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Metal Accumulation without Enhanced Oxalate Secretion in Wood Degraded by Brown Rot Fungi

Jonathan S. Schilling* and Jody Jellison

Department of Biological Sciences, University of Maine, Orono, Maine 04469

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Brown rot fungi were incubated in agar and agar-wood microcosms containing metallic or hydroxide forms of Al, Cu, and Fe. Metal dissolution was associated with elevated oxalate concentrations in agar, but metals translocated into wood did not affect oxalate accumulation, crystal production, or decay rate, demonstrating a substrate-dependent oxalate dynamic.

Brown rot fungi initiate wood decay by demethylating lignin (9) and oxidatively depolymerizing holocellulose via Fe-dependent Fenton chemistry (12, 17). These fungi characteristically produce oxalic acid during metabolism (20). Secreted oxalate may promote brown rot by weathering soil minerals (2), mobilizing Fe^{3+} (3, 11), and detoxifying metals (21).

Oxalate secretion in response to metals has been demonstrated for brown rot fungi in artificial media (26), and Cu tolerance in wood has been associated with Cu oxalate crystallization (24). Similarly, oxalate has been theorized to chelate excess Fe^{3+} near hyphae, minimizing radical formation (16), although excessive Fe chelation in wood could impede Fenton-based depolymerization (15, 25). It remains uncertain whether metal detoxification in wood is attributable to increased oxalate secretion (resistance) (5), incidental to oxalate production (tolerance) (6, 13), or related to other mechanisms (4, 10). Oxalate dynamics in artificial media often do not reflect those in the wood matrix, where brown rot fungi may decarboxylate (18) and control surplus oxalate (7, 23).

This in situ experiment tests the hypotheses that Al, Cu, and Fe translocated into wood from metallic and soil-relevant hydroxide forms during brown rot enhance oxalate secretion and metal-oxalate crystallization, that Fe enrichment accelerates decay, and that enhanced wood oxalate results in elevated Ca^{2+} accumulation.

The brown rot fungi *Gloeophyllum trabeum* (strain ATCC 11539) and *Fomitopsis pinicola* (strain FP-105877R) were grown for 10 weeks in agar-wood microcosms. Microcosm setup, processing, and analysis were as in previous reports (23), except as follows: two birch strips supported two spruce blocks (cut on the tangential plane), a separate strip between inoculum and blocks supported metal treatments, inoculum and microcosm plates contained 20 ml agar to standardize volume, only acid-extractable oxalate was measured in agar-only microcosms, and the ergosterol analysis column temperature was 35°C.

Treatments were metallic Al shot (99% pure), Cu wire (99.9% pure), and Fe chips (99.98% pure) (Sigma), plus two hydroxide treatments: $Fe(OH)_3$, precipitated from equimolar NaOH and $FeCl_3$ at pH 7, and 100 mM $Al(OH)_3$ in type A agar (Sigma). Metal treatments and their birch supports had no contact with other strips or with spruce.

Positive controls contained no metal treatments, negative controls contained no inoculum, and agar-only controls contained metals on glass coverslips and no wood. Corrosion controls were with or without metallic Fe in direct contact with spruce, with weight loss assessed at week 6. All treatments and controls, except corrosion controls ($n = 4$), included five replicates. Statistical analyses were analysis of variance with protected Tukey's tests and t tests ($\alpha = 0.05$).

Crystals in wood were evaluated with scanning electron microscopy and energy-dispersive spectrometry. Wood was dehydrated, freeze fractured, critical point dried, and Au coated to 300 nm. A Cameca SX 100 microscope at 25 kV, 150 pA beam, and 25 s per frame was used for imaging. X-ray spectra were collected with a Rontec flash EDS detector probing for 100 s.

By week 10, both fungi often produced colored exudates (e.g., green droplets in Cu treatments) where mycelia contacted metals. In agar-wood microcosms, blocks decayed with some treatment metals were darkened.

Metal enrichment in wood blocks was confirmed by cation analysis of milled wood (Table 1). For both fungi, wood incubated with a particular metal treatment was significantly enriched in that metal versus the controls with no added metals, with the exception of *G. trabeum* cultures with metal hydroxides.

Accumulation of treatment metals in wood during decay did not influence wood weight loss at week 10 for either test fungus (Table 2). Ergosterol, a proxy for fungal biomass (22), was similarly unaffected by metals. Wood pH was characteristically lowered during decay by both species and was lower in metallic Fe treatments than in other treatments or positive controls. For each week 10 variable, the overall mean for one test fungus was significantly different than that of the other. For both fungi, wood in direct contact with metallic Fe had higher weight loss at week 6 than did wood with no added Fe.

Soluble and acid-extractable oxalate levels in metal-enriched wood were not different from each other or from positive controls (Fig. 1A and D). For both soluble and

* Corresponding author. Mailing address: 311 Hitchner Hall, Department of Biological Sciences, University of Maine, Orono, ME 04469. Phone: (207) 581-3032. Fax: (207) 581-2537. E-mail: jonathan@maine.edu.

