Effect of Ammonium Hydroxide on Thiamine and Available Micronutrients in Ponderosa Pine Sapwood

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Accepted for publication 28 November 1977.

ABSTRACT

AMBURGEY, T. L., and B. R. JOHNSON. 1978. Effect of ammonium hydroxide on thiamine and available micronutrients in ponderosa pine sapwood. Phytopathology 68: 951-954.

The amounts of thiamine and micronutrients available for fungal metabolism was decreased, but not rendered completely unavailable, in ponderosa pine sapwood treated with either ammonia gas or ammonium hydroxide and heated to 76.7 C. Growth of *Phycomyces blakesleeanus* and sporulation of *Aspergillus niger* were inhibited on media containing hydroxide-treated wood. This response suggested that this treatment affected other growth factors in the wood as well. *Gloeophyllum trabeum* grew on media containing hydroxide-treated wood. Results suggested that the increased decay resistance of alkali-treated wood may be due to factors that inhibit germination of basidiospores, not thiamine or micronutrient depletion alone.

Additional key words: wood preservation, wood decay, fungi.

Wood has been protected from decay by preservation with toxic substances. Because treating wood with alkali at high temperatures increases decay resistance in both the laboratory and field (1, 5), alkali treatments have been proposed as an alternative method of wood protection. The alkali treatment may destroy thiamine (3), which is essential for the growth of many wood-decay fungi. The treatment also may increase decay resistance in wood by reducing the availability of micronutrients essential for fungal growth (1) or by increasing the pH or ammoniacal nitrogen content (6). The objectives of this study were to determine whether alkali-treated wood contained sufficient thiamine to support fungal growth, had fewer available micronutrients than nontreated wood, and was able to support the growth of the wood-decay fungus *Gloeophyllum trabeum* (Pers. ex Fr.) Murr.

MATERIALS AND METHODS

The presence of thiamine or micronutrients was determined by measuring the thallus weight and amount of sporulation of test fungi after 7 days of growth on a chemically defined medium used either alone or supplemented with treated or nontreated wood meal. Bioassays were performed for thiamine on *Phycomyces blakesleeanus* Burgeff (7), a thiamine-requiring fungus, and for micronutrients on *Aspergillus niger* Van Tieghem, a fungus sensitive to changes in micronutrients (9, 10). Isolates of *P. blakesleeanus* (ATCC 8743a, plus strain) and *A. niger* (ATCC 10581) were obtained from the American Type Culture Collection. The brown-rot fungus, *G. trabeum* (Madison 5031 isolate), was used to determine the effects of treated wood on the growth of a wood-decay fungus.

Wood samples.—One yr before testing began, three kinds of treatments were applied to wood samples prepared from freshly cut ponderosa pine (*Pinus ponderosa* Laws.) sapwood furnished by the National Woodwork Manufacturers Association: nontreated, treated with ammonium hydroxide, and treated with ammonia gas. The two alkali treatments are both commercially feasible. Wood to be treated with ammonium hydroxide was kiln dried to approximately 10% moisture content before being pressure treated with 3.2% aqueous ammonium hydroxide by means of the full cell process, heated at 76.7 C for 6 hr, and redried in a kiln. Wood to be treated with ammonia was infused with a 16.02 kg/m^3 (1 lb/ft^3) retention of ammonia gas to give an ammonium hydroxide concentration within the green wood of approximately 3.2% and then was heated to 76.7 C and kiln dried.

Six to 8 mo after preparation, samples of nontreated and treated wood were macerated separately in a Wiley mill to furnish a wood meal that passed a 420 × 420 μm (40-mesh) sieve.

Media.—All media were prepared in acid-washed glassware that had been rinsed in double-distilled water. The chemically defined medium of Houston et al. (7), minus thiamine and agar, was used in all tests. The ingredients per liter were: macronutrients—KH_2PO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, asparagine 5.0 g, glucose 50.0 g; micronutrients—$Fe_2(SO_4)_3$ 0.19 mg, $ZnSO_4 \cdot 7H_2O$ 0.40 mg, $CuSO_4$ 0.05 mg, $MnSO_4 \cdot H_2O$ 0.03 mg, H_3BO_3 0.03 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.02 mg; and glass-distilled water 976 ml.

Micronutrients were added to the medium as aqueous solutions; 24 ml were required. In one test with *A. niger*, a composite micronutrient solution was added to the medium in varying quantities. In all tests, 5.0 g activated charcoal (Norite-A) was added per liter of medium and boiled for 5 min before it was filtered to remove vitamins

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that could mask test results.

