

# High-performance liquid chromatographic analysis of soluble and total oxalate in Ca- and Mg-amended liquid cultures of three wood decay fungi

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## Abstract

Two brown-rot wood decay fungi, *Fomitopsis pinicola* and *Meruliporia incrassata*, and the white-rot species *Phanerochaete chrysosporium* were grown for 4 weeks in liquid culture at 0.35, 0.70, 1.05, and 5.00 mM calcium (Ca) and 1.35 and 2.70 mM magnesium (Mg) concentrations. Soluble and total oxalate levels were quantified using a revised ion-exchange HPLC protocol developed specifically for resolving oxalate and other organic acid anions from medium components. Total oxalate concentrations in brown-rot filtrate were not significantly different among treatments; however, soluble oxalate decreased significantly with increasing Ca concentration. Higher Mg concentrations increased soluble oxalate levels only slightly. There was a significant decrease in medium pH at 5.00 mM Ca for all species, as well as an apparent increase in decarboxylation activity in brown-rot fungi. Total and soluble oxalate levels in the white-rot cultures were generally below detection for all treatments. The results show a significant influence of Ca on soluble oxalate concentrations not seen previously in the brown-rot species *Postia placenta*.

**Keywords:** biodegradation; brown-rot; calcium; HPLC; oxalic acid; white-rot.

## Introduction

Wood biodegradation is brought about primarily by brown- and white-rot basidiomycete fungi (Dutton et al. 1993). These fungi characteristically produce oxalic acid ( $pK_1=1.23$ ,  $pK_2=4.26$ ) which has been proposed to be a metabolic product of incomplete glucose oxidation in the tricarboxylic acid (TCA) cycle (Munir et al. 2001). Further catabolism of the oxalate anion via decarboxylation is common in white-rot fungi (Shimazono 1955; Dutton et al. 1994; Makela et al. 2002), while brown-rot fungi often accumulate oxalate extracellularly (Shibamoto et al. 1952; Takao 1965; Green et al. 1991; Green and Clausen 1999).

Higher extracellular concentrations of oxalic acid in brown-rot fungi may help lower the extracellular pH in wood and mobilize ferric iron, and these effects may play an important role in non-enzymatic wood decay (Goodell et al. 1997; Hyde and Wood 1997; Diouf et al. 2002; Qian et al. 2002). Oxalate may also precipitate with copper in wood preservatives, resulting in increased copper tolerance in some brown-rot species (Collett 1992; Woodward and DeGroot 1999; Clausen et al. 2000). Oxalic acid production is therefore a familiar aspect of wood decay research.

After oxalic acid is produced, however, functional oxalate concentrations may change extracellularly if the oxalate anion binds with a divalent cation to form a salt. The most common of these precipitates in wood degraded by fungi are calcium oxalate crystals ( $K_{sp}=2.6 \times 10^{-9}$ ). These crystals are effectively insoluble and often contain calcium translocated from other areas of the mycelium (Dutton et al. 1993; Connolly and Jellison 1995; Ghariieb et al. 1998). Calcium translocation and accumulation in wood has been demonstrated both in soil-block experiments and in field decay studies (Cromack et al. 1975; Jellison et al. 1992; Connolly 1996; Ostrofsky et al. 1997).

The reason for calcium translocation and accumulation is not known. Oxalic acid secretion may simply create a sink for passively diffused calcium ions. In addition to calcium, accumulation of other divalent cations such as magnesium has been observed in wood decay studies (Tyler 1982; Ostrofsky et al. 1997). Functional roles for calcium oxalate formation during wood decay have also been hypothesized, including hydrolysis of pectin in pit membranes (Green et al. 1996), decrease in buffering capacity around hyphae (Connolly and Jellison 1994), and neutralization of excess oxalate (Bech-Andersen 1987). In all cases, precipitation of calcium with oxalate should theoretically decrease the soluble fraction of extracellular oxalate.

