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In vitro oxalic acid production by the brown-rot fungus *Postia placenta*

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Inhalt · Contents · Contenu

MICALES, J. A.: *In vitro* oxalic acid production by the brown-rot fungus *Postia placenta* 159

MICALES, J. A.: Oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta* 177

COOKSON, L. J. and PHAM, K.: Relative tolerance of twenty basidiomycetes to boric acid 187

LARSEN, M. J., WINANDY, J. E. and GREEN, F., III: A proposed model of the tracheid cell wall of southern yellow pine having an inherent radial structure in the S_2 layer 197

EATON, R. A. and CRAGG, S. M.: Evaluation of creosote fortified with synthetic pyrethroids as wood preservatives for use in the sea. Part 1: Efficacy against marine wood-boring molluscs and crustaceans 211

UNGER, W. and UNGER, A.: On the bioresistance of waterlogged wood impregnated with sucrose 231

In vitro oxalic acid production by the brown-rot fungus *Postia placenta*

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K e y w o r d s: Wood decay, brown rot, oxalic acid, oxalate decarboxy - lase, ***Postia placenta***

1. Introduction

Decay caused by brown-rot fungi is a major cause for the failure of wooden structures throughout the world. Brown-rot fungi metabolize the hemicellulose and cellulose of wood. They also cause a rapid depolymerization of cellulose early in the decay process resulting in serious strength losses with minimal decay (E. B. COWLING, 1961). The exact mechanism for this rapid depolymerization of cellulose is not well understood. The pore structure of wood is too small to allow rapid diffusion of carbohydrate-degrading enzymes and other high molecular weight compounds (D. S. FLOURNOY et al., 1991; E. SREBOTNIK and K. MESSNER, 1991). The agent (or agents) that initially depolymerizes cellulose is therefore believed to be a highly reactive compound of low molecular weight.

Many brown-rot fungi accumulate large quantities of oxalic acid (S. TAKAO, 1965) causing the pH of decayed wood to be extremely low, F. GREEN III et al. (1991) recorded a pH of 1.7 by inserting a pH probe into wood inoculated with the brown-rot fungus ***P. placenta*** (Fr.) M. Lars. et Lomb. The exact role of oxalic acid in the decay process is widely debated, and it probably performs several different functions simultaneously. Brown-rot fungi can detoxify certain metals, such as copper, by forming oxalate precipitates (H. SUTTER et al., 1983). M. SHIMADA et al. (1992) proposed that oxalic acid also serves as a metabolic sink to dis-

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pose of the large quantities of excess carbon that is present in wood. M. SHIMADA et al. (1991) and M. SHIMADA et al. (1992) have also suggested that oxalic acid can directly initiate the depolymerization of amorphous cellulose. Most researchers assign oxalic acid a more indirect role in cellulose depolymerization. Oxalic acid is associated with the Fenton reaction where it is thought to reduce ferric ions to the more active ferrous form (J. W. KOENIGS, 1974; J. W. KOENIGS, 1975; C. J. SCHMIDT et al., 1981). The ferrous ions then react with hydrogen peroxide to form extremely reactive hydroxyl radicals that depolymerize cellulose. In an alternative hypothesis oxalic acid is oxidized to form hydrogen peroxide, thus generating hydroxyl radicals (E. ESPEJO and E. AGOSIN, 1991). Oxalic acid has been shown to hydrolyze the hemicellulose in wood, thus making the cellulose fibers more accessible to cellulases (J. BECH-ANDERSON, 1987). Expanding on this hypothesis F. GREEN III et al. (1991, 1992) suggested that the acidic conditions in wood, initiated by oxalic acid production, are responsible for early acid hydrolysis and depolymerization of hemicellulose and amorphous cellulose, thus increasing wood porosity. Enzymes and other degrading agents in the hyphal sheath would then have access to the remaining structural carbohydrates and cause their final removal. J. A. MICALES (1994) showed that certain carbohydrates associated with hemicellulose degradation stimulated oxalic acid production, forming a self-potentiating system that would rapidly decrease the pH of wood.

Introductory studies in our laboratory with the brown-rot fungus ***Postia placenta*** have shown that oxalic acid accumulation can be quite variable, resulting in large standard errors of the mean and data that are not normally distributed (J. A. MICALES, 1992, 1994). A low-decay isolate of ***Postia placenta,*** strain ME 20, seems to have a particularly atypical pattern of oxalic acid accumulation. The goal of this study was to identify parameters that affect ***in vitro*** production and accumulation of oxalic acid in high- and low-decay isolates of ***P. placenta.***

2. Materials and methods

2.1 Isolate storage

All isolates were maintained on 2% malt-extract agar tubes at 4°C for the duration of the study. Culture collection information is listed in Table 1.