Test chambers.—The glassware used in the growth studies was acid-washed and rinsed in double-distilled water. To prepare the test chambers, 50 ml of the chemically defined medium plus amendments was measured into 250-ml Erlenmeyer flasks. Then varying amounts of either thiamine (hydrochloride), treated or nontreated wood meal, or thiamine combined with treated or nontreated wood meal were dispensed into separate flasks. Flasks were covered with 50-ml beakers and autoclaved at 1.05 kg/cm^2 (15 psi) for 20 min.

Inoculum.—Test fungi were grown on the chemically defined medium supplemented with thiamine and 15 g Difco purified agar per liter. *Phycomyces blakesleeanus* was incubated according to a modification of Houston's (7) procedure to avoid carryover of thiamine from the inoculum to the test media. Incubation was for 24 hr at 15 C, 5 days at 25 C, 9 days at 8 C, and 5 days at 27 C. *Aspergillus niger* and *G. trabeum* were incubated at 27 C for 2 wk.

In most tests, inocula of *P. blakesleeanus*, *A. niger*, and *G. trabeum* were spore suspensions; in one test, the inoculum was fragmented mycelium of *G. trabeum*. Spores and sporangia of *P. blakesleeanus* were collected aseptically, placed in sterile distilled water, and agitated to release the spores. Conidia of *A. niger* were removed from actively growing cultures and placed in sterile distilled water. Basidiospores of *G. trabeum* were obtained by inverting petri dishes of sporulating cultures, collecting spores for 24 hr on the petri dish lids, and placing spores in sterile distilled water. Mycelium of *G. trabeum* was fragmented in a Waring Blendor, washed, and suspended in sterile distilled water.

Flasks of test media were inoculated with 1 ml of the *P. blakesleeanus* spore suspension, 1 loop of the *A. niger* spore suspension, 1 ml of the *G. trabeum* spore suspension, or 1 ml of the fragmented mycelium suspension of *G. trabeum*.

Growth tests.—Inoculated flasks were incubated in an environment controlled at 27 ± 1 C and 70 ± 2% RH for 7 days, the incubation time that resulted in maximum thallus production by *P. blakesleeanus* in a previous study (4). All flasks in both *P. blakesleeanus* (thiamine) tests and *A. niger* (micronutrient) tests were placed on one shelf which was divided into three sections. One of the three replicates per medium was placed in each section at random. The flasks inoculated with *G. trabeum* were agitated on a reciprocating shaker on another shelf during incubation to promote growth because *G. trabeum* did not grow well in liquid culture without agitation.

At the conclusion of a test, thallus weights were obtained from one section at a time by filtering the contents of each flask through an appropriate filter paper disk and washing with distilled water. Filter papers were tared before and after use by equilibration at 27 C and 70% RH. Thallus weights from cultures grown in media containing wood meal were determined by measuring the final weights of both thallus and wood meal and deducting the weight of the wood. Noninoculated media were processed at the conclusion of the tests to determine the weight of the wood.

The statistical significance of differences in growth on the various media within each experiment was tested by analysis of variance ($\alpha = 0.05$). Differences between means were tested for significance using Duncan's test ($\alpha = 0.05$). Growth responses to varying quantities of wood meal, thiamine, or minor nutrients in media were analyzed by linear regression.

RESULTS AND DISCUSSION

Depletion of thiamine.—*Phycomyces blakesleeanus.*—Growth of the thaimine-requiring fungus, *P. blakesleeanus*, was dependent on the amount of thiamine added to the chemically defined medium. There was a significant positive correlation ($r^2 = 0.99$, $n = 18$) between thallus weight and thiamine concentration over the range of 1 to 15 μg thiamine per liter. There was no growth when there was less than 1 μg thiamine per liter, and maximum thallus weight was produced when the medium contained 50 μg thiamine per liter.

