

# Oxalate regulation by two brown rot fungi decaying oxalate-amended and non-amended wood

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## Abstract

Oxalic acid secretion by brown rot wood-degrading fungi has been proposed to function in pH control and non-enzymatic biodegradation. Although oxalate production in liquid cultures of brown rot fungi commonly correlates with glucose oxidation, excess oxalate accumulation in wood during oxidative decay could impede  $\text{Fe}^{3+}$  reduction by fungal-derived chelators and thus inhibit brown rot. In this study, we pre-treated spruce wood with various oxalate concentrations and subjected it to brown rot decay by *Fomitopsis pinicola* and *Meruliporia incrassata* in agar- and soil-block trials. In agar-block microcosms containing wood pre-treated with 0, 1, 10 or 100 mM sodium oxalate, test fungi equalized wood oxalate and pH at week 12 of decay by either increasing or reducing wood oxalate, depending on the pre-treatment. Oxalate reductions in wood were not accompanied by increases in agar oxalate. During soil-block decay of wood pre-treated with 0 or 50 mM oxalate, oxalate and pH regulation were time-dependent and more variable. Wood oxalate levels did not increase with increasing fungal biomass (per ergosterol); however, decreases in oxalate were not explained by enhanced oxalate catabolism activity,  $\text{Ca}^{2+}$  import, or translocation of oxalate into the soil. Our results suggest that brown rot fungi may optimize extracellular oxalate during wood decay, and that soil characteristics may influence this dynamic.

**Keywords:** biodegradation; cations; decarboxylation; dry rot; iron; oxalic acid.

## Introduction

During wood degradation, brown rot fungi circumvent the lignin barrier in wood primarily by demethylating aryl methoxy groups on lignin rings (Filley et al. 2002). This modification provides access to holocellulose without fully degrading lignin, and brown rot fungi may subsequently initiate depolymerization oxidatively (Cowling and Brown 1969). Non-enzymatic decay likely involves  $\text{HO}^{\cdot}$  produced from  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  via the Fenton reaction (Koenigs 1974), with  $\text{Fe}^{3+}$  reduction mediated by a fun-

gal-derived compound (Goodell et al. 1997; Hyde and Wood 1997; Kerem et al. 1998). Although fungal secretion of oxalic acid may enhance  $\text{Fe}^{3+}$  availability during brown rot by reducing pH and solubilizing soil-bound  $\text{Fe}^{3+}$  (Varela and Tien 2003), it is unclear if this facilitative role is mechanistic or incidental.

Oxalate accumulation has historically been a distinguishing physiological characteristic between brown and white rot fungi (Takao 1965). Unlike white rot fungi, which often decarboxylate extracellular oxalate (Shimazono 1955; Makela et al. 2002), brown rot fungi typically accumulate oxalate in culture media. In addition to solubilizing soil iron, it has been theorized that oxalate produced by brown rot fungi functions in acid-hydrolyzing cellulose (Green et al. 1991), detoxifying metals (Collett 1992; Clausen et al. 2000; Clausen and Green 2003), reducing  $\text{Fe}^{3+}$  (Schmidt et al. 1981), and chelating calcium from wood pit membranes (Green et al. 1996).

Research into the physiology of oxalate production by brown rot fungi suggests that oxalic acid is a metabolic product of glucose oxidation (Munir et al. 2001). Generating oxalate instead of  $\text{CO}_2$  from TCA and GLOX cycles may be efficient in high carbon/nitrogen wood. Without oxalate decarboxylation or alternative oxalate regulation, oxalate would accumulate as a function of fungal respiration. A direct correlation between glucose metabolism and oxalate accumulation has been demonstrated in liquid culture for brown rot fungi (Micales 1994; Munir et al. 2001).

Emergent brown rot decay theory, however, suggests that oxalate could impede non-enzymatic degradative mechanisms at high physiological concentrations (Varela and Tien 2003). Oxalate improves dissolution of soil  $\text{Fe}^{3+}$  (Stone 1997), bound primarily to hydroxides in aerobic soils (Jellison et al. 1997). In the dark, however, oxalate does not reduce  $\text{Fe}^{3+}$  (Henry 2003), and thus oxalate cannot initiate Fenton chemistry. Instead, iron is likely reduced by a separate fungal-derived chelator such as 2,5-dimethoxybenzoquinone (2,5-DMBQ) (Paszczynski et al. 1999). Because oxalate could interfere with chelator-mediated iron reduction when oxalate/ $\text{Fe}^{3+}$  ratios are higher than 1:1, brown rot fungi may benefit by regulating excess oxalate. Varela and Tien (2003) have demonstrated that 2,5-DMBQ-mediated iron reduction and subsequent lipid peroxidation are inhibited at higher oxalate levels.