Table 1: Collection data on *Postia placenta* isolates[a]

Isolate	Date of acquisition	Source	Weight loss[b] (%)
TRL2556	03/50	Mineshaft support, Transvaal, Africa	68.2
RLG3760R	07/63	*Betula allegheniensis* Britton, Newcomb, NY	65.9
L9138sp	08/58	*Pinus ponderosa* Laws., Coronado National Forest, AZ	65.4
MAD575	10/20	*Pseudotsuga menziesii* (Mirb.) Franco, OR	60.8
JM1	02/88	Stored wood chips, *Picea* sp., Fairbanks, AK	60.5
MAD698	12/41	Yacht "America" decking, *Pseudotsuga menziesii*	60.4
FP100086-sp	03/49	Conifer sills and piles, Fairfield, OH	59.5
MD281	11/57	Utility pole, *Pseudotsuga menziesii*, OR	59.4
JPL335	09/73	*Pinus ponderosa*, Coronado National Forest, AZ	58.2
MD506	04/62	Test stake, *Pinus* sp., Madison, WI	57.2
Hybrid	03/87	Forest Products Laboratory, Madison, WI, derived from a cross of ME20 and L8035spA	55.3/1.0[c]
L12538-sp	08/62	*Tsuga* sp., Ashville, NC	50.4
RLG12516	02/81	No information available	48.0
L8035spA	09/57	Conifer log, Olympic Peninsula, WA; monokaryon	41.6
FP100910sp	12/70	Laurel, MD	36.2
Frankl 5	09/51	Flight deck of carrier "Franklin," *Pseudotsuga menziesii*	32.2
ME20	unknown	Floor planking, Pleasant Hill, CA	5.1

[a] Culture collection maintained by the Center for Forest Mycology, Forest Products Laboratory, U.S. Department of Agriculture, Forest Service, Madison, WI.
[b] Weight loss of southern yellow pine (*Pinus* sp.) test blocks (25.4 mm by 25.4 mm by 3.2 mm; long axis parallel to the grain) as described by the ASTM soil-block technique (ASTM, 1971). Values represent the average of five replicate soil-block bottles.
[c] In 1989, initial test resulted in 55% weight loss (J. A. MICALES and T. L. HIGHLEY, 1989). When the test was repeated in 1992, weight loss was 1.0% and the morphology of the isolate had changed to resemble the ME20 parent.

2.2 Effect of carbohydrate on oxalic acid production

Isolates were grown in 0.055 M (1% w/v) glucose or 0.055 M glucuronic acid in a basal salts solution (T. L. HIGHLEY, 1973) with 0.05 M ammonium phosphate as the nitrogen source, The pH of the medium was adjusted to 5.5 by the addition of 1.0 N NaOH before autoclaving. Five mls of media were dispensed in 50-ml Erlenmeyer flasks, with three replicate flasks per isolate per treatment. The media were inoculated with 5-mm-diameter agar plugs taken from the margins of 7-day-old cultures grown on 0.5 -1.0% glucose or cellobiose (w/v) in basal salts (T. L. HIGHLEY, 1973). Cultures were incubated at 27°C and aseptically sampled for oxalic acid and glucose concentration on days 5, 8, 11, 15, 18, 25, 32, and 40.

2.3 Effect of initial pH on oxalic acid production

Strain MAD 698 was grown in 0.055 M glucose and 0.05 M ammonium phosphate in a basal salts solution (T. L. HIGHLEY, 1973). The initial pH (before autoclaving) of the growth medium was adjusted to 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5 with either 0.1 N HCl or 0.1 N NaOH. Four replicate flasks were prepared for each treatment per isolate. The cultures were incubated at 27°C. Oxalic acid concentration was determined on day 14.

2.4 Effect of phosphate concentration on oxalic acid production

The effect of phosphate concentration on oxalic acid production by *P. placenta* was determined by growing isolates MAD 698 and ME 20 in increasing concentrations of potassium phosphate (monobasic) in the presence of 0.055 M glucose or 0.055 M glucuronic acid in a basal salts solution (T. L. HIGHLEY, 1973) with 0.05 M ammonium nitrate. Final concentrations of phosphate were 0, 15 mM, 50 mM, 100 mM, and 250 mM. There were three replicate cultures per treatment. The initial pH of the media was adjusted to 5.5 by the addition of 1 M NaOH. The cultures were incubated for 21 days at 27°C due to slow growth. Dry weight, culture filtrate pH, and oxalic acid concentration were then determined.

2.5 Effect of calcium concentration on oxalic acid production

Strain MAD 698 was grown in 50-ml flasks containing 5 ml of 0.055 M glucuronic acid and 0.05 M ammonium phosphate and basal salts solution with the following concentrations of calcium chloride: 0, 0.1 mM, 0.5 mM, 0.68 mM (the standard amount in the basal salts solution), 1.00 mM, and 5.00 mM. The initial pH of the media was adjusted to 5.5 by the addition of 1 M NaOH. There were five replicates per concentration. The cultures were incubated for 14 days at 27°C then tested for pH and oxalic acid concentration.

The possible interference of the oxalic acid assay by calcium was tested by combining an equal volume of the noninoculated culture media with 3 mM oxalic acid. The oxalic acid assay was performed and the results were compared to values obtained from culture media that contained no calcium.

