

# Localization and Induction of Oxalate Decarboxylase in the Brown-Rot Wood Decay Fungus *Postia placenta*<sup>1</sup>

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Oxalate decarboxylase (ODC), the enzyme that converts oxalic acid to formic acid and carbon dioxide, was recently isolated from high- and low-decay isolates of the brown-rot wood decay fungus *Postia placenta*. The enzyme is induced in the presence of concentrations of oxalic acid that inhibit growth and is associated with the hyphal surface and hyphal sheath. A probable role for ODC in *P. placenta* is to prevent the overaccumulation of oxalic acid, forming a nontoxic, buffered, low-pH environment that facilitates the decay process. Published by Elsevier Science Limited

## INTRODUCTION

One of the long-standing beliefs of wood-decay research is that oxalic acid is produced and accumulated in large quantities by many brown-rot fungi due to their inability to form oxalate decarboxylase (ODC), the enzyme that converts oxalic acid into formic acid and carbon dioxide (Shimazono, 1955; Takao, 1965; Green & Highley, 1997). This generalization was recently challenged when Micales (1995) detected ODC activity in liquid cultures and mycelial extracts of both high- and low-decay strains of the brown-rot fungus *Postia placenta*. How do ODC formation and oxalic acid production interrelate, and how do both processes facilitate the biodeterioration of wood by *P. placenta*?

The roles of oxalic acid in brown-rot decay are still speculative (Dutton & Evans, 1996) but may involve iron chelation, pH gradient, and free radical formation (Goodell *et al.*, 1996; Hyde & Wood, 1995; Shimada *et al.*, 1996), calcium precipitation and pectin hydrolysis (Green *et al.*, 1995a,b), and acid-catalyzed hydrolysis of hemicellulose and/or cellulose (Schmidt *et al.*, 1981; Green *et al.*, 1991, 1992; Highley *et al.*, 1994; Shimada *et al.*, 1994, 1996; Green & Highley, 1997). Wood test blocks inoculated with *Postia placenta* dropped to a pH of 1.6 after only 1 week of fungal growth (Green *et al.*, 1991). The decrease in pH was correlated with a sharp increase in extractable oxalate (1.2 mg oxalate extracted from 50 mg of wood). Oxalic acid, with a dissociation coefficient ( $K_A$ ) of  $9 \times 10^{-2}$  for its first hydrogen atom, is one of the strongest organic acids (Conant, 1944). In comparison, the  $K_A$  values of formic acid and acetic acid are  $2.14 \times 10^{-4}$  and  $1.86 \times 10^{-5}$ , respectively. How does the fungus protect itself from high concentrations of this reactive compound?

Oxalate decarboxylase may be involved in active pH regulation by the fungus (Micales, 1995). The optimum pH for ODC activity is 1.75–2.20 (Micales, 1995), which corresponds well with the pH of brown-rotted wood (Green *et al.*, 1991). Oxalate decarboxylase may serve as a safety mechanism that prevents the pH of the micro-environment around the fungus from becoming overly acidic. Alternatively, the actual concentration of oxalic acid may be more important than the pH. The localization of the enzyme is important to either hypothesis. If the enzyme is extracellular, it could have an important effect on the micro-environment of the hyphae.

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The objective of this study was to determine whether ODC is primarily an extracellular or intracellular enzyme and whether elevated quantities of oxalic acid and/or extremely low pH can induce ODC production. Strain ME20 was used for the localization experiments because it overproduces ODC, forming as much as 50 times that of other strains (Micales, 1995). It also forms low levels of extracellular carbohydrates (Micales *et al.*, 1990; Micales & Highley, 1990), thus facilitating the removal of extracellular enzymes from the aberrant hyphal sheath. Strain MAD698 was used for the induction studies because it has retained the ability to decay wood and is more representative of the species.

## MATERIALS AND METHODS

### Isolate storage and cultural condition

*Postia placenta* strains MAD698, a standard high-decay isolate, and ME20, a low decay isolate, were maintained in 2% malt-extract agar slants at 4°C for the duration of the study. Subcultures were made as required. Cultures of the isolates were stored in liquid nitrogen in the culture collection of the Center for Forest Mycology Research at the Forest Products Laboratory in Madison, Wisconsin.