There is also observational evidence that brown rot fungi may be capable of regulating extracellular oxalate. *Gloeophyllum trabeum*, for example, can cause significant cellulose depolymerization while accumulating less oxalate than other fungi (Connolly and Jellison 1994). Espejo and Agosin (1991) demonstrated significant  $^{14}\text{C}$  evolution from  $^{14}\text{C}$ -labeled oxalic acid in cultures of *G. trabeum* and *Postia placenta*, indicative of enzymatic oxalate catabolism. The oxalate-catabolizing enzyme

oxalate decarboxylase (ODC) has also been purified from *Postia placenta* (Micales 1995). Combined, these data suggest that brown rot fungi may be capable of regulating extracellular oxalate during wood decay and that these fungi may benefit mechanistically by doing so.

For our study, we amended spruce wood with different oxalate concentrations and subjected it to colonization by two brown rot fungi in agar- and soil-block microcosms. We tested a *Fomitopsis pinicola* isolate that accumulates significant amounts of oxalate in liquid culture, and a *Meruliporia incrassata* isolate that produces less oxalate and may import  $\text{Ca}^{2+}$  to bind oxalate, as theorized for "dry rot" fungi (Palfreyman et al. 1996). Our study had three goals: (1) to test if wood oxalate accumulated linearly with increasing fungal biomass during decay; (2) to determine if test fungi would equalize different initial wood oxalate levels; and (3) to explore the fate of oxalate when levels in wood were reduced, either via catabolism, translocation out of the wood matrix, or "neutralization" with translocated  $\text{Ca}^{2+}$ .

## Materials and methods

### Treatments, growth, and harvest

Brown rot isolates *Meruliporia incrassata* (Berk. et Curt.) Murr. (isolate No. mfstoner1) and *Fomitopsis pinicola* (Sw.:Fr) P. Karst. (isolate No. FP-105877R) were maintained on 2% (w/v) malt extract (Difco, Detroit, MI, USA) solidified with 2% Bacto agar (Difco). Inoculum for microcosm trials was obtained by cutting agar squares (1 cm<sup>2</sup>) from the mycelial margin in 2-week-old cultures.

For agar-block trials, inoculum was placed on 20 ml of solidified medium between two parallel 5-mm-O.D. glass rods embedded in the agar. Spruce heartwood blocks (transverse, 25×25×7 mm) were placed on the rods and away from inoculum to avoid direct agar contact. For modified standard soil-block trials (ASTM 1986), jars were filled half-full with 1:1:1 potting soil/peat/vermiculite soil mix moistened with distilled water. Four inoculum squares were placed on two birch feeder strips resting on the soil surface. After 2 weeks of growth, two spruce blocks (transverse, 25×25×12.5 mm) were added transverse-side-down to each jar.

Prior to inoculation, spruce blocks were soaked in sodium oxalate (Sigma, St. Louis, MO, USA) solutions for 48 h in the dark at 21°C. Oxalic acid was not used due to possible acid hydrolysis. Oxalate levels for agar-block treatments were 0, 1, 10, and 100 mM ( $n=6$  per treatment), and were 0 and 50 mM for soil-block treatments ( $n=4$  per treatment/harvest). Blocks were oven-dried at 95°C to determine dry weights.

Control treatments consisted of both time-zero wood treatments and incubated microcosms without fungal inoculum. A post-hoc control trial was also used to compare treatment efficacy between soaking and vacuum aspirating blocks of both sizes ( $n=4$  per treatment) over 48 h. In addition, wood treated with 0 or 50 mM  $\text{NaSO}_4$  instead of sodium oxalate was decayed in soil-block jars ( $n=4$  per treatment) by both fungi for 12 weeks to test ionic strength and sodium effects on decay. All microcosms were maintained at room temperature during growth.

Agar-block treatments were harvested in week 12, while soil-block treatments were harvested in a time series in weeks 2, 4, 8 and 16. In both agar and soil harvests, a block was removed, weighed, dried at 95°C and re-weighed to quantify moisture content, weight loss, and density. The dried block was milled in

a Wiley mill through a 20-mesh filter, and the wood flour was used for organic acid and cation analysis.

In soil-block jars, the additional paired block was thoroughly cleaned of surface hyphae, weighed, and milled without drying. Exactly 0.4 g of flour was stored immediately in 5 ml of MeOH at 4°C as per Newell et al. (1988) prior to ergosterol analysis. The remaining flour was stored at -70°C for oxalate catabolism assays.

Two vertical 15 mm I.D. soil cores were taken adjacent to the blocks in soil-block jars. This soil was oven-dried and homogenized. Agar from agar-block microcosms was stored at -70°C along with soil for subsequent organic acid analysis.

### Oxalate and pH

Dried wood flour and deionized water at a ratio of 1:20 (w/v) were allowed to equilibrate for 2 h. After measuring the pH, 2 ml was removed, filtered through polyethersulfone (PES) to 0.22  $\mu\text{m}$ , and analyzed by HPLC as "soluble" oxalate. The remaining sample was acidified with HCl to 0.2 N, extracted overnight, and buffered with phosphate buffer (pH 1.35). The pH was adjusted to 1.35 with 1 N NaOH and samples were filtered as above. This oxalate fraction was considered "acid-extractable" and was designed to include both membrane-bound oxalate and crystallized calcium oxalate.