Because there is interest in total oxalic acid production and soluble oxalate concentration, accurate quantification of both insoluble and soluble oxalate fractions is valuable. Soluble oxalate determinations have been common, and analytical techniques include calorimetric assay (Espejo and Agosin 1991; Micales 1994), high-performance liquid chromatography (HPLC) (Dutton et al. 1993; Jordan et al. 1996; Urzua et al. 1998), and gas chromatography (Munir et al. 2001). Ion-exchange HPLC is the most widely used of these techniques, but methods have been unreliable due to interfering peaks and poor sensitivity. Total oxalate (acid-extractable) has generally been ignored in previous wood decay studies due either to the acidic conditions required during analysis or to the difficulty in using traditional  $KMnO_4$  titrations (Bateman and Beer 1965).

We designed this study to quantify both soluble and total oxalate production by several wood decay fungi growing in various calcium and magnesium concentrations. We wanted to develop an ion-exchange HPLC method that could resolve oxalate from medium components and that used an acidic mobile phase suitable for total oxalate determination. Our immediate goal was to test the effects of these base cations on oxalic acid production and oxalate solubility, and to monitor the effects on pH and oxalate catabolism. We also wanted to compare brown-rot species with low decarboxylation activity to a decarboxylating white-rot species, and compare our results to a previous experiment with *Postia placenta* (Micales 1995a) where similar calcium treatments had no detectable effect on soluble oxalate.

## Materials and methods

### Treatments and growth conditions

Brown-rot isolates *Meruliporia incrassata* mfstoner1 and *Fomitopsis pinicola* FP-105877R, and the white-rot isolate *Phanerochaete chrysosporium* ATCC 24725 were grown for 2 weeks on 2% (w/v) malt extract broth agar plates solidified with 2% Bacto agar (Difco, Detroit, MI, USA). Inoculum blocks with a top surface area of 1 cm<sup>2</sup> were cut from the outer edge of the growing cultures. Two blocks were floated on the surface of 200 ml sterile modified basal salts solution (Highley 1973) per 500 ml flask. The sole carbon source was 2% (w/v) glucose.

Magnesium (Mg) treatments were amended to 1.35 mM (low) and 2.70 mM (high) with MgSO<sub>4</sub>·7 H<sub>2</sub>O (Sigma Chemical Co., St. Louis, MO, USA). Calcium (Ca) treatments were amended with CaCl<sub>2</sub>·2 H<sub>2</sub>O (Sigma) to 0.35, 0.70, 1.05, and 5.00 mM, similar to Micales (1995a). There were 8 treatments representing each Ca/Mg combination, and there were 4 replicate flasks per treatment and per test species. Replicated non-inoculated cultures for each treatment were included as controls, with oxalate and pH measurements after the incubation period. Cultures were incubated without shaking for 4 weeks at room temperature, approximately 21°C.

### Soluble oxalate and pH

At the time of harvest, duplicate 5 ml aliquots of unfiltered culture filtrate were removed from each flask, one for soluble oxalate and pH and the other for the decarboxylation assay. The oxalate/pH aliquot was transferred to a sealable PTFE tube, the pH was measured on an Accumet Research AR10 pH meter, and the samples were stored at -70°C. The decarboxylation assay was performed immediately on the second 5 ml aliquot in glass test tubes as described later.

For soluble oxalate determination, filtrate samples were thawed to 4°C, and filtered through 2.2 µm PES syringe filters into autosampler tubes for immediate HPLC analysis. After verifying the absence of oxalate in non-inoculated cultures, positive controls for each treatment were created by amending with sodium oxalate (Sigma) to a final concentration of 2 mM. These were analyzed for soluble oxalate in triplicate to determine the effect of treatment on oxalate solubility in the absence of the fungal isolates. Positive controls were then extracted for total oxalate for use as positive standards described below.

### Total oxalate

Total oxalate extractions were achieved by acidifying the remaining flask cultures with HCl to a final concentration of 0.2 N. Acid-

ified cultures were shaken gently, extracted overnight, shaken again, and buffered with phosphate buffer (pH 1.35). The pH was adjusted to 1.35 with 1 N NaOH. Samples were then filtered through 0.22 µm filters and analyzed immediately.