### Oxalate assay

Oxalate concentrations were quantified using a microtiter plate adaptation (Micales, 1994) of a diagnostic test kit for oxalate (Sigma Chemical Company, St. Louis, MO).<sup>2</sup> This test kit enzymatically detects the oxalate anion, so results are expressed as concentrations of oxalate rather than oxalic acid. Oxalate concentration was measured in millimoles, based on a standard curve that was constructed using known concentrations of oxalate (Sigma oxalate standards). Samples that resulted in absorbance values greater than 0.7 were re-diluted and re-assayed to be within the logarithmic portion of the standard curve.

### Oxalate decarboxylase assay

Oxalate decarboxylase activity was quantified by determining the rate of oxalate decomposition.

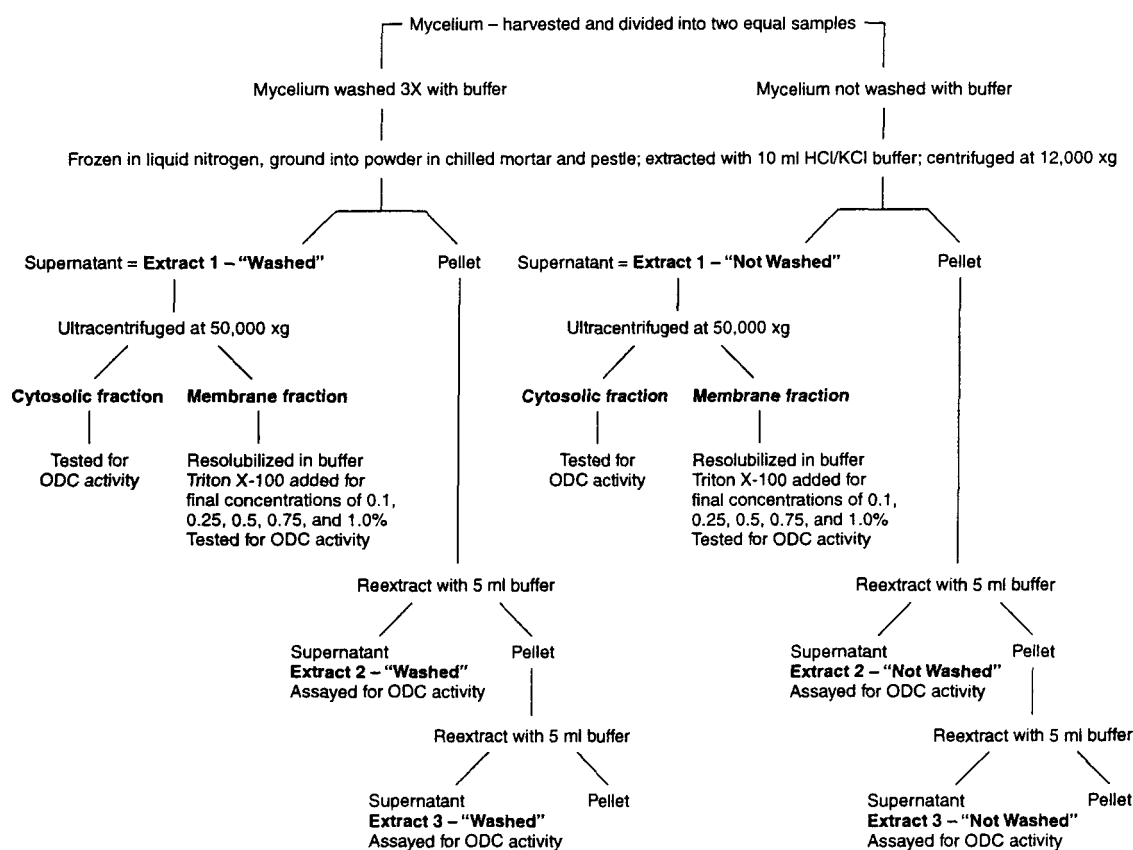
<sup>2</sup>The use of trade or firm names in this report is for reader information and does not imply endorsement by the US Department of Agriculture of any product or service.