For agar media analyses, agar was added to deionized water at a ratio of 1:1 (v/v). Soil analysis of the soil-block was also in deionized water at a ratio of 1:15 (w/v). Both agar and soil samples were acid-extracted as above and analyzed by HPLC.

HPLC organic-acid separations were at 30°C on an Aminex HPX-87H (Bio-Rad, Richmond, CA, USA) ion-exchange column (9  $\mu\text{m}$ , 300×7.8 mm I.D.), with a mobile phase of 25 mM  $\text{H}_2\text{SO}_4$  + 10 mM  $\text{NaSO}_4$  pumped at 0.6 ml min<sup>-1</sup>. Injections were of 20  $\mu\text{l}$ , detection was at 210 nm, and quantification was by peak area. Oxalate was verified by peak loss after incubation with oxalate oxidase (Sigma). Extraction efficiency was measured as the coefficient of variation (CV) among 12 extractions of a single 10 mM-treated wood block. Variation among 10 separate injections was also quantified.

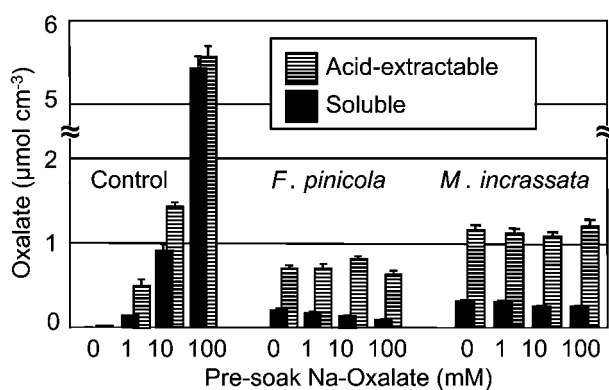
### Ergosterol analysis

To assess fungal biomass in decaying wood, wood flour stored in MeOH was extracted for ergosterol similar to the method of Tank and Webster (1998). Samples were refluxed, saponified with 4% KOH in ethanol, refluxed again and centrifuged twice with one MeOH wash. To the combined supernatant, 1 ml of deionized water and 2 ml of pentane were added and mixed. After 15 min, the pentane layer was removed and evaporated overnight. The precipitate was dissolved in 2 ml of MeOH, filtered through PTFE to 0.2  $\mu\text{m}$ , and analyzed immediately by HPLC.

Ergosterol separations were at 40°C on a Synergi Hydro-RP (Phenomenex, Torrance, CA, USA) reverse-phase column (5  $\mu\text{m}$ , 250×4.6 mm I.D.) with MeOH eluent pumped at 1 ml min<sup>-1</sup>. Injections were of 20  $\mu\text{l}$ , detection was at 282 nm, quantification was by peak area, and peak resolution was verified against 7-dehydrocholesterol. Ergosterol → biomass conversions were avoided due to historic variability (Charcosset and Chauvet 2001), and ergosterol concentration ( $\mu\text{g g}^{-1}$ ) was used instead.

### Oxalate catabolism activity

Frozen wood flour was warmed and 0.2 g was added to 4 ml of 2 mM sodium oxalate in deionized water. Baseline samples were immediately boiled for 5 min. Duplicate samples were incubated at 40°C for 2 h prior to boiling. Samples were acid-extracted and analyzed by HPLC, and activity was determined as the con-



**Figure 1** Mean oxalate concentration ( $\pm$  SE) in dried wood flour after 12 weeks of decay in agar-block microcosms with no inoculum (control) or with *Fomitopsis pinicola* or *Meruliporia incrassata*, and as a function of wood pre-treatment with 0, 1, 10, and 100 mM sodium oxalate. Oxalate expressed per approximate wood volume.

centration of oxalate reactant loss during incubation. Due to peak interference in the wood matrix, we could not include data on formate production after incubation.

#### Wood cation analysis

Dried wood flours from time-zero and week-16 soil-block jars were dry-ashed at 550°C, dissolved in dilute HCl, and analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES) for calcium (Ca), potassium (K), magnesium (Mg), aluminum (Al), iron (Fe), manganese (Mn), zinc (Zn), and sodium (Na).

#### Statistics

One- and two-way ANOVA were used to analyze oxalate treatment and treatment/species effects in agar-block trials, and treatment/time effects in soil-block trials. Treatment interactions were tested for significance after transformation. Prior to analysis, percentage and ratio data were log-transformed, and pH data were converted to  $[H^+]$ . All analyses were performed using SYSTAT, with the significance level set at 0.05.