Positive standards for total oxalate were included in triplicate in order to determine the efficacy of the total oxalate extraction procedure with these treatments.

### Decarboxylation assay

Decarboxylation activity was measured indirectly and only for brown-rot fungi. Ten µmol sodium oxalate was added to 5 ml culture filtrate to achieve a baseline oxalate concentration of 2 mM. Samples were incubated at 40°C for 2 h and immediately frozen to -70°C to slow enzymatic activity. These samples were then thawed to 4°C, boiled for 5 min, filtered, and analyzed for soluble oxalate. Oxalate catabolism is expressed as µmol min<sup>-1</sup>.

### HPLC

All oxalate HPLC analyses were performed using a Hitachi system with an L6000 reciprocating piston pump, an AS-4000 autosampler, and an L4500A-DAD detector. Separations were at 30°C on an Aminex HPX-87H (Bio-Rad Laboratories, Richmond, CA, USA) strong ion-exchange column (9 µm, 300×7.8 mm I.D., pH range 1–3), protected with a SecurityGuard (Phenomenex, Torrance, CA, USA) guard column. The mobile phase for all analyses was 20 mM H<sub>2</sub>SO<sub>4</sub> (pH 1.40) pumped at a rate of 0.6 ml min<sup>-1</sup>. Injection volume was 20 µl, detection was at 210 nm, and quantification was by peak area.

Oxalate retention time (t<sub>r</sub>), baseline resolution, and column efficiency were determined using sodium oxalate standards both in HPLC-grade water and in basal salts solution. The mobile phase was also compared to a 5 mM H<sub>2</sub>SO<sub>4</sub>/15 mM Na<sub>2</sub>SO<sub>4</sub> mobile phase to clarify the role of pH versus sulfate in oxalate retention and peak efficiency. Medium components were added individually to determine the source of any interfering peaks, and oxalate oxidase (Sigma) was added both to basal salts standards and to fungal culture filtrate to test peak purity by monitoring the loss of the oxalate peak.

Soluble oxalate standards were prepared both in basal salts medium without Ca or Mg sources and in deionized distilled water. All soluble oxalate standards were acidified with HCl to pH 3.00. Total oxalate standard curves were generated for each treatment combination by preparing standards in basal salts media at every concentration of Ca and Mg, with subsequent buffering with phosphate buffer and NaOH as in filtrate samples.

### Data analysis

Post-hoc LSD means comparisons between treatments were performed using SYSTAT after two-way ANOVA. Grouping variables were for both cation concentrations.

## Results

### HPLC detection

Oxalate retention was affected by sulfate concentration as opposed to pH, but peak efficiency was higher in 20 mM H<sub>2</sub>SO<sub>4</sub> than in the sodium sulfate mixture. The H<sub>2</sub>SO<sub>4</sub> mobile phase allowed baseline resolution of oxalate (t<sub>r</sub>=6.95–7.00) from a large NO<sub>3</sub><sup>-</sup> peak (t<sub>r</sub>=5.95–6.00) in the culture medium (Figure 1). Peak loss upon addition of oxalate oxidase was complete in basal salts and in fungal cultures, verifying oxalate peak

purity. System variability (CV) among replicated standards was less than 2%, and retention time did not change during the analyses.

Standard curves for soluble oxalate were replicated at different times with total variability less than 1%. There was no difference between chromatographs from the salts medium and from water other than the  $\text{NO}_3^-$  peak. Total oxalate standard curve slope decreased 13% between the lowest and highest calcium concentrations, but there was no significant effect on  $R^2$  values which averaged 0.98. Magnesium concentrations did not affect total oxalate extraction efficiency.