TABLE 1. Cation content in spruce blocks decayed for 10 weeks by brown rot fungi in agar-wood microcosms containing metal treatments

Species	Treatment	Mean cation content (confidence interval) (μmol cm^{-3}) with indicated metal treatment[a]			
		Ca	Al	Cu	Fe
None	Negative control (no fungus)	4.77 (0.25)	<DL	<DL	0.10 (0.04)
F. pinicola	Positive control (no metal)	2.84 (0.30)	0.04 (0.05)*	0.01 (0.00)*	0.09 (0.02)*
	Metallic Al (Al^0)	3.09 (1.29)	1.09 (0.20)‡	0.01 (0.00)*	0.09 (0.01)*
	$Al(OH)_3$	2.36 (0.07)	0.32 (0.21)†	0.01 (0.00)*	0.06 (0.01)*
	Metallic Cu (Cu^0)	3.16 (0.91)	0.04 (0.07)*	0.06 (0.04)†	0.07 (0.01)*
	Metallic Fe (Fe^0)	2.71 (0.61)	<DL	0.01 (0.00)*	0.31 (0.08)†
	$Fe(OH)_3$	2.84 (0.41)	0.06 (0.05)*	<DL	0.43 (0.11)†
G. trabeum	Positive control (no metal)	3.37 (0.43)	0.03 (0.06)*	<DL	0.18 (0.14)*
	Metallic Al (Al^0)	3.07 (0.37)	0.37 (0.20)†	<DL	0.17 (0.11)*
	$Al(OH)_3$	3.04 (0.49)	0.10 (0.15)*	0.01 (0.00)*	0.07 (0.01)*
	Metallic Cu (Cu^0)	3.38 (3.10)	0.04 (0.05)*	0.07 (0.03)†	0.11 (0.03)*
	Metallic Fe (Fe^0)	3.11 (0.31)	<DL	<DL	1.19 (0.38)†
	$Fe(OH)_3$	3.81 (0.54)	0.03 (0.05)*	0.01 (0.01)*	0.20 (0.03)*

[a] Data are adjusted for wood mass loss as cations per wood volume. For each species, values followed by identical symbols are not significantly different; symbols are shown only when treatment effects occur. <DL, below detection limit.

acid-extractable wood oxalate, the pooled mean ($n = 30$) in *G. trabeum* cultures was significantly higher than in *F. pinicola* cultures. This pattern was reversed in the agar of agar-wood (Fig. 1B and E) and agar-only microcosms (Fig. 1C and F), where *F. pinicola* produced substantially more oxalate. Oxalate production in agar was enhanced by some metals, particularly Fe in agar-only microcosms. Soluble oxalate fractions were generally higher in agar than in wood, although soluble agar oxalate in agar-wood *G. trabeum* microcosms was below detection.

Microscopy and X-ray analysis revealed copious Ca oxalate crystals in wood degraded by *F. pinicola*, while crystals were not observed in wood degraded by *G. trabeum*. Import of Ca^{2+} consequent to wood oxalate secretion was not observed for either fungus, and wood Ca^{2+} concentrations were not affected by treatments (Table 1). No Cu oxalate was observed, and crystal microanalysis generated only Ca and Au peaks, with no associated treatment metals.

Reduced oxalate solubility in wood may be due to esterification with wood fibers (14) instead of crystallization with Ca^{2+} or Cu^{2+}. Because metal enrichment did not affect the decay rate or fungal biomass, these fungi may have detoxified metals via intracellular sequestration or perhaps by pH modulation (10), evident in this study during degradation of Fe-enriched wood.

Only *F. pinicola*, a more common forest floor decomposer than *G. trabeum* (8), mobilized metals from soil-relevant hydroxides. For both fungi, enhanced decay with Fe in contact with wood suggests electrolytic corrosion (1) and encourages caution when interpreting Fe-promoted brown rot (19).

TABLE 2. Weight loss, ergosterol (fungal biomass), and pH in spruce decayed by brown rot fungi in agar-wood microcosms containing metal treatments in contact with (week 6) or separate from (week 10) wood blocks

Fungus	Wk	Treatment	Mean (CI)[a]		
			% Weight loss	Ergosterol (μg/g)	pH (upper CI)
None		Negative control (no fungus)	0.5 (0.0)	0.0 (0.0)	4.4 (0.0)*
F. pinicola	10	Positive control (no metal)	27.2 (5.7)	7.6 (1.2)	2.9 (0.1)*
		Metallic Al (Al^0)	23.5 (4.9)	9.3 (1.0)	3.2 (0.1)*
		$Al(OH)_3$	32.2 (2.9)	10.2 (1.4)	3.1 (0.2)*
		Metallic Cu (Cu^0)	20.8 (5.5)	8.0 (0.7)	3.0 (0.1)*
		Metallic Fe (Fe^0)	20.4 (6.0)	9.4 (1.4)	2.8 (0.1)†
		$Fe(OH)_3$	26.6 (3.1)	8.7 (0.6)	3.0 (0.1)*
	6	Metallic Fe (wood contact)	30.1 (9.3)		
		Positive control (no Fe)	12.8 (2.7)		
G. trabeum	10	Positive control (no metal)	10.6 (1.2)	19.5 (9.0)	3.5 (0.1)*
		Metallic Al (Al^0)	13.0 (3.1)	24.4 (10.3)	3.4 (0.1)*
		$Al(OH)_3$	12.7 (4.4)	21.9 (9.5)	3.5 (0.1)*
		Metallic Cu (Cu^0)	12.1 (5.2)	31.9 (11.4)	3.5 (0.1)*
		Metallic Fe (Fe^0)	16.2 (2.5)	24.8 (12.1)	3.2 (0.1)†
		$Fe(OH)_3$	9.5 (1.8)	21.7 (3.5)	3.4 (0.1)*
	6	Metallic Fe (wood contact)	22.4 (7.4)		
		Positive control (no Fe)	10.5 (2.8)		