## Results

### Agar-block experiment

**Controls** Increasing sodium oxalate pre-soak concentration increased the oxalate levels in wood prior to fungal degradation, and increased the wood pH due to the nature of sodium oxalate as a basic salt. After 12 weeks in non-inoculated control microcosms, these oxalate (Figure 1) and pH (Table 1) patterns were unchanged, and no oxalate was detectable in the agar. The coefficient of variation (CV) among 12 separate extractions of a 10 mM control wood flour was 2.7%, and among 10 replicate HPLC injections was 1%.

**Wood exposed to fungi** After 12 weeks of decay by *Fomitopsis pinicola*, wood weight-loss was moderate (Table 1), with no effect of oxalate pre-treatment concentration ( $P=0.852$ ). There was also no oxalate pre-treatment effect on final wood pH ( $P=0.416$ ) or on final oxalate concentration in the agar medium ( $P=0.881$ ). Week-12 wood acid-extractable oxalate levels were statistically the same, regardless of wood oxalate pre-treatment ( $P=0.142$ ), while week-12 soluble oxalate was treatment-dependent ( $P<0.001$ ), being higher in 0 and 1 mM than in 10 and 100 mM oxalate pre-treatments (Figure 1).

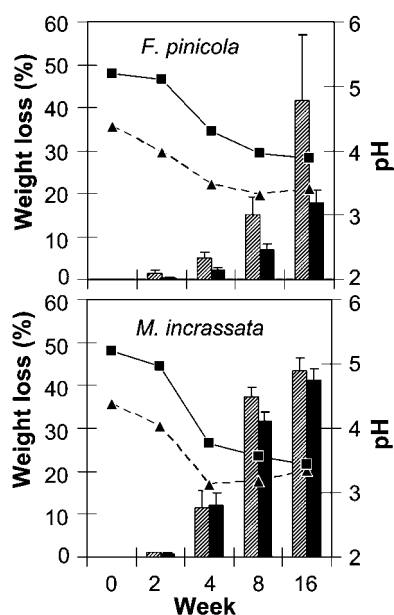
In wood decayed for 12 weeks by *Meruliporia incrassata*, oxalate pre-treatment had no effect on wood weight-loss ( $P=0.472$ ), wood pH ( $P=0.547$ ), or agar oxalate concentration ( $P=0.448$ ) (Table 1). Final acid-extractable oxalate levels were statistically the same, irrespective of oxalate pre-treatment levels ( $P=0.614$ ), and soluble wood oxalate was dependent on oxalate pre-treatment ( $P=0.003$ ), again being higher in 0 and 1 mM than in 10 and 100 mM pre-soaks (Figure 1).

Two-way ANOVA showed significantly more wood weight-loss in *F. pinicola* cultures than for *M. incrassata*, as well as lower pH, less soluble and acid-extractable oxalate, and less oxalate in the agar medium at week 12 ( $P<0.001$  in each case).

**Table 1** Week 12 agar-block wood weight-loss, wood pH, and agar oxalate according to wood oxalate pre-treatment and test fungus.

Test fungus	Oxalate pre-treat (mM)	Wood		Agar oxalate (mM)
		Weight loss (%)	pH	
Control (no inoculum)	0	0.04 (0.03) <sup>a</sup>	4.43 <sup>a</sup>	0.00 (0.00) <sup>a</sup>
	1	0.01 (0.03) <sup>a</sup>	4.74 <sup>b</sup>	0.00 (0.00) <sup>a</sup>
	10	0.03 (0.01) <sup>a</sup>	5.59 <sup>c</sup>	0.00 (0.00) <sup>a</sup>
	100	0.00 (0.01) <sup>a</sup>	5.64 <sup>c</sup>	0.00 (0.00) <sup>a</sup>
<i>F. pinicola</i>	0	15.98 (1.13) <sup>b</sup>	3.43 <sup>d</sup>	0.85 (0.13) <sup>b</sup>
	1	15.10 (1.66) <sup>b</sup>	3.40 <sup>d</sup>	0.77 (0.10) <sup>b</sup>
	10	16.50 (2.30) <sup>b</sup>	3.40 <sup>d</sup>	0.83 (0.13) <sup>b</sup>
	100	16.97 (0.70) <sup>b</sup>	3.36 <sup>d</sup>	0.94 (0.28) <sup>b</sup>
<i>M. incrassata</i>	0	7.20 (1.38) <sup>c</sup>	3.76 <sup>e</sup>	2.05 (0.64) <sup>c</sup>
	1	6.28 (0.95) <sup>c</sup>	3.84 <sup>e</sup>	2.96 (0.47) <sup>c</sup>
	10	5.25 (0.99) <sup>c</sup>	3.73 <sup>e</sup>	2.20 (0.07) <sup>c</sup>
	100	5.23 (0.30) <sup>c</sup>	3.77 <sup>e</sup>	2.22 (0.54) <sup>c</sup>

Means ( $\pm$  SE) followed by same letter are not significantly different.



**Figure 2** Mean wood weight-loss ( $\pm$ SE) and pH per time in 0 or 50 mM oxalate pre-treated wood decayed in soil-block microcosms by *Fomitopsis pinicola* or *Meruliporia incrassata*. Weight loss: ▨ 0 mM; ■ 50 mM; wood pH: ---▲--- 0 mM; ---■--- 50 mM.