Phycomyces blakesleeanus grew when either treated or nontreated wood meal was the sole thiamine source in the medium (Table 1), an indication that neither wood treatment depleted all of the thiamine necessary for fungal metabolism. However, the amount of thiamine remaining in the treated wood could not be determined. When the amount of wood meal added to the medium was doubled, the thallus weight did not increase proportionately (Table 1). The coefficient of determination (r^2) between the amount of wood meal in the medium and fungal growth was 0.81, 0.67, and 0.57, with ammonia gas-treated, ammonium hydroxide-treated, and nontreated wood, respectively. Similar results were obtained when the tests were repeated. In other studies, when tissue (2) or wood extracts (8) were added to growth media, it was observed that decreased thallus production was associated with increased

TABLE 1. Thallus production by *Phycomyces blakesleeanus* on a chemically defined medium[a] supplemented with thiamine and ammonia-treated or nontreated wood meal

Wood treatment (wood meal/liter) (g)	Thiamine (μg/liter)	Thallus weight[b] (mg)
Ammonia gas		
10	0	5.2 ab
20	0	7.9 ab
40	0	26.4 d
10	10	153.4
Ammonium hydroxide		
10	0	2.1 a
20	0	14.5 bc
40	0	19.4 cd
10	10	96.9
Nontreated		
10	0	12.9 bc
20	0	18.0 cd
40	0	27.0 d
10	10	164.5

[a]The chemically defined medium of Houston (Houston et al. 1969. Mycologia 6:1168-1171) minus thiamine and agar.

[b]Average of three replicates. Means followed by the same letter do not differ significantly. Those not followed by letters were not included in the analysis.

amounts of wood. Therefore, growth on media containing wood meal was not controlled solely by depletion of thiamine in the wood, but it appeared to be influenced by other substances as well.

When both 10 μg thiamine and 10 g wood meal were added per liter of medium (Table 1), thallus weight on the medium containing gas-treated wood (153.4 mg) or nontreated wood (164.5 mg) was not significantly greater than that on the medium containing only 10 μg thiamine (151.3 mg). However, thallus weight on a medium containing hydroxide-treated wood (95.8 mg) was significantly less than the growth on media containing 10 μg thiamine and no wood meal. Similar results were obtained when the tests were repeated, an indication that the hydroxide treatment altered more than the thiamine content of the wood.

Spore production of *P. blakesleeanus* was not abundant in media without wood meal when the thiamine concentration was less than 15 μg per liter; no spores were produced on media without thiamine added or when treated or nontreated wood meal was the sole source of thiamine. Sporulation increased when 10 μg thiamine per liter was included in addition to 10 g per liter of treated or nontreated wood meal, another indication that thiamine was available in the treated wood.

Gloeophyllum trabeum. —The wood decay fungus *G. trabeum* responded positively to increasing thiamine concentrations in the chemically defined medium, but it also grew when no thiamine was added (Table 2). Growth in flasks inoculated with fragmented mycelium increased progressively ($r^2 = 0.75$) from 8.0 to 26.8 mg as the concentration of thiamine increased from 0.2 to 10.0 μg per liter. However, growth in flasks inoculated with basidiospores was not significantly different when 0.2 to 3.0 μg thiamine per liter was added to the media. This indicates that basidiospores are less sensitive to changes in thiamine concentration than is fragmented mycelium. Changes in growth due to difference in thiamine concentration in media containing wood meal would be reflected more accurately in flasks inoculated with

TABLE 2. Thallus production by *Gloeophyllum trabeum* on a chemically defined medium[a] containing various concentrations of thiamine

Thiamine (μg/liter)	Thallus weight[b]	
	Basidiospore inoculum (mg)	Fragmented mycelium inoculum (mg)
10.0	29.6	26.8
5.0	48.7	20.0
3.0	23.5	13.4
2.0	24.0	15.5
1.0	22.1	10.4
0.8	18.8	11.3
0.6	19.4	8.7
0.4	19.7	8.1
0.2	20.5	8.0
0.0	15.6	5.5

[a]The chemically defined medium of Houston (Houston et al. 1969. Mycologia 6:1168-1171) minus thiamine and agar.
[b]Average of three replicates.

TABLE 3. Thallus production by *Gloeophyllum trabeum* on a chemically defined medium[a] containing various concentrations of wood meal

Wood treatments[b] and wood meal supplements (g/liter)	Thallus weight[c]	
	Basidiospore inoculum (mg)	Fragmented mycelium inoculum (mg)
Ammonia gas		
10	15.7	36.8
20	13.9	23.1
40	10.6	21.9
10[d]	24.3	86.3
Ammonium hydroxide		
10	8.4	56.5
20	16.8	45.0
40	16.1	29.0
10[d]	21.6	96.9
Nontreated		
10	10.3	45.3
20	0.0	0.0
40	5.7	0.3
10[d]	23.1	116.2

[a]The chemically defined medium of Houston (Houston et al. 1969. Mycologia 6:1168-1171) minus thiamine and agar.
[b]Ammonia gas infusion (16.02 kg/m^3) that produced an ammonium hydroxide concentration within the green wood of ~ 3.2%; kiln-drying to a moisture content of 10%, pressure treatment (full cell process) with 3.2% aqueous ammonium hydroxide, heating to 76.7 C for 6 hr, and redrying in a kiln; and nontreated controls.
[c]Average of three replicates.
[d]Media contained 5 μg thiamine per liter.