2.6 Effect of inoculum on oxalic acid production

Strains MAD 698 and ME 20 were grown in 1 % glucose and 0.05 M ammonium phosphate and basal salts (T. L. HIGHLEY, 1973) with an initial pH of 5.5 (adjusted by the addition of 1 M NaOH). The medium was inoculated with 5-mm-diameter agar plugs taken from the margins of 14-day-old cultures growing on 1 % cellobiose and basal salts agar (T. L. HIGHLEY, 1973), a mycelial suspension made by adding 100 ml sterile distilled water to the mycelium from a 14-day-old culture growing in 1 % cellobiose and basal salts, or a 1:10 or 1:100 dilution of the same mycelial suspension. The cultures were incubated at 27°C. Oxalic acid production and glucose concentration were determined on day 9.

2.7 Assays

The amount of oxalic acid in culture filtrates was determined using a microtiter plate adaptation of a diagnostic test kit for oxalic acid (Sigma Chemical Company, St. Louis, MO) (J. A. MICALES, 1994). The assay was incubated at room temperature for 5 min and then read in a Dynatech MR 5000 microplate reader. Millimolar oxalic acid concentration was measured based on a standard curve that was calculated using known concentrations of oxalic acid (Sigma oxalic acid standards).

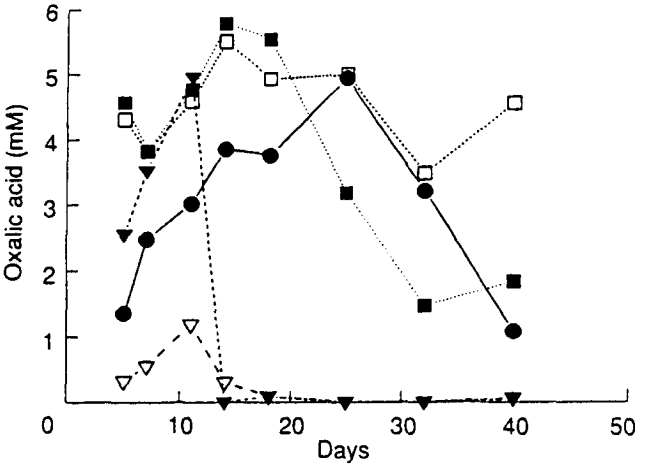
Millimolar glucose concentration was determined with the Sigma glucose assay (procedure number 510) using Sigma glucose standard solution, 5.56 mM, as the control.

3. Results

3.1 Effect of carbohydrate

Figure 1 shows the production and accumulation of oxalic acid by *P. placenta* grown in 0.055 M glucose in basal salts. Most of the isolates (L8035spA, RLG12516R, JM1, Frank15, MD575, TRL2556, and FP100910) accumulated oxalic acid until approximately day 14; the oxalic acid levels then gradually declined. Isolate MAD698 accumulated

Figure 1: Oxalic acid production (mM) by isolates of *Postia placenta* grown in 1% (w/v) glucose and 0.05 M ammonium phosphate and basal salts (T. L. HIGHLEY, 1973).



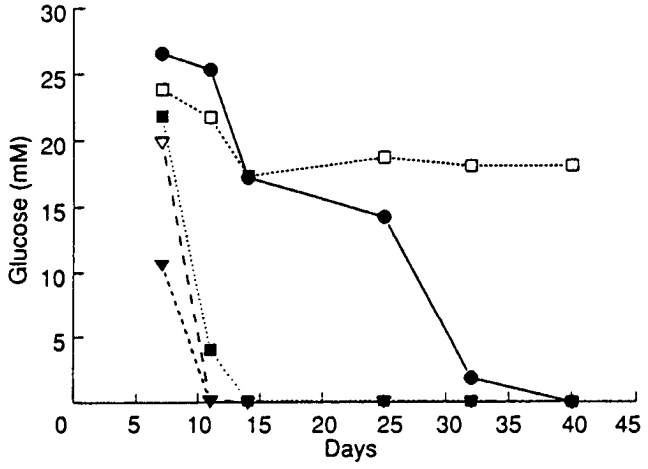
●——● MAD698; ▽-----▽ Hybrid; ▼------▼ ME20; □·····□ L9138sp; ■·····■ L8035spA.

oxalic acid more slowly and did not reach its maximum level until day 25. Several isolates (L9138sp, MD 281, L1253sp, L8035spA, and FP100086sp) appeared to resume oxalic acid production on day 40. Isolate ME20 produced an atypical curve, reaching its maximum accumulation of oxalic acid on day 12. The concentration of oxalic acid then declined rapidly to 0 by day 14. A hybrid strain, derived from a cross of ME 20 and L8035spA, produced low levels of oxalic acid throughout the growth curve.

The utilization of glucose by *P. placenta* isolates is shown in Figure 2. Most isolates (MAD 698, L8035spA, RLG12516R, TRL2556, MD575, MD281, MD506, L12538sp, FP100910) showed a steady utilization of glucose throughout the 40-day period. Several isolates (JM1, Frankl5, and FP100086sp) utilized little glucose during this time but were still able to grow. Isolates ME20, L9138spA, and the hybrid rapidly utilized all of the glucose in the culture medium and by day 12 or 15 all of the glucose was gone. This timing corresponds to the disappearance of oxalic acid in the culture filtrates of isolates ME20 and the hybrid.