Samples were combined with an oxalic acid stock solution to give a final concentration of 2 mM oxalic acid. Water or an appropriate buffer was used in place of the culture filtrate or mycelial extract for a negative control. Preliminary tests with low pH buffers confirmed that the test was not pH-sensitive. The solutions were initially incubated at 40°C for 2 h. Depending upon the enzymatic activity, this test could be repeated for shorter periods (3–90 min) so that the substrate was not totally degraded. The oxalate concentration of the sample following incubation was determined using the Sigma oxalate assay. One unit of ODC activity was the amount of enzyme needed to degrade 1 mg oxalate per minute at 40°C.

### Localization of oxalate decarboxylase

Cultures of ME20 were grown in nine 500-ml flasks that contained 300 ml 0.055 M (= 1% w/v) glucose in a basal salts solution (Highley, 1973) with 0.05 M ammonium phosphate as the nitrogen source. The pH of the medium was adjusted to 4.0 by the addition of 1.0M HCl before autoclaving. The medium in each flask was inoculated with 20 ml of a mycelial suspension made from 7-day-old shake cultures of the fungus growing in the same medium. After 11 days on a rotary shaker, the mycelia were collected, pooled by vacuum filtration through Miracloth<sup>®</sup> (Calbiochem, LaJolla, CA), and divided into two samples of equal mass (86 g each). One sample was resuspended in 750 ml 0.1-M HCl/KCl buffer, pH 1.75. The suspension was stirred for 30 min on a magnetic stir plate. The buffer was then removed by vacuum filtration and centrifugation (5000 g for 20 min). An additional 200 ml buffer was added to the mycelium, the suspension was stirred, and the buffer was removed by centrifugation. This was repeated once more. The unwashed mycelial sample and the centrifuged pellet of washed mycelium were then wrapped separately in aluminum foil, frozen in liquid nitrogen, and stored over liquid nitrogen overnight.

The frozen mycelia from both samples were ground into a frozen, fine powder with liquid nitrogen in a mortar and pestle. Ten milliliters of cold 0.1-M HCl/KCl buffer, pH 1.75, was added to each powder, and the sample was allowed to thaw. The suspensions were centrifuged at 12000 g for 20 min. The supernatants were collected, and the pellets were resuspended in 5 ml 0.1 M HCl/KCl buffer and recentrifuged. This was repeated



**Fig. 1.** Protocol for obtaining mycelial extracts from mycelia that were washed or not washed with 0.1 M HCl/KCl buffer, pH 1.75.

one additional time, forming a total of six separate extracts (Fig. 1). Each extract was dialyzed overnight at 4°C in 0.1 M HCl/KCl buffer, pH 1.75, and then tested for ODC activity.

Extract 1 from both the washed and unwashed mycelium was ultracentrifuged to separate the cytosolic from the membrane fractions. Two replicate tubes of each treatment, each holding 11.7 ml of extract, were ultracentrifuged at 50000 g for 90 min. The supernatant served as the cytosolic fraction. The pellets were resuspended in 300 µl 0.1 M HCl/KCl buffer, pH 1.75, to form the membrane fraction. Triton X-100 was added to aliquots of the resuspended membrane fraction to make final concentrations of 0%, 0.1%, 0.25%, 0.5%, 0.75%, and 1.0% (w/v). Control tubes contained detergent and buffer alone to determine whether Triton X-100 interfered with the ODC assay.

### Sensitivity to oxalic acid

Cultures of MAD698 and ME20 were grown in liquid culture containing increasing quantities of oxalic acid to determine the sensitivity of these fungi to this compound. Crystalline oxalic acid was

added before autoclaving to 25 ml of 0.28 M (= 0.5%) glucose (w/v) + basal salts (Highley, 1973) + 0.05 M ammonium phosphate, initial pH 4.0, in 250-ml flasks. The final concentrations of oxalic acid were 0, 100, 200, 300, 400, and 500 mM. The pH of the media was not adjusted after the addition of oxalic acid. The media were inoculated with 1 ml of hyphal suspension, prepared as described above, forming three replicate flasks per treatment. The cultures were incubated at 27°C, without shaking, for 14 days. Mycelia were collected by vacuum filtration through tared glass-fiber filter paper. Mycelial dry weights were determined after drying overnight at 60°C. The pH of the culture filtrate was determined before and after incubation.