### Soluble/total oxalate and pH

Culture filtrate pH decreased over time in all three species (Figure 2). Significance between treatments was based on hydrogen ion concentration, and protected LSD means comparisons revealed a significantly lower pH at 5.00 mM calcium treatments for *F. pinicola* ( $p < 0.001$ ) and for *P. chrysosporium* ( $p < 0.001$ ).

While *F. pinicola* total oxalate concentrations were not significantly different among treatments, soluble oxalate concentrations (Figure 3) were significantly different as a function of calcium concentration (Table 1). Magnesium concentration did not significantly affect oxalate levels.

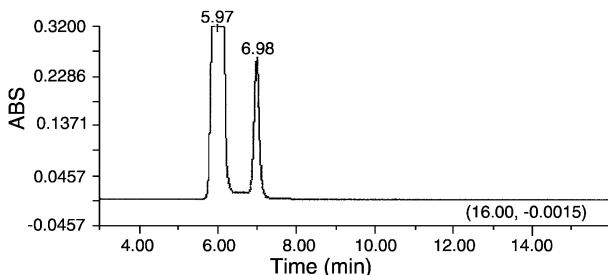
*M. incrassata* total oxalate concentrations were also unaffected by treatment, and levels were notably lower than those for *F. pinicola*. Soluble oxalate (Figure 3) was significantly lower in the 5 mM calcium treatments (Table 1). Magnesium appeared to positively affect oxalate solubility, but the difference was not significant ( $p = 0.091$ ).

Small amounts of soluble and total oxalate were detected in several *P. chrysosporium* replicates among several treatments, although most were below the minimum concentration of the standard curve ( $< 0.01$  mM). There were no significant differences between any treatments. Significant concentrations of another organic acid anion, possibly malate, were also observed and showed no treatment effect.

Non-inoculated positive standards for soluble and total oxalate revealed both a significant effect of calcium on solubility and a significant increase in solubility at the higher magnesium concentration (Figure 4).

### Decarboxylation assay

The decarboxylation assay suggested more oxalate metabolism in *M. incrassata* than in *F. pinicola* (Figure 5).



**Figure 1** HPLC chromatograph showing the separation of oxalate ( $t_R = 6.98$ ) from medium components (nitrate,  $t_R = 5.97$ ), with detection at 210 nm.

**Table 1** Protected\*\* LSD means comparisons of soluble oxalate concentration between fungal cultures with different Ca treatments.

	<i>Fomitopsis pinicola</i>				<i>Meruliporia incrassata</i>			
	0.35 mM	0.70 mM	1.05 mM	5.00 mM	0.35 mM	0.70 mM	1.05 mM	5.00 mM
0.35 mM	-				-			
0.70 mM		-				-		
1.05 mM	**	*	-				-	
5.00 mM	**	**	**	-	**	*	*	-

\* $p < 0.05$ ; \*\* $p < 0.001$ .

Rates of decarboxylation for both brown-rot fungi were significantly higher in the 5.00 mM calcium treatments than at other calcium concentrations.