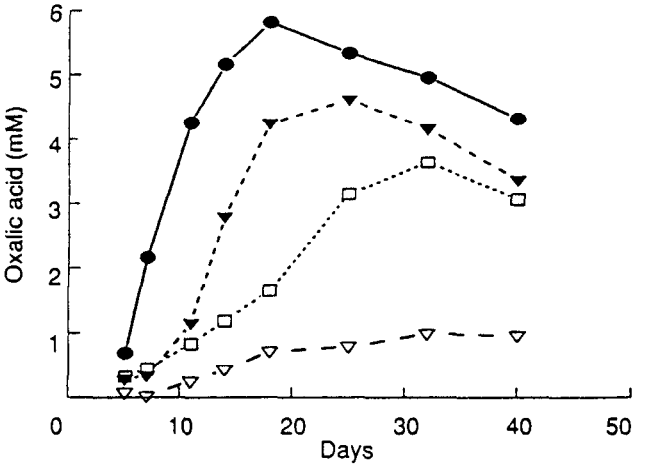
Figure 3 shows oxalic acid production and accumulation when glucuronic acid was the carbon source. All of the isolates except ME 20, the hybrid, and FP100910 produced, accumulated, and broke down oxalic

Figure 2: Glucose utilization (mM) by isolates of *Postia placenta* grown in 1% (w/v) glucose and 0.05 M ammonium phosphate and basal salts (T. L. HIGHLEY, 1973).



●——● MAD698; ▽-----▽ Hybrid; ▼------▼ ME20; □·····□ FP100086sp; ■·····■ L9138spA.

Figure 3: Oxalic acid production by isolates of *Postia placenta* grown in 0.055 M glucuronic acid 0.05 M ammonium phosphate and basal salts (T. L. HIGHLEY, 1973).



●——● MAD698; ▼------▼ FP100910; □-----□ ME20; ▽-----▽ Hybrid.

acid in a uniform pattern. Isolate FP100910 was slower to accumulate oxalic acid, but its concentration in the culture filtrate eventually reached that of the other isolates. Isolate ME20 also accumulated oxalic acid more slowly than did the other isolates and produced lesser amounts. The concentration of oxalic acid in the culture filtrate of ME20 decreased much more gradually in the presence of glucuronic acid than it did when glucose was the carbon source. The hybrid isolate produced only low concentrations of oxalic acid throughout the growth period.

The production of oxalic acid and pH of the culture filtrate of ME20 and MAD698 grown in the presence of different carbon sources for 7 days are shown in Table 2. Oxalic acid production by MAD698 was induced in the presence of glucuronic acid, galacturonic acid, glucose, cellobiose, mannose, laminarin, xylose, glycolic acid, and sodium formate. It was not produced in significant quantities in the presence of malic acid, sodium pyruvate, glyoxylic acid, succinic acid, sodium citrate, soluble xylan, arabinose, sodium fumarate, or sodium acetate. Oxalic acid accumulated in cultures of ME20 only when sodium glucuronate, galacturonic acid, cellobiose, and lactose were the carbon sources. Strain ME20 apparently metabolized galacturonic acid and sodium glucuronate differently than MAD698. The pH of the culture filtrate of ME20 increased (to 6.29 and 6.60 from 5.50) in the presence of these car-

Table 2: Effect of carbon source on pH of culture filtrate and oxalic acid production of *MAD698* and *ME20*[a]

Carbon Source	MAD698		ME20	
	pH[b]	Oxalic acid[c] (mM)	pH[b]	Oxalic acid[c] (mM)
Sodium glucuronate	3.99	3.89 (0.26)	6.29	0.14 (0.05)
Galacturonic acid	3.12	3.84 (0.13)	6.60	1.12 (0.25)
Glucose	2.49	1.16 (0.33)	2.64	0.00 (0.00)
Cellobiose	2.48	0.90 (0.20)	2.51	0.65 (0.52)
Mannose	2.35	0.61 (0.18)	2.72	0.00 (0.00)
Laminarin	2.39	0.53 (0.01)	2.77	0.00 (0.00)
Xylose	2.57	0.53 (0.02)	2.59	0.00 (0.00)
Glycolic acid	6.10	0.43 (0.09)	6.02	0.00 (0.00)
Sodium formate	6.29	0.20 (0.04)	6.52	0.04 (0.00)
Lactose	2.58	0.19 (0.05)	2.49	1.12 (0.60)
Malic acid	5.32	0.06 (0.04)	5.12	0.00 (0.00)
Sodium pyruvate	5.65	0.04 (0.02)	5.75	0.00 (0.00)
Glyoxylic acid	5.71	0.04 (0.01)	5.18	0.00 (0.00)
Succinic acid	5.20	0.04 (0.02)	5.14	0.00 (0.00)
Sodium citrate	4.47	0.03 (0.03)	4.48	0.00 (0.00)
Soluble xylan	6.58	0.02 (0.02)	6.51	0.00 (0.00)
Arabinose	2.81	0.01 (0.01)	3.02	0.00 (0.00)
Sodium fumarate	5.02	0.00 (0.00)	4.83	0.00 (0.00)
Sodium acetate	4.88	0.00 (0.00)	4.82	0.00 (0.00)

[a] After 7 days incubation at 27°C.
[b] All values represent the average of three replicate flasks. Standard errors of the mean did not exceed 0.05.
[c] All values represent the average of three replicate flasks followed by the standard error of the mean.

bon sources. The pH of MAD698 culture filtrate decreased (to 3.99 and 3.12) under the same conditions. The pH of the culture filtrate did not always correlate with the amount of oxalic acid in the medium.