### Induction of oxalate decarboxylase by oxalic acid

Isolate MAD698 was grown in 30 ml of the basal salt medium, as described above, in 300-ml flasks on a rotary shaker at room temperature (25°C) for 6 days. At this time, crystalline oxalic acid (which is inherently sterile) was added to the medium for final concentrations of 50, 100, 200, or 400 mM. There were three replicate flasks per treatment. In

a separate experiment, an additional set of cultures growing in the same medium was supplemented with 1 N HCl to provide pH reductions equivalent to the oxalic acid concentrations described above. Final concentrations of HCl were 0.06, 0.09, 0.12, and 0.14 N. Control cultures were not supplemented. The cultures were incubated for a further 2 days. The mycelia were then collected by vacuum filtration through Miracloth®, frozen in liquid nitrogen, and ground into a fine powder with mortar and pestle. The crushed mycelia were extracted with 2 ml 0.2 M citrate-phosphate buffer, pH 2.2. The resultant slurry was centrifuged at 13800 g for 3 min. The supernatant was dialyzed in 0.2 M citrate-phosphate buffer, pH 2.2, for 20 h at 4°C with three changes of buffer using a total volume of 4l. The dialyzed samples were assayed for protein (Lowry *et al.*, 1951) and ODC activity. The dry weight was estimated by collecting the hyphal pellets on tared glass-fiber filter paper by vacuum filtration and reweighing them after overnight drying at 60°C.

## RESULTS

The ODC activities detected in the culture filtrate and the mycelial extracts from washed and unwashed mycelia of ME20 are presented in Table 1. The highest level of activity was found in the first extract of the unwashed mycelia. Repeated extractions produced some additional enzyme activity, but this amount was fairly low when expressed on a per weight basis. Washing the mycelium with buffer reduced the amount of ODC activity in the mycelial extracts to negligible levels. Microscopic examination showed that the washed hyphae were intact and had not been ruptured by the treatment. Use of the 0.1 M HCl/KCl buffer, pH 1.75, had resulted in high enzyme activities in a previous

study (Micales, 1995), so exposure to this buffer should not adversely affect enzyme activity.

The levels of ODC activity found in the cytosolic and membrane fractions of the unwashed and washed mycelial extracts are summarized in Table 2. Low levels of activity were detected in all extracts, with the higher activity in those from the unwashed mycelium. The cytosolic fraction from the unwashed mycelium showed more activity than did the membrane fraction, but the activities of both cytosolic and membrane fractions from the washed mycelium were similar. The addition of Triton X-100, a detergent which solubilizes certain membrane proteins, did not significantly increase enzyme activity in the membrane fraction of either preparation.

The effect of oxalic acid concentration on the dry weight of strains MAD698 and ME20 is shown in Fig. 2. The growth of MAD698 was stimulated by lower levels of oxalic acid (100 mM), whereas concentrations of 200 mM and above inhibited, but did not totally prevent, growth. Strain ME20 was

**Table 1.** Oxalate Decarboxylase Activity in Culture Filtrate and Extracts from Washed and Unwashed Mycelia of Strain ME20

Treatment	Extract number	ODC <sup>a</sup> (units ml <sup>-1</sup> )	ODC <sup>a</sup> (units g <sup>-1</sup> mycelium)
Culture filtrate		0.34 (0.35)	6.05 <sup>b</sup> (6.12)
Unwashed mycelia	1	7.75 (0.26)	5.19 <sup>c</sup> (0.18)
	2	5.21 (0.31)	0.39 <sup>c</sup> (0.02)
	3	4.57 (0.26)	0.32 <sup>c</sup> (0.02)
Washed mycelia	1	0.61 (0.66)	0.22 <sup>c</sup> (0.24)
	2	1.04 (0.80)	0.08 <sup>c</sup> (0.06)
	3	0.00 (0.00)	0.00 <sup>c</sup> (0.00)

<sup>a</sup>Each value represents average of three replicate assays. Standard errors are shown in parentheses.