TABLE 4. Thallus production by *Aspergillus niger* on a chemically defined medium[a] supplemented with various amounts of ammonia-treated or nontreated wood meal

Wood treatments and wood meal supplements (g/liter)	Thallus weight[b] (mg)
Ammonia gas	
1	205 a
5	431 b
10	557 cd
20	883 f
Ammonium hydroxide	
1	227 a
5	456 b
10	613 d
20	744 e
Nontreated	
1	259 a
5	500 bc
10	784 ef
20	970 g

[a]The chemically defined medium of Houston (Houston et al. 1969. Mycologia 6:1168-1171) with 50 μg thiamine per liter and minus the micronutrients and agar.
[b]Average of three replicates. Means followed by the same letter do not differ significantly.

fragmented mycelium. Also, thiamine depletion would more likely increase the decay resistance of wood if fragmented mycelium were the natural source of inoculum, but basidiospores are the principal source of inoculum in field exposures.

Gloeophyllum trabeum was able to grow on media with treated wood meal as the only source of thiamine, whether the inoculum was basidiospores or fragmented mycelium, despite the inhibition of growth that occurred as the concentration of wood meal increased (Table 3). However, contrary to the results obtained on media containing thiamine hydrochloride and no wood meal (Table 2), greater thallus weight was produced on media inoculated with fragmented mycelium than with basidiospores. Also, the addition of both 5 μg thiamine and 10 g wood meal per liter of medium resulted in a large increase in growth when fragmented mycelium was the inoculum, but not when it was spores (Table 3). This difference first indicates that something in wood other than depleted thiamine is inhibiting basidiospore germination and, second, it suggests that this inhibitor may account for some of the resistance usually attributed to the alkali treatment.

Depletion of micronutrients.—The sensitivity of *A. niger* to changes in the micronutrient content of media was measured by determining the weight of the thallus produced on media consisting of the macronutrients, 50 μg thiamine per liter, and 0, 12.5, 25, 50, or 100% of the previously described micronutrients. As the micronutrient content increased from 0 to 100%, thallus weight increased linearly from 254 mg to 786 mg ($r^2 = 0.98$, $n = 15$). This result confirmed that *A. niger* responds to changes in the micronutrient content of media (9, 10) but is able to grow (254 mg) on media without micronutrients.

When *A. niger* was grown on media containing treated or nontreated wood meal as the source of micronutrients, growth was not significantly different on media containing 1 or 5 g of gas-treated, hydroxide-treated, or nontreated wood (Table 4). However, growth was significantly less on treated than on nontreated wood when 10 g or more of wood meal per liter of media was the source of micronutrients. Also, adding 12.5 or 25% of the micronutrients normally used in the medium along with 1 g of wood meal per liter increased thallus production from 205 mg to 261 mg or 368 mg with gas-treated wood, from 227 mg to 324 or 430 mg with hydroxide-treated wood, and from 259 to 336 or 398 mg with nontreated wood. The pattern of these increases suggests that the amount of available micronutrients was less in treated than in nontreated wood and that the gas-treated wood had fewer available micronutrients than the hydroxide-treated wood.

Abundant conidia were produced on all media except those containing 10 g or more of hydroxide-treated wood meal per liter. Such inhibition of sporulation suggests, too, that the hydroxide treatment altered some unknown growth factor in the wood.

CONCLUSIONS

The amount of thiamine and micronutrients in ponderosa pine sapwood treated with ammonia gas or ammonium hydroxide and heated to 76.7 C was sufficient to support fungal growth. *Phycomyces blakesleeanus*, a thiamine-requiring fungus, grew on media containing gas- or hydroxide-treated wood and no added thiamine. Thallus production by *A. niger* indicated that although the amount of available micronutrients was less in treated than in nontreated wood, there were sufficient micronutrients to support greater growth than that which occurred on media without micronutrients. Growth of *P. blakesleeanus* and sporulation of *A. niger* were inhibited on media containing hydroxide-treated wood, suggesting that this treatment affected growth factors other than thiamine content. *Gloeophyllum trabeum* grew on media containing gas- or hydroxide-treated wood. Results suggested that the increased decay resistance of alkali-treated wood may be due to factors that inhibit the germination of basiodiospores, and not to thiamine or micronutrient depletion alone.

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