3.2 Effect of initial pH

The effect of initial pH on oxalic acid production by MAD698 is shown in Table 3. Oxalic acid production was greatest when the initial pH was 5.5. Little or no oxalic acid formed when the initial pH of the medium was lower than 5.0.

Table 3: Effect of initial pH on oxalic acid production by MAD698[a]

Initial pH	Oxalic acid (mM)[b]
3.0	0.04 a
3.5	0.03 a
4.0	0.09 a
4.5	0.13 a
5.0	1.30 b
5.5	3.02 c

[a] Cultures grown for 12 days at 27°C in 1% glucose, 0.05 M ammonium phosphate, and basal salts (T. L. HIGHLEY, 1973). Similar results obtained on days 5, 8, and 15.
[b] Each value represents the average of four replications. Values with the same letter are not significantly different at $p < 0.05$ by the Student-Newman-Keuls test.

3.3 Effect of phosphate

The effect of phosphate on growth, pH, and oxalic acid production is shown in Table 4. Phosphate concentrations of 100-250 mM resulted in the highest accumulation of oxalic acid when glucuronic acid was the carbon source. A concentration of 250 mM was required for the accumulation of oxalic acid when glucose was the carbon source. This is higher than what is normally used in the basal salts solution (T. L. HIGHLEY, 1973) used in this study.

3.4 Effect of calcium

The effects of added calcium on oxalic acid production are shown in Table 5. Calcium concentrations of 1 mM and below did not effect the production of oxalic acid nor did they interfere with the oxalic acid assay. A concentration of 5 mM calcium strongly inhibited the oxalic acid assay so that reliable oxalic acid readings could not be made.

3.5 Effect of inoculum

The effect of inoculum type on oxalic acid production and glucose utilization is shown in Table 6. Strain MAD698 produced oxalic acid when agar plugs, a mycelium suspension, or a 1:10 dilution of the mycelium suspension was used as inoculum. Most of the carbon was metabolized in

Table 4: Effect of phosphate concentration on oxalic acid accumulation by MAD 698[a]

Carbon source	Phosphate concentration (mM)	pH[b]	Dry weight. (mg)[b]	Oxalic acid (mM)[b]
Glucose	0	2.83 (0.03)	20.7[b] (1.2)	0.011[b] (0.01)
	15	3.40 (0.06)	56.3 (4.8)	0.070 (0.01)
	50	2.90 (0.00)	52.7 (6.3)	0.00 (0.00)
	100	3.00 (0.00)	62.3 (2.4)	0.00 (0.00)
	250	2.93 (0.03)	71.0 (0.6)	0.24 (0.13)
Glucuronic acid	0	3.53 (0.03)	10.3 (1.9)	0.00 (0.00)
	15	3.87 (0.03)	29.0 (1.5)	0.00 (0.00)
	50	3.57 (0.09)	26.7 (0.3)	0.00 (0.00)
	100	3.23 (0.19)	19.7 (0.3)	2.93 (0.13)
	250	3.17 (0.07)	19.0 (0.6)	2.99 (0.69)

[a] Cultures grown in 0.055 M glucose or 0.055 M glucuronic acid and basal salts without phosphate (T. L. HIGHLEY, 1973) for 21 days at 27 °C.
[b] Each reading represents the average of three replications followed by the standard error of the mean.

Table 5: Effect of calcium concentration on pH and oxalic acid production by MAD 698

Concentration (mM)	14 days pH[a]	14 days Oxalic acid (mM)[a]	Oxalic acid controls (mM)[a,b]
0.00	3.88	0.26	1.37
0.10	3.85	0.18	1.38
0.50	3.86	0.22	1.37
0.68	3.86	0.22	1.32
1.00	3.81	0.19	1.35
5.00	3.80	0.06*	0.27*

[a] Each value represents the average of 5 replications. Values marked with an asterisk (*) are significantly different ($p > 0.05$) from the control (0 mM) using the Bonferroni t-test.
[b] Obtained by mixing equal volumes of 3 mM oxalic acid with equal volumes of noninoculated media for a final theoretical value of 1.5 mM oxalic acid.