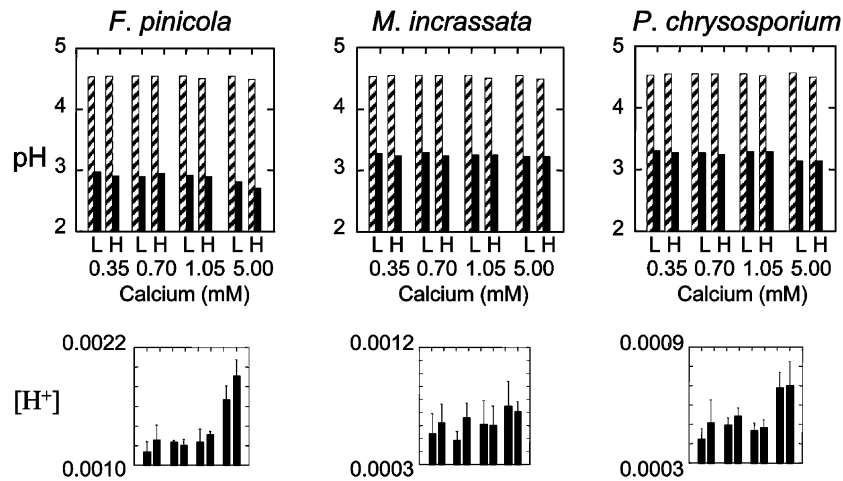
### Discussion

Our HPLC method allows resolution of a pure oxalate peak from an interfering nitrate peak, something which was not achievable using many previously described procedures. Because oxalate elutes before most other organic acid anions during ion-exchange, elution of oxalate after nitrate will also allow determination of other organic acids of interest. The use of an acidic mobile phase is also an important component of our method. This will increase total oxalate accuracy both by avoiding calcium oxalate reprecipitation in the column and by keeping oxalic acid protonated to allow detection of a more homogeneous phase (Dutton et al. 1991). Because of historical problems using ion-exchange HPLC for oxalate determination, these methodological advances are an important contribution of our study.

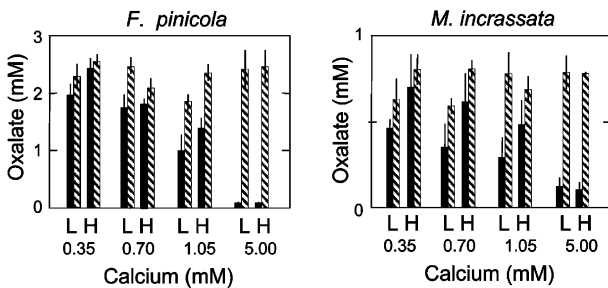
In our fungal cultures, calcium and magnesium availability did not significantly affect total oxalate concentrations in filtrate. For brown-rot species with negligible decarboxylation activities, this suggests that these cation treatments did not affect oxalic acid production.

Solubility of the oxalate anion, however, is clearly affected by calcium. The decrease in soluble oxalate with increasing calcium likely reflects a Le Chatelier shift toward calcium oxalate product as calcium concentration is increased. This is supported by the similarity of oxalate graphs from fungal cultures to the graph of oxalate solubility in non-inoculated standards (Figure 4). The pattern among *M. incrassata* treatments, however, was not as pronounced as in *F. pinicola*, and one explanation could be active regulation of extracellular soluble oxalate concentration by *M. incrassata*. Statistical confidence in this experiment, however, would have been increased with more evenly-spaced treatments over a broader range of concentrations.

Our treatment structure was adapted from a previous study of oxalate in calcium-amended cultures of *Postia placenta* (Micales 1995a). In this earlier study, media calcium concentrations did not affect soluble oxalate levels after two weeks of growth in liquid culture, although low



**Figure 2** Filtrate pH as a function of species and Ca and Mg concentration at time zero and at week 4. Graphs of [H<sup>+</sup>] mirror pH graphs, but include standard deviation (S.D.). L 1.35 mM Mg; H 2.70 mM Mg; ▨ time zero; ■ week 4.



**Figure 3** Soluble and total oxalate ( $\pm$ S.D.) in *F. pinicola* and *M. incrassata* at harvest. ■ soluble; ▨ total.

oxalate levels in the 5 mM treatment were disregarded due to possible calcium interference with the calorimetric kit. The lack of significance may be due to calorimetric kit resolution problems or may be the result of a shorter growth period. *Postia placenta* is also known to produce oxalate decarboxylase (ODC) and catabolize oxalate (Micales 1995b), and this may have complicated the observed effects of calcium on soluble oxalate.

Although a significant effect of calcium on brown-rot filtrate pH was present only in *F. pinicola*, a decrease in pH may be more apparent at discrete sites along growing hyphae. As oxalic acid binds with calcium near the hyphal wall, H<sup>+</sup> will be released and the buffering capacity of the oxalate anion will be lost. Therefore, calcium-oxalate formation may decrease pH in the area immediate to the crystals. Maintenance of a pH differential between the area of crystal formation along the hyphal wall and the wood cell would have direct implications for non-enzymatic oxidative decay mechanisms (Goodell et al. 1997; Jellison et al. 1997).