<sup>b</sup>Value based on 172 g mycelia (fresh weight) growing in culture medium.

<sup>c</sup>Value based on 86 g mycelia (fresh weight).

**Table 2.** Oxalate Decarboxylase Activities in Cytosolic and Membranous Fractions of Mycelial Extracts from Washed and Unwashed Mycelia of Strain ME20

Treatment	Fraction	ODC activity (units ml <sup>-1</sup> ) <sup>a</sup> at various Triton X-100 concentrations (%)					
		0	0.10	0.25	0.50	0.75	1.00
Unwashed mycelia	Cytosolic	6.28 <sup>b</sup> (0.37)	1.40 (0.35)	1.02 (0.32)	1.44 (0.32)	1.41 (0.24)	1.54 (0.36)
	Membrane	1.21 <sup>c</sup> (0.40)					
Washed mycelia	Cytosolic	0.40 <sup>d</sup> (0.46)	0.36 (0.26)	0.29 (0.11)	0.35 (0.12)	0.21 (0.09)	0.12 (0.13)
	Membrane	0.31 <sup>c</sup> (0.15)					

<sup>a</sup>Standard errors appear in parentheses.

<sup>b</sup>Each value represents average of three replicate assays of four replicate samples.

<sup>c</sup>Each value represents average of two replicate assays of two replicate samples.

<sup>d</sup>Each value represents average of three replicate assays of two replicate samples.

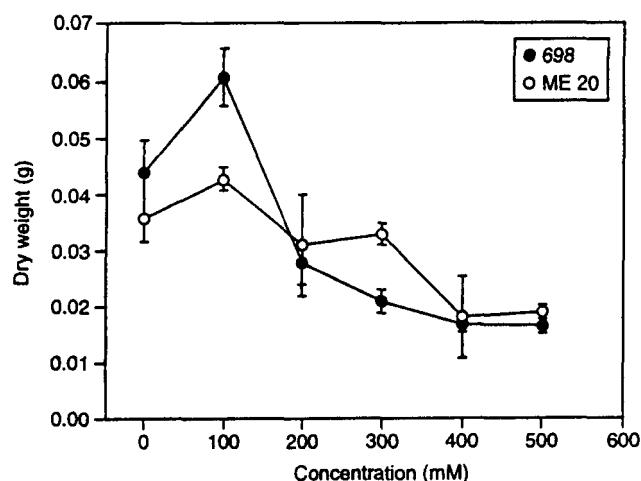


Fig. 2. Sensitivity of *P. placenta* isolates MAD698 and ME20 to oxalic acid. Each point represents the mean of three replications with standard error bars.

slightly more tolerant to oxalic acid than was MAD698 and required a concentration of 400 mM for growth inhibition.

The effect of adding oxalic acid to the culture media of MAD698 is presented in Table 3. The addition of 200 mM oxalic acid appeared to induce ODC production (approximately 50 $\times$ ), as shown by increased ODC activity and decreased oxalate concentration compared to the initial quantity of oxalic acid added to the medium. Enzyme activity was also detected in certain samples with 100 mM oxalic acid, but this was highly variable among replicate cultures. In a separate experiment, ODC activity was also detected when 400 mM oxalic acid

was added to certain 6-day cultures (average specific activity = 0.60 units mg<sup>-1</sup> protein; activities of control cultures without oxalic acid supplementation = 0.04 units mg<sup>-1</sup> protein). However, results were highly variable among cultures. Concentrations of oxalic acid less than 100 mM (1, 5, 10, and 50 mM) did not result in ODC production (data not shown). The addition of increasing concentrations of HCl, which reduced the pH of the culture medium but which did not increase the concentration of oxalic acid to the culture, did not result in ODC formation (Table 4).

The addition of oxalic acid to the culture media lowered the pH (Table 3). The pH of the media continued to decrease from day 6 to day 8, even though the level of oxalate in the media declined.

## DISCUSSION

Most of the ODC activity was associated with the surface of the fungal cell wall in ME20. Activity was also detected in the culture filtrate, but this was quite dilute in a per milliliter basis and highly variable among cultures. Enzyme activity in the culture filtrate has been reported previously, but it was always detected erratically and in rather low concentrations (Micales, 1995). The pH of the culture media, which is usually about 2.8–3.4 after fungal growth, probably inactivates the enzyme as it is released into the media. The optimum pH for ODC activity is 1.8–2.2 (Micales, 1995).

Table 3. Influence of Oxalic Acid on ODC Activity by *Postia placenta* Strain MAD698<sup>a</sup>

Concentration of oxalic acid added to medium on day 6 (mM)	pH (day 6)	pH (day 8)	Oxalate remaining in medium on day 8 (mM)	ODC-specific activity (units mg <sup>-1</sup> protein)	ODC-specific activity (units g <sup>-1</sup> mycelial weight)
0 (control)	3.00 (0.02)	2.48 (0.03)	2 (0)	0.01 (0.00)	0.01 (0.01)
50	2.04 (0.01)	1.84 (0.01)	20 (2)	0.00 (0.00)	0.00 (0.00)
100	1.67 (0.00)	1.52 (0.00)	36 (1)	0.42 (0.38)	0.31 (0.27)
200	1.38 (0.01)	1.27 (0.01)	82 (2)	0.51 (0.16)	0.24 (0.06)

<sup>a</sup>All values represent average of four replications. Numbers in parentheses represent standard errors.

Table 4. Influence of pH Change on ODC Activity by *Postia placenta* Strain MAD698<sup>a</sup>

Concentration of HCl added to medium (day 6) (N)	pH (day 6)	pH (day 8)	Oxalate (mM) day 8	ODC-specific activity (units mg <sup>-1</sup> protein)	ODC-specific activity (units g <sup>-1</sup> mycelial weight)
0.00	3.20 (0.15)	3.13 (0.16)	0.89 (0.30)	0 (0)	0 (0)
0.06	1.88 (0.02)	1.88 (0.01)	0.60 (0.12)	0 (0)	0 (0)
0.09	1.70 (0.02)	1.78 (0.08)	0.54 (0.16)	0 (0)	0 (0)
0.12	1.44 (0.01)	1.47 (0.03)	0.63 (0.11)	0 (0)	0 (0)
0.14	1.33 (0.01)	1.36 (0.00)	0.52 (0.09)	0 (0)	0 (0)

<sup>a</sup>All values represent average of three replications. Numbers in parentheses represent standard errors.

Low levels of enzyme activity were also detected in intracellular preparations. It is likely that the intracellular preparations from the unwashed hyphae were contaminated with enzyme from the hyphal surface that had not been completely removed from the previous buffer extractions. The intracellular preparations from the washed hyphae had much less activity. This activity was divided fairly evenly between the cytosolic and the membrane-bound fractions. This low activity could represent an internal pool of enzyme that is responsible for intracellular pH regulation, or it could reflect the activity of enzymes that are being synthesized intracellularly in an active state, which are then secreted. Solubilizing the cell membranes with Triton X-100 did not substantially increase enzyme activity, demonstrating that the enzymes are not formed in an inactive, membrane-bound form, as described for some fungal cellulases (Hill & Mullins, 1979).

The presence of ODC on the fungal surface or in the hyphal sheath supports the hypothesis that the fungus actively buffers and regulates the pH and/or oxalic acid concentration of its environment. The enzyme appears to be produced constitutively in very low levels, but it is more strongly induced (approximately 50×) in the presence of very high quantities of oxalic acid (100–200 mM and above). These concentrations are far higher than what is normally detected in wood during brown-rot decay (Hyde & Wood, 1995), but the actual concentration of oxalic acid in close proximity to the hyphae could be significantly higher than in the remainder of the wood. White-rot fungi, in which ODC is more frequently reported, respond to much lower concentrations of oxalic acid. Dutton et al. (1993) reported that the white-rot fungus *Trametes versicolor* developed a 50-fold increase in ODC specific activity in the presence of 5 mM oxalic acid. Micales (unpublished data) was able to extract oxalate (0.5–0.6 mM ml<sup>-1</sup> water) from crushed, southern yellow pine wood blocks decayed by MAD698 that were more than 12 weeks old. This indicates that ODC is probably not produced in great quantities by *P. placenta*, even late in the decay process, since oxalate is still present in the wood in advanced stages of decay. It is more likely that the enzyme is a safety mechanism that prevents potentially toxic quantities of oxalate from accumulating close to the surface of the hyphae. The fungus apparently produces ODC in response to the presence of oxalate, not the decline in pH, since the low pH levels formed by the addition of HCl did not result in increased enzyme production. This observation is

consistent with other experiments in which ODC was produced, albeit with low activity, when oxalic acid was present, even though the pH of the culture medium was greater than 3.0 (Micales, 1995).

Although the amount of oxalate declined in the cultures, the actual pH of the medium did not increase. This may be an artifact of liquid culture, or the decrease in pH may also result from the production of other organic acids, such as citric acid. It is also possible that the formation of formic acid by ODC may combine with the remaining oxalic acid to form a strong buffer that maintains a low wood pH. Beth-Anderson (1989) hypothesized that the presence of two organic acids, in this case oxalic acid and formic acid, buffers the pH of the wood more efficiently than would a single organic acid alone. The wood is thus maintained at a pH low enough to facilitate decay (Agosin *et al.*, 1989), but without causing hyphal damage. Jellison *et al.* (1997) speculated that factors other than organic acid production, including ATP hydrolysis, also may be involved in generating and maintaining acidic conditions, so a reduction in the amount of oxalic acid may not necessarily result in increased pH. This observation provides further support to the idea that ODC protects the cell from oxalic acid toxicity rather than excessive hydrogen ion concentration.

The concentration of oxalic acid around the fungal hyphae appears to be tightly regulated and may be essential to the decay process. Synthetic oxalic acid enzymes are produced constitutively, but they can also be induced with basic compounds such as sodium carbonate and sodium hydroxide. The synthetic enzymes require neutral pH for activity and seem to be formed early in the decay process when the pH of the wood is not as acidic (Akamatsu *et al.*, 1993; Akamatsu & Shimada, 1994). Large quantities of oxalate are induced in the presence of pectin and galacturonic acid (Micales, 1994; Green *et al.*, 1995a). The free oxalate precipitates with the calcium from the pectin in the pit membranes, allowing the pectinases better accessibility to the polygalacturonic acid, which can be used as a carbohydrate source (Green *et al.*, 1995a). Oxalate continues to accumulate beyond the level that precipitates with the calcium. This accumulation, perhaps in concert with other mechanisms such as ATP hydrolysis, drops the acidity of the wood to extremely acidic levels. To prevent oxalic acid accumulation from reaching toxic levels, ODC is produced. The formic acid produced by ODC then combines with the remaining oxalic acid to form a strong buffer,

which maintains the wood at a pH low enough to facilitate decay (Agosin *et al.*, 1989) but without causing hyphal damage. Thus, the interaction of oxalic acid synthetic enzymes, calcium precipitation, and oxalic acid catabolism is essential for the successful establishment of decay. When one of these components is missing or aberrant, decay does not develop. This has been demonstrated for ME-20, a low-decay isolate of *P. placenta* that overproduces ODC and does not accumulate significant levels of oxalic acid in wood (Micales, 1995), and by the decay inhibition properties of *N,N*-naphthaloylhydroxylamine, a chemical that precipitates the calcium of the pit membranes *in situ* and prevents calcium chelation by oxalic acid (Green *et al.*, 1997).

All brown-rot fungi do not accumulate oxalic acid equally during decay (Green *et al.*, 1991). *Gloeophyllum trabeum*, for example, fails to accumulate oxalic acid, whereas *Serpula lacrimans* and *Fomitopsis palustris* produce extremely high concentrations of the compound (Shimada *et al.*, 1996). The ability to form ODC, and its possible role in the decay process, still need to be examined for other brown-rot species.

## ACKNOWLEDGMENTS

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