Table 6: Effect of inoculum type on oxalic acid production and glucose utilization by MAD698 and ME20[a]

Isolate	Inoculum	Oxalic acid (mM)	Glucose (mM)[b]
MAD698	Agar plug	1.51 (0.18)	20.70 (0.08)
	Mycelial suspension	1.76 (0.18)	3.70 (2.61)
	Mycelial suspension 1:10 dilution	0.94 (0.48)	20.84 (0.05)
	Mycelial suspension 1:100 dilution	0.00 (0.00)	20.85 (0.04)
ME20	Agar plug	0.39 (0.39)	2.31 (1.08)
	Mycelial suspension	0.00 (0.00)	0.29 (0.01)
	Mycelial suspension 1:10 dilution	0.00 (0.00)	1.24 (0.27)
	Mycelial suspension 1:100 dilution	0.00 (0.00)	21.56 (0.08)

[a] Cultures grown in 1% glucose and 0.05 M ammonium phosphate and basal salts (T. L. HIGHLEY, 1973) for 9 days at 27°C. Similar results were obtained on days 5 and 14. Initial pH of the medium was 5.5. Values represent the average of three replications followed by the standard error of the mean.
[b] Concentration of glucose remaining in the culture filtrate from an initial concentration of 55 mM (1% w/v).

the cultures inoculated with the mycelial suspension. Carbon levels were still high when agar plugs were used as inoculum or when the mycelial suspension was diluted. Cultures of ME20 produced oxalic acid only when agar plugs were used as inoculum. Strain ME20 consumed most of the glucose in the medium except when the inoculum was highly diluted (1:100) and there was little growth.

4. Discussion

In vitro oxalic acid production by the brown-rot fungus *P. placenta* depends on the cultural conditions of the growth medium. Carbon source, nitrogen source (J. A. MICALES, 1992, 1994), phosphate concentration, the initial pH of the medium, and the type of inoculum can significantly impact the amount of oxalic acid present in the culture filtrate at any given time.

The influence of carbon source on oxalic acid formation in plant pathogenic fungi is well documented (A. R. BENNET and D. F. HINDAL, 1989; P. KURIAN and D. A. STELZIG, 1979; D. P. MAXWELL and D. F. BATEMAN, 1968; D. P. MAXWELL and R. D. LUMSDEN, 1970; P. E. PIERSON

and L. H. RHODES, 1992; Z. K. PUNJA and S. F. JENKINS, 1984). This also occurs in *P. placenta,* as shown in this study and others (F. GREEN III, 1991; J. A. MICALES, 1992, 1994). Many of the components of hemicellulose, including xylose, mannose, and the uronic acids, induce the accumulation of oxalic acid in strain MAD698. Glucose and some of its polymers, including laminarin, cellobiose, and lactose, also resulted in the production of oxalic acid. This confirms the conclusions of previous studies (J. A. MICALES, 1992, 1994) and supports the hypothesis of M. SHIMADA et al. (1992) that oxalic acid can act as a metabolic sink to dispose of large quantities of excess glucose. Oxalic acid is formed in many fungi *via* the glyoxylic shunt of the tricarboxylic acid cycle (H. M. MULLER, 1975a, 1975b). Some of the carbon sources that are intermediates in this pathway, including malic, pyruvic, glyoxylic, succinic, citric, and fumaric acid, did not result in oxalic acid formation in MAD 698. Under the described conditions, these carbon sources were unable to support significant growth, as indicated by the lack of change in the pH of the growth medium. The same carbon sources at different pHs, in different concentrations, or with supplemental carbon sources may induce oxalic acid formation, as has been shown for acetic acid (J. A. MICALES, 1994). Similar results were reported by D. P. MAXWELL and D. F. BATEMAN (1970a) in *Sclerotium rolfsii* Sacc.

Strain ME20, a low-decay isolate that does not cause significant weight loss in wood, apparently failed to accumulate large quantities of oxalic acid in the presence of some of the same carbon sources that were strong inducers in MAD698, including sodium glucuronate, glucose, mannose, laminarin, xylose, and glycolic acid. Sampling the culture filtrate on a single day can be misleading. Strain ME20 produced large quantities of oxalic acid when grown on glucose and sodium glucuronate in other experiments. The acid was rapidly broken down at a certain point in the growth curve. This shows that the concentration of oxalic acid in the growth medium depends on the physiological stage of the fungus (i.e., its relative position on the growth curve) rather than its chronological age. In all of the strains tested, a decline in oxalic acid concentration appeared to correlate with the depletion of glucose (or other carbon source) and the onset of autolysis. This usually occurred around day 32-40 for most strains under the specific set of culture conditions described. Like hydrogen peroxide, oxalic acid appears to be formed transiently from hyphae at a specific hyphal age (T. L. HIGHLEY et al., 1994). It is later oxidized (E. ESPEJO and E. AGOSIN, 1991) and does not accumulate in wood (C. J. SCHMIDT et al., 1981) or liquid culture indefinitely. Oxalic acid production has also been associated with the logarithmic phase of growth in *Sclerotium rolfsii* (D. P. MAXWELL and D. F. BATEMAN, 1968).

