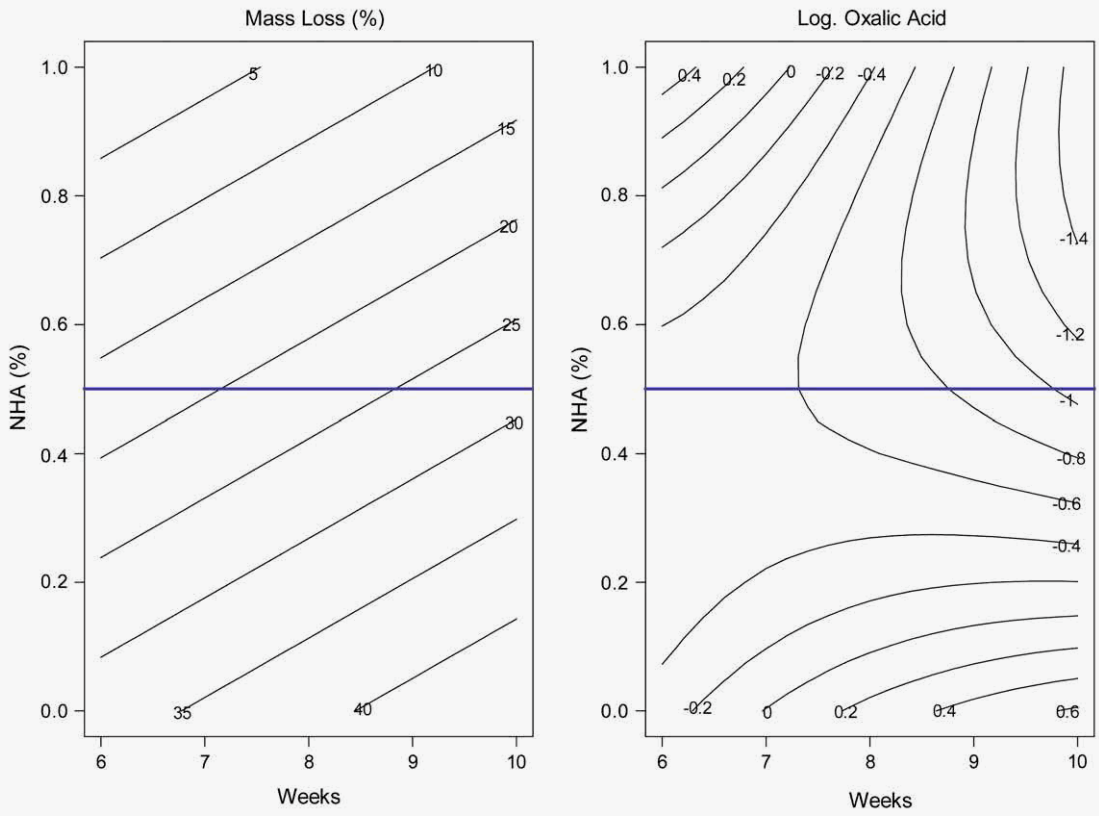

Fig. 4. Contour plots for mean mass loss (%)and logarithmic Oxalic acid. The horizontal liner indicate the lowest percentage NHA tested.

RMSE = 0.38, $p < 0.0001$, but there is insufficient evidence to drop the 6-week controls and the resulting surface appears similar to the above model except in the low NHA (<0.5%) and control region. Contour plots for each of the fitted models are shown in Fig. 4 and illustrate similar patterns as given in the boxplots. Eased on the quadratic response of logarithmic oxalic acid, the oxalic acid model predicts a minimum occurs at 10 weeks with a 0.92% NHA. At 10 weeks, considering mass loss increases with decreased NHA (percentages), concentrations above the 0.92% NHA lead to reduced mass loss.

Trends seen in the boxplots support the hypothesis that sustained production of oxalic acid is a key event in the ability of fungus to metabolize the wood as a food source (Green et al., 1991; Green and Clausen. 2005). Tyromyces palushis TYP-6137 was at least in part suppressed by the preservative treatment, and therefore not able to degrade the wood, causing the fungus to utilize oxalic acid production earlier in the process in an effort to metabolize the wood. It is possible that the fungus exhausts its energy reserves to produce the oxalic acid in an effort to break down or circumvent the NHA treatment. *T. palustris* was shown to be successful in altering the efficacy of the preservative in the 0.5–0.8% NHA-treated groups, but appears to be less successful on 0.9% and 1% treated blocks (Fig. 1). In the untreated control groups, the fungus was easily able to metabolize the wood, resulting in early mass loss to the wood, and thus able to produce a more soluble oxalic acid later in the decay process (Fig. 3). It may also be possible that the total amount of oxalic acid produced may not be as different as shown if the oxalic acid is bound in the NHA-treated blocks (Schilling and Jellison. 2005).

4. Summary

Tyromyces palustris TYP-6137 is tolerant to 0.5–0.8%NHA. Wood blocks show an increase in phosphate-buffer-soluble oxalic acid production by the fungus in response to increased NHA concentration at an earlier time period (6weeks) than the control blocks. It appears that early production of soluble oxalic acid represents a metabolic response of the fungus to the preservative-treated substrate. Mass loss is observed at concentrations between 0.5 and 0.8% NHA increasingly at later exposures, indicating that oxalic acid production is part of a successful strategy of the fungus in utilizing the wood substrate. This relationship, however, is not proof that the two independent variables are directly correlated, but they do appear to coincide in a time-delayed manner. Schilling and Jellison (2005) recognized the fact that adding oxalic acid facilitated degradation of wood by acid hydrolysis and therefore utilized sodium oxalate in the decay system. It is well accepted that oxalic acid production is a major part of the decay process. Numerous papers have shown T palushis TYP-6137 to be copper-tolerant and a prolific producer of oxalic acid. Some strains of *Tyromyces* palushis. in spite of accumulation of oxalic acid, were inhibited by the NHA treatment even at 0.5% (i.e., FP100004-T). Copper tolerance may be similar to NHA tolerance in that NHA precipitates out below pH 6.0, and may begin by lowering the pH of the substrate by oxalic acid accumulation. This study showed oxalic acid to play a key role in fungal metabolism of preservative-treated wood, but it was not confirmed as the sole component responsible for tolerance of *T. palustris* TYP-6137 seen in NHA-treated wood. Individual strain variation should be examined before determining tolerance or inhibition to wood preservatives in brown-rot decay fungi.

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