Wood decayed by both test fungi had significantly less soluble and acid-extractable oxalate than at time zero in 100 mM pre-soaks ( $P < 0.001$  for each), demonstrating that oxalate was reduced in certain treatments to achieve the final level.

### Soil-block experiment

**Controls** Wood pre-soaked in 50 mM sodium oxalate had higher soluble ( $P = 0.002$ ) and acid-extractable oxalate levels ( $P = 0.003$ ) than in 0 mM treatments at time zero. Wood pH was higher ( $P < 0.001$ ) in 50 mM treatments. These values are presented with oxalate data from the decay trial.

Mean oxalate ( $\pm$ S.D.) in vacuum-aspirated post-hoc control blocks was 1.23 (0.07) mmol g<sup>-1</sup> in large blocks

and 1.79 (0.40) mmol g<sup>-1</sup> in small experimental blocks versus 0.78 (0.04) mmol g<sup>-1</sup> in soaked large blocks and 1.15 (0.09) mmol g<sup>-1</sup> in small experimental blocks. Blocks soaked in sodium oxalate solution without aspiration accumulated approximately 64% of oxalate compared to vacuum aspiration.

Pre-treatment with 50 mM NaSO<sub>4</sub> did not affect wood weight-loss during 12 weeks of decay by *M. incrassata* ( $P = 0.133$ ) or *F. pinicola* ( $P = 0.371$ ). Weight loss in all 0 and 50 mM treatments was 39–51%, with CV  $\leq 4\%$ , with the exception of 50 mM treatments decayed by *F. pinicola* (CV = 24.3%).

**Weight loss and pH** Weight loss in wood decayed by *F. pinicola* increased over time ( $P < 0.001$ ), with significance at each time interval (Figure 2, top). Weight loss was higher in 0 mM sodium oxalate treatments ( $P = 0.015$ ). Time/oxalate treatment interaction was not significant ( $P = 0.914$ ). Wood pH decreased significantly over time ( $P = 0.003$ ) and was lower in 0 mM treatments ( $P < 0.001$ ).

Weight loss in wood decayed by *M. incrassata* increased over time ( $P < 0.001$ ) and at each time interval, except week 16, when decay slowed (Figure 2, bottom). Oxalate pre-treatment did not affect weight loss ( $P = 0.816$ ). Wood pH had significant interactions between time and oxalate pre-treatment ( $P < 0.001$ ). Wood pH was significantly different between pre-treatments at each harvest except week 16, and did not change significantly between week 4 and 8 for either treatment.

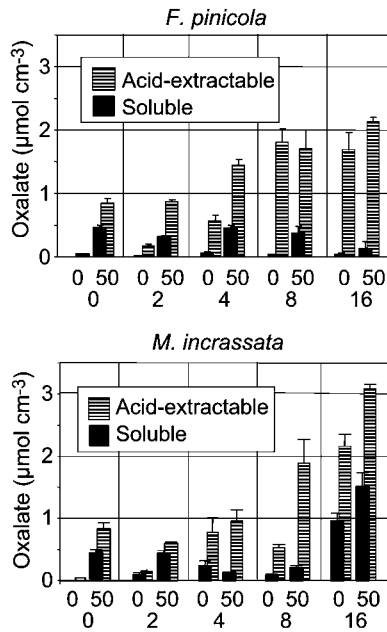
**Ergosterol** Wood ergosterol concentration increased over time ( $P < 0.001$ ), with no oxalate pre-treatment effect on *F. pinicola* ( $P = 0.830$ ) or *M. incrassata* ( $P = 0.380$ ) (Table 2). Regression analysis of ergosterol as a function of pre-incubation wood oxalate from catabolism assays generated weak linear correlations for *F. pinicola* ( $y = 1 \times 10^{-4}x + 2.9149$ ,  $R^2 = 0.097$ ) and *M. incrassata* ( $y = 2 \times 10^{-5}x + 0.7342$ ,  $R^2 = 0.267$ ).

**Oxalate** In wood decayed by *F. pinicola*, soluble oxalate levels had time/oxalate pre-treatment interactions

**Table 2** Ergosterol, soil oxalate, and wood oxalate catabolism activity in soil-block decayed wood according to time, wood oxalate pre-treatment (0 or 50 mM Ox), and test fungus.