[a] The week 6 treatment effect is significant for both fungi. For each species, values followed by identical symbols are not significantly different; symbols are shown only when treatment effects occur. CI, confidence interval.

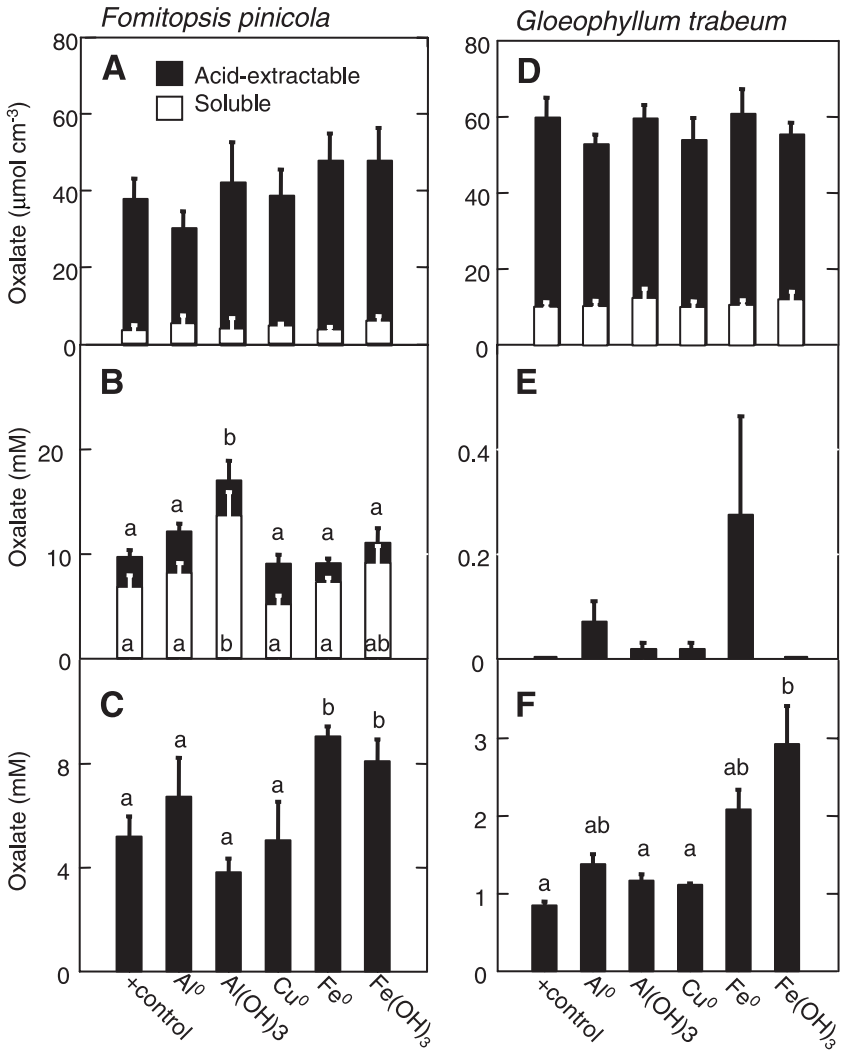

FIG. 1. Oxalate at week 10 in microcosms containing either *F. pinicola* (A to C) or *G. trabeum* (D to F). Oxalate in agar-wood microcosms containing spruce blocks is shown from both the wood component (A and D) and the agar component (B and E), and oxalate (acid extractable only) from agar-only microcosms is shown from agar (C and F). Wood oxalate data are adjusted for mass loss as oxalate per wood volume. In each graph, bars with the same fill pattern and same letter are not significantly different. Letters are included only when treatment effects occur.

The difference in agar oxalate patterns between microcosms with and without wood blocks suggests that wood may influence oxalate secretion in soil mycelia. Although this could influence nutrient acquisition and forest biogeochemistry, Ca^{2+} translocation was not necessarily dependent on oxalate concentrations, as hypothesized, and was against the wood-agar Ca gradient.

This work demonstrates a capacity for brown rot fungi to control the extracellular environment differently inside and outside the wood matrix. Metal tolerance among brown rot fungi, including Cu intolerance in *G. trabeum*, has been associated with oxalate production patterns in artificial media (13). These correlations have been inconsistent (4), and differences observed in this study suggest that in situ trials are valuable for evaluating the role of oxalate both in metal tolerance/resistance and in oxidative brown rot.

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