Strains ME20 and the hybrid metabolized the glucose in the medium much more rapidly than the other strains of *P. placenta*. The carbon source was depleted by day 12 in one experiment and day 7 in a second test. Strains ME20 and the hybrid both have an appressed morphology compared to the floccose nature of other *P. placenta* strains. This is probably due to structural irregularities in the hyphal sheath (F. GREEN III et al., 1994). Strain ME20 forms only low levels of extracellular carbohydrate in liquid culture, unlike other strains of *P. placenta* (J. A. MICALES and T. L. HIGHLEY, 1989; J. A. MICALES and T. L. HIGHLEY, 1990; J. A. MICALES et al., 1990). One proposed role of the hyphal sheath is storage of excess carbon for the fungus in a carbon-rich environment (B. BES et al., 1987). A defective hyphal sheath might prevent ME20 and the hybrid from being able to store excess glucose. If these strains have elevated metabolic rates, as described for similarly appressed isolates of *Schizophyllum commune* Fr.: Fr. (J. G. H. WESSELS, 1978), they would rapidly deplete all of the available carbon in the medium and enter the autolytic phase with its associated breakdown of oxalic acid. This is a different situation than in mutants of *Sclerotinia sclerotiorum* (Lib.) de Bary where decreased virulence is associated with a defective synthetic pathway for oxalic acid production (P. MARCIANO et al., 1989). In ME20, oxalic acid is formed initially but does not accumulate for very long. Future research will determine the mechanism of oxalic acid decomposition.

The initial pH of the growth medium and the presence of calcium and phosphate also influenced the amount of oxalic acid detected in cultures of *P. placenta*. Low pH conditions (< 5.0) inhibited oxalic acid formation, but the addition of phosphate greatly increased it. This corresponds to observations by D. P. MAXWELL and D. F. BATEMAN (1968a, b) for cultures of *Sclerotium rolfsii*. Addition of phosphate apparently buffers the medium, maintaining a more neutral pH, which induces the fungus to produce more acid. The concentration of calcium in the medium is also important. Excess calcium can precipitate oxalic acid as calcium oxalate (Z. K. PUNJA and S. F. JENKINS, 1984), although this did not seem to occur at calcium concentrations of 1 mM and below. Excess quantities of calcium (5 mM and above) and other metal ions and chelators, including copper, zinc, potassium, and EDTA, inactivated the enzymatic assay for oxalic acid (J. A. MICALES, unpublished data). High performance liquid chromatography should be used to assay oxalic acid in the presence of these compounds. It is particularly important to run appropriate controls when using the enzymatic assay.

The type and amount of inoculum also has a significant effect on oxalic acid accumulation in culture. This is probably due to the effect of the inoculum on the initial growth rate. A large quantity of inoculum results

in immediate growth and a more rapid depletion of the carbon source than a smaller quantity of inoculum. Agar plugs may also supply additional nutrients to the flask, delaying the onset of autolysis. Small variations in inoculum may be responsible for large variations among replicate cultures. B. L. ILLMAN has shown that different growth rates can result from adjacent inoculum plugs taken from a single fungal colony (T. L. HIGHLEY et al., 1994). This would result in slightly different growth curves and thus variable levels of oxalic acid in the growth medium.

The influence of cultural parameters on oxalic acid production and accumulation is very significant. Researchers who study oxalic acid metabolism must take great care in standardizing the media and inoculum. A single reading of oxalic acid concentration from a single flask on a single day will not provide much useful information and can be greatly misleading. Whenever possible, the accumulation of oxalic acid should be traced by sampling over a period of time. For *P. placenta,* culture media should have an initial pH of at least 5.0 and contain high quantities of phosphate and low quantities of calcium.

Although it is difficult to extrapolate the results from liquid culture to *in vivo* activity in wood (C. A. CLAUSEN et al., 1994), this study raises some interesting questions about the mechanisms of decay by brown-rot fungi. Strain ME20 does not lower the pH of wood (F. GREEN III et al., 1994). In liquid culture, ME20 and the hybrid are unable to accumulate large quantities of oxalic acid due to their rapid depletion of carbon source and early onset of autolysis. This was also demonstrated with nonsoluble substrates as the carbon source (F. GREEN III et al., 1991). Strain ME20 is unable to depolymerize cellulose even though it can cause strength loss (F. GREEN III et al., 1994). This organism may be able to solubilize and depolymerize only the hemicellulose in wood, thus causing strength loss without significant weight loss (F. GREEN III et al., 1994). The inability of ME20 to accumulate oxalic acid may prevent it from depolymerizing cellulose, thus stopping the decay process. This hypothesis asserts a key role for oxalic acid in the physiology of brown-rot decay.

5. Summary

Production and accumulation of oxalic acid by the brown-rot fungus *Postia placenta* depended on cultural parameters including carbon source, nitrogen source, phosphate concentration, initial pH of the medium, and inoculum load. Net oxalic acid concentration depended on the stage of the fungal growth curve. Concentrations increased during the logarithmic phase and tended to decline when the fungus reached the autolytic stage. Subtle variations in culture parameters resulted in large differences in oxalic acid concentration among replications due to varia-

tion in growth rate. Thus, absolute values of oxalic acid concentration obtained under a particular set of conditions are difficult to replicate. Low-decay strains of *P. placenta* entered autolysis more rapidly than those that cause high decay. The inability of low-decay isolates to sustain a prolonged build-up of oxalic acid may have prevented them from depolymerizing cellulose, thus halting the decay process before significant strength loss occurred. This hypothesis asserts *an* important role for oxalic acid in the physiology of brown-rot decay.