Test fungus	Week	Ergosterol ( $\mu\text{g g}^{-1}$ )		Soil oxalate ( $\mu\text{mol g}^{-1}$ )		Oxalate catabolism ( $\mu\text{mol cm}^{-3}$ )	
		0 mM Ox	50 mM Ox	0 mM Ox	50 mM Ox	0 mM Ox	50 mM Ox
Control (no inoculum)	0	0.00 (0.00) <sup>a</sup>	0.00 (0.00) <sup>a</sup>	0.11 (0.03)	0.11 (0.03)	0.07 (0.03)	0.02 (0.04)
	16	0.00 (0.00) <sup>a</sup>	0.00 (0.00) <sup>a</sup>	0.04 (0.06)	0.10 (0.03)	0.07 (0.01)	0.03 (0.05)
<i>F. pinicola</i>	0	0.00 (0.00) <sup>a</sup>	0.00 (0.00) <sup>a</sup>	0.11 (0.03)	0.11 (0.03)	0.07 (0.03)	0.02 (0.04) <sup>a</sup>
	2	0.88 (0.51) <sup>a</sup>	0.00 (0.00) <sup>a</sup>	5.81 (1.97)	4.21 (0.62)	5.41 (1.22)	6.02 (0.42) <sup>a</sup>
	4	2.19 (0.32) <sup>a</sup>	1.85 (0.29) <sup>a</sup>	5.77 (2.75)	5.94 (1.99)	2.78 (1.00)	3.07 (0.51) <sup>a</sup>
	8	1.76 (0.86) <sup>ab</sup>	3.24 (1.56) <sup>ab</sup>	2.94 (1.71)	0.18 (0.05)	0.11 (2.81)	2.66 (1.11) <sup>a</sup>
	16	7.75 (3.03) <sup>b</sup>	6.94 (1.40) <sup>b</sup>	11.40 (7.25)	0.09 (0.03)	0.94 (2.79)	6.47 (1.85) <sup>a</sup>
	0	0.00 (0.00) <sup>a</sup>	0.00 (0.00) <sup>a</sup>	0.11 (0.03) <sup>a</sup>	0.11 (0.03) <sup>a</sup>	0.07 (0.03)	0.02 (0.04)
<i>M. incrassata</i>	2	1.84 (1.83) <sup>a</sup>	0.50 (0.50) <sup>a</sup>	0.16 (0.05) <sup>a</sup>	1.75 (0.95) <sup>a</sup>	4.63 (0.18)	4.18 (0.27)
	4	9.70 (4.45) <sup>b</sup>	7.29 (0.90) <sup>b</sup>	7.24 (2.60) <sup>b</sup>	10.02 (3.53) <sup>b</sup>	3.87 (0.82)	4.04 (0.51)
	8	14.75 (2.51) <sup>c</sup>	12.96 (2.02) <sup>c</sup>	1.56 (1.05) <sup>b</sup>	6.96 (1.60) <sup>b</sup>	4.95 (0.90)	5.28 (1.12)
	16	17.39 (2.86) <sup>c</sup>	18.68 (3.82) <sup>c</sup>	9.15 (1.36) <sup>b</sup>	7.95 (0.82) <sup>b</sup>	6.71 (1.77)	5.71 (2.19)

Means ( $\pm$ SE) followed by the same letter per treatment are not significantly different, and may reflect pooled treatment means if there are no significant interactions. No letters are given for unprotected ANOVA.



**Figure 3** Mean soluble and acid-extractable oxalate ( $\pm$ SE) according to oxalate wood pre-treatment (0 or 50 mM) and time (week 0, 2, 4, 8 or 16) in wood decayed in soil-block microcosms by *Fomitopsis pinicola* or *Meruliporia incrassata*.

( $P=0.042$ ) (Figure 3, top). Soluble oxalate did not change over time in 0 mM wood pre-soaks and significantly decreased at week 16 in 50 mM wood pre-soaks, resulting in no difference in final soluble oxalate levels. Acid-extractable oxalate also had time/oxalate pre-treatment interactions ( $P=0.05$ ). Acid-extractable oxalate increased significantly at week 4 for 50 mM treatments, and at weeks 2, 4, and 8 for 0 mM treatments. Pre-treatment effects were not significant at week 8 or 16.

In wood decayed by *M. incrassata*, soluble oxalate had time/oxalate pre-treatment interactions ( $P=0.046$ ) (Figure 3, bottom). In 0 mM wood pre-soaks, soluble oxalate increased significantly only at week 16. In 50 mM wood pre-soaks, soluble oxalate significantly decreased at

week 4, and significantly increased at week 16, with no change between weeks 4 and 8.

Acid-extractable wood oxalate in *M. incrassata* cultures had time/oxalate pre-treatment interactions ( $P=0.029$ ). In 0 mM wood pre-soaks, oxalate increased significantly over time, except between weeks 4 and 8. In 50 mM treatments, oxalate increased significantly over time at weeks 8 and 16. The effects of oxalate pre-treatment on both soluble and acid-extractable wood oxalate were not significant at week 4.

Soil oxalate (Table 2) was not dependent on incubation time ( $P=0.214$ ) or wood oxalate pre-treatment ( $P=0.214$ ) in *F. pinicola* microcosms and was highly variable. Soil oxalate in *M. incrassata* treatments was not dependent on wood oxalate treatment ( $P=0.131$ ), but was significantly dependent on time ( $P<0.001$ ), increasing significantly at week 4.