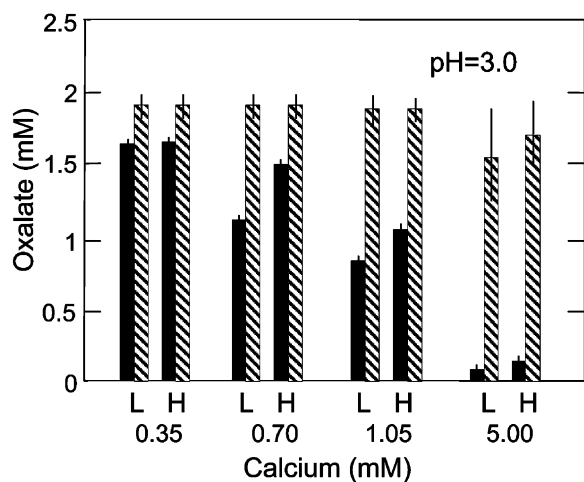
Similar pH dynamics in *P. chrysosporium*, however, complicate our interpretations of oxalate and pH maintenance. *Phanerochaete chrysosporium* did not accumulate any soluble or insoluble oxalate. This fungus may be producing oxalate but decarboxylating it prior to secretion; however, formate, an organic acid anion produced during decarboxylation by oxalate decarboxylase (ODC), was not detected in any treatment. These observations may reflect a mechanism other than oxalic acid

production for pH regulation in cultures of *P. chrysosporium*.

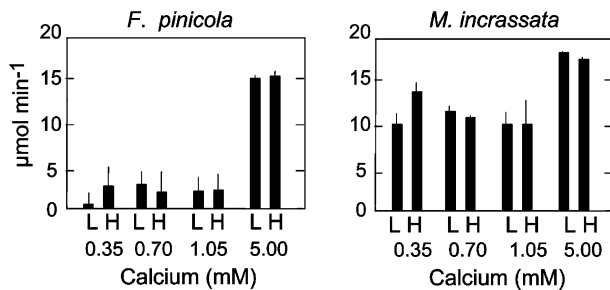
The decarboxylation assay of brown-rot species suggests more oxalate catabolism at high calcium concentrations, but this pattern may be a function of free Ca<sup>2+</sup> in the medium. If oxalic acid production is constant among various calcium treatments, the higher calcium treatments will have more free calcium ions to bind oxalate as it is added, leading to unreliable results when measuring soluble oxalate. This may also explain why decarboxylation activity appears higher for *M. incrassata* which produced less oxalate than *F. pinicola*. Based on these results, we suggest including measures of both soluble and total oxalate when using loss of oxalate reactant as a measure of decarboxylating enzyme activity.

## Conclusion

We have developed an HPLC method for use on fungal culture filtrate that efficiently resolves oxalate from medium components and uses an acidic (pH 1.4) mobile



**Figure 4** Soluble and total oxalate ( $\pm$ S.D.) in non-inoculated basal medium amended to 2.00 mM oxalate with sodium oxalate. ■ soluble; ▨ total.



**Figure 5** Decarboxylation activity at week 4 as a function of species and Ca and Mg concentration. Rate is oxalate consumed per minute over 2 h at 40°C.

phase, allowing a simple and more accurate quantification of both soluble and total oxalate as a function of culture variables. In our study, calcium and magnesium did not affect brown-rot oxalic acid production while calcium significantly affected oxalate solubility. Effects on oxalate solubility are important because soluble oxalate may play an essential role in non-enzymatic brown-rot decay of wood (Goodell et al. 1997). Research is underway to explore this calcium-dependent oxalate partitioning in the wood matrix and as a function of species. Understanding the dynamics of calcium oxalate accumulation in decaying wood is important both for understanding the role of calcium in wood decay mechanisms and for understanding the nature of calcium retention in forest soils increasingly stressed by acidic deposition (Likens et al. 1996).

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