Zusammenfassung

In vitro Oxalsäure-Produktion des Braunfäulepilzes *Postia placenta*

Die Produktion und Speicherung von Oxalsäure durch den Braunfäulepilz *Postia placenta* hängt von verschiedenen Kulturbedingungen, wie der Kohlenstoffquelle, der Stickstoffquelle, der Phosphat-Konzentration, dem anfänglichen pH-Wert des Substrats und der Menge des Inoculums ab. Bei den durchgeführten Versuchen hing die Netto-Oxalsäure-Konzentration von dem jeweiligen Stadium des Wachstums ab. Die Konzentration erhöhte sich während der logarithmischen Phase und nahm eher ab, wenn sich der Pilz der autolysischen Phase näherte. Geringe Veränderungen der Kulturbedingungen führten während unterschiedlicher Wachstumsphasen zu erheblichen Unterschieden in der Oxalsäure-Konzentration. Die absolute Oxalsäure-Konzentration, die unter bestimmten Versuchsbedingungen erreicht wird, ist schlecht reproduzierbar. Stämme von *P. placenta*, die eine geringe Fähigkeit zum Holzabbau zeigten, erreichten das Stadium der Autolyse schneller als Stämme mit höherer Abbaufähigkeit. Die Unfähigkeit der Stämme mit geringem Abbauvermögen, die Oxalsäureproduktion über einen längeren Zeitraum aufrechtzuerhalten, könnte der Grund dafür sein, daß sie die Cellulose nicht abbauen konnten, was den Holzabbau beendet, bevor ein erheblicher Festigkeitsverlust einsetzt. Nach dieser Hypothese kommt der Oxalsäure bei der Physiologie der Braunfäulepilze eine wichtige Rolle zu.

Résumé

La production d'acide oxalique *in vitro* par le champignon de la pourriture brune *Postia placenta*

La production et l'accumulation d'acide oxalique par le champignon de la pourriture brune *Postia placenta*, dépendent des paramètres de culture incluant la source de carbone, la source d'azote, la concentration de phosphate, le pH initial du milieu et la quantité d'inoculum. La concentration net d'acide oxalique dépend du stade de la courbe de croissance du champignon. Les concentrations augmentent durant la phase exponentielle de croissance et tendent à diminuer quand le champignon atteind la phase autolytique. Des variations subtiles dans les paramètres de culture, entrainent alors une large différence dans la concentration d'acide oxalique entre les essais à cause des variations dans les taux de croissance. Par conséquent, les valeurs absolues d'acide oxalique obtenues sous une série particulière de conditions sont difficiles à reproduire. Les souches de *P. placenta* à faible pouvoir de pourriture entrent en autolyse plus rapidement que les souches plus agressives. L'incapacité des souches isolées à faible pouvoir de pourriture de maintenir une accumulation prolongée d'acide oxalique, a dû éviter une dépoly-

mérisation de la cellulose, ce qui arrête le processus de pourriture avant qu'une perte importante de solidité se produise. Cette hypothèse donne un rôle important à l'acide oxalique dans la physiologie de la pourriture brune.

References

ASTM (1971): Standard test method for Standards. American Society for Testing and Materials, Philadelphia, PA.

BECH-ANDERSON, J. (1987): Production, function, and neutralization of oxalic acid produced by the dry rot fungus and other brown-rot fungi. Internat. Res. Group on Wood Pres., Doc. No. IRG/WP/1330.

BENNET, A. R. and HINDAL, D. F. (1989): Mycelial growth and oxalic acid production by five strains of *Cryphonectria parasitica* in selected liquid culture media. Mycologia 81: 554 - 560.

BES, B., PETTERSSON, B., LENNHOLM, H., IVERSON, T., and ERIKSSON, K. E. (1987): Synthesis, structure, and enzymatic degradation of an extracellular glucan produced in nitrogen-starved cultures of the white rot fungus *Phanerochaete chrysosporium*. Biotechnol. and Appl. Biochem. 9: 310 - 318.

CLAUSEN, C. A., GREEN F. III and HIGHLEY, T. L. (1994): Extracellular polysaccharide degrading enzymes of *P. placenta* isolated from wood or artificial media. Biodeterior. Res. 4: 247 - 260.

COWLING, E. B. (1961): Comparative biochemistry of the decay of sweetgum sapwood by white-rot and brown-rot fungi. Techn. Bull. 1258. U.S. Dep. Agric., DC., 79 pp.

ESPEJO, E. and AGOSIN, E. (1991): Production and degradation of oxalic acid by brown-rot fungi. Appl. Environ. Microbiol. 57: 1980 - 1986.

FLOURNOY, D. S., KIRK, T. K. and HIGHLEY, T. L.(1991): Wood decay by brown-rot fungi: changes in pore structure and cell wall volume. Holzforschung 45: 383 - 388.

GREEN, F. III, LARSEN, M. J., WINANDY, J