**Oxalate catabolism** Oxalate reducing activity (Table 2) was not affected by oxalate pre-treatment ( $P=0.109$ ) or time ( $P=0.220$ ) in wood decayed by *F. pinicola*. Wood decayed by *M. incrassata* also showed no time ( $P=0.116$ ) or pre-treatment effect ( $P=0.873$ ).

**Cation analysis** Interactions between decay stage (week 0 vs. week 16) and oxalate pre-treatment existed for all cations examined. Decay stage/oxalate treatment combinations are compared individually in Table 3.

Sodium levels in time-zero wood were higher in 50 mM than in 0 mM treatments, and did not change after 16 weeks in non-inoculated control jars. Both *F. pinicola* and *M. incrassata* reduced Na levels in 50 mM-treated wood by week 16 of decay. *Meruliporia incrassata* reduced Na significantly more than *F. pinicola*.

Wood decayed by *F. pinicola* accumulated Mg, Al, and Fe above time-zero concentrations in 0 mM wood pre-soaks, and only Mg in 50 mM pre-soaks. Wood decayed by *M. incrassata* accumulated Ca, Mg, K, Zn, Al, and Fe in 0 and 50 mM pre-soaks; however, Ca, Mg, Al, and Fe accretion was higher in 50 mM pre-soaks.

**Table 3** Week 16 cation analysis of wood pre-treated with 0 or 50 mM oxalate and incubated with no inoculum (C), *F. pinicola* or *M. incrassata*.

Test fungus	Oxalate (mM)	Analyte concentration ( $\mu\text{mol cm}^{-3}$ )			
		Na	Ca	Mg	K
Control	0	0.55 (0.05) <sup>a</sup>	8.35 (0.74) <sup>a</sup>	1.54 (0.13) <sup>a</sup>	2.39 (0.26) <sup>a</sup>
	50	26.00 (2.24) <sup>b</sup>	8.59 (0.658) <sup>a</sup>	1.16 (0.13) <sup>a</sup>	2.10 (0.41) <sup>a</sup>
<i>F. pinicola</i>	0	1.44 (0.18) <sup>a</sup>	7.12 (0.91) <sup>b</sup>	4.43 (1.76) <sup>b</sup>	4.26 (3.01) <sup>b</sup>
	50	15.86 (3.41) <sup>c</sup>	9.15 (0.43) <sup>a</sup>	2.82 (0.47) <sup>c</sup>	3.25 (1.07) <sup>ab</sup>
<i>M. incrassata</i>	0	1.13 (0.13) <sup>a</sup>	11.10 (0.50) <sup>c</sup>	6.77 (0.65) <sup>d</sup>	4.29 (1.06) <sup>b</sup>
	50	6.48 (1.50) <sup>d</sup>	12.87 (0.52) <sup>d</sup>	10.43 (1.19) <sup>e</sup>	4.31 (0.91) <sup>b</sup>
Control	0	1.12 (0.13) <sup>a</sup>	0.05 (0.02) <sup>a</sup>	0.24 (0.15) <sup>a</sup>	0.05 (0.02) <sup>a</sup>
	50	0.84 (0.09) <sup>b</sup>	0.04 (0.00) <sup>a</sup>	0.18 (0.21) <sup>a</sup>	0.04 (0.00) <sup>a</sup>
<i>F. pinicola</i>	0	0.96 (0.12) <sup>ab</sup>	0.05 (0.01) <sup>a</sup>	1.53 (1.76) <sup>b</sup>	0.65 (0.65) <sup>ab</sup>
	50	0.83 (0.12) <sup>b</sup>	0.03 (0.00) <sup>a</sup>	0.12 (0.08) <sup>a</sup>	0.11 (0.03) <sup>a</sup>
<i>M. incrassata</i>	0	0.96 (0.15) <sup>ab</sup>	0.09 (0.01) <sup>b</sup>	2.95 (0.74) <sup>c</sup>	1.89 (0.62) <sup>c</sup>
	50	0.92 (0.12) <sup>b</sup>	0.09 (0.02) <sup>b</sup>	6.93 (0.82) <sup>d</sup>	3.24 (0.80) <sup>d</sup>

Results are expressed per approximate wood volume. Means ( $\pm$ SE) in each analyte group followed by the same letter are not significantly different.

## Discussion

Pre-treating spruce blocks with increasing levels of sodium oxalate increased wood oxalate and pH. *Fomitopsis pinicola* and *M. incrassata* equalized these initial discrepancies in agar-block microcosms at week 12 of decay. Oxalate was either accumulated or reduced in the wood matrix to achieve the final concentration, and translocation into the agar medium did not account for wood oxalate decreases. Final oxalate levels in wood decayed by both fungi were lower in 10 and 100 mM wood pre-treatments. These data suggest that oxalate was actively regulated by the decay fungi.

It is notable that wood and agar oxalate levels were higher in *M. incrassata* cultures. In liquid cultures of these isolates, *F. pinicola* reliably produces more oxalate than *M. incrassata* (Schilling and Jellison 2004). This suggests that oxalate regulation may be elicited by a wood component not present in liquid media.

Both isolates characteristically acidified wood during decay, with a consistent final wood pH, regardless of initial pH. The largest fungus-mediated pH decrease occurred in 100 mM wood pre-soaks, and was accompanied by a decrease in wood oxalate, suggesting that oxalic acid may not be solely responsible for the pH reduction.

In sodium oxalate pre-treated blocks used in the soil-block trial, we confirmed a concomitant increase in wood Na, in addition to oxalate and pH increases. Uptake efficiency in these larger spruce blocks, however, was lower, likely due to the increased volume without an increase in transverse-face surface area. Lower initial oxalate levels could make fungus-mediated changes less pronounced and could dilute localized patterns if oxalate is heterogeneously distributed in the wood. Oxalate treatments in this experiment, however, were efficient enough to increase wood oxalate above typical physiological concentrations for the test fungi.

During soil-block decay, wood weight-loss was predictably higher than in agar-block microcosms (e.g., Smith et al. 1996). Decay rate in *M. incrassata* cultures was not affected by sodium oxalate pre-treatments; however, *F. pinicola* showed reduced decay in 50 mM wood pre-treatments and variable decay in wood treated with NaSO<sub>4</sub>. Reduced decay in soil-block jars may be related to stress due to ionic strength or Na<sup>+</sup> that are perhaps less inhibitive in higher-humidity agar-block microcosms.

While both fungi reduced wood pH during soil-block decay, neither equalized pH early in decay, unlike in agar-block cultures. It is unclear if this discrepancy was caused by differences in block volume, wood buffering capacity, or physiological changes in fungal pH regulation.

Acid-extractable wood oxalate patterns also did not demonstrate the tight regulation demonstrated in agar-block microcosms, and may reflect greater complexation of extracellular oxalate. Water-soluble oxalate, however, was equalized by both fungi at intermediate weight loss (15–35%) and at similar levels as in agar-block microcosms. Fungal regulation of soluble oxalate during brown rot may be more critical than regulation of the bound fraction, as bound oxalate may not participate in solu-

bilizing the soil-bound ferric iron necessary to initiate oxidative degradation. Quantifying acid-extractable oxalate likely incorporates crystallized calcium oxalate (Connolly and Jellison 1995) and esterified oxalate (Hunt et al. 2004).

Ergosterol/acid-extractable oxalate correlations were very weak, and soil oxalate did not reflect oxalate export from wood into soil. Furthermore, acid-extractable oxalate accumulation over time was not additive to initial pre-treatment levels. Combined, these data suggest that wood oxalate was metabolized or otherwise reduced during decay, but that these patterns were not consistent among microcosms.

Oxalate decreases in wood decayed in soil-block jars by *F. pinicola* and *M. incrassata* are not clearly explained by oxalate catabolism activity. This test for catabolic activity could be problematic by not adjusting pH to optimize enzymatic catabolic activity and by attempting to measure small oxalate losses after a relatively large oxalate addition. If our oxalate and catabolism data accurately reflect wood oxalate dynamics, however, an alternative fate for oxalate would be necessary.

One alternative sink for excess soluble oxalate, proposed by Bech-Andersen (1987), is via crystallization with translocated Ca<sup>2+</sup>. Our ICP data showed an increase in wood Ca after decay by *M. incrassata*; however, cations with similar chemistry such as Mg are also accumulated, as observed in other studies (Cromack et al. 1975; Jellison et al. 1992; Ostrofsky et al. 1997). In *M. incrassata* cultures, cation uptake was higher in 50 mM pre-soaks, in which Na levels decreased significantly over time, suggesting that cation translocation countered a Na<sup>+</sup> gradient. In *F. pinicola*, for which cation accumulation was less pronounced and less treatment-dependent, Na export was lower. In 0 mM pre-soaks decayed by *F. pinicola*, however, cation accumulation was more prevalent, suggesting that pH may also play a role in cation accumulation.

Overall, we have demonstrated that brown rot fungi can regulate wood oxalate and pH during decay, but that regulation may depend on the decay environment. We were not able to fully explain the cause of oxalate decreases in wood during decay, primarily due to variability in our soil-block decay data. Soil-block trials commonly result in more variable wood decay and higher threshold values than agar-block trials when testing wood preservatives (Cockroft 1974; Wazny and Cookson 1994; Jamsa and Viitaniemi 1998), particularly those targeting oxidative decay mechanisms (Backa et al. 1992; Green and Schultz 2003), and soil type has been shown to affect preservative efficacy (Machek et al. 2004). By affecting oxalate regulation, soil characteristics may affect brown rot oxidative decay, and could also alter forest floor processes influenced by wood oxalate levels, such as base cation accumulation. Our current work is focusing on soil matrix characteristics, particularly soil metals, that may affect oxalate regulation and decay efficacy during brown rot wood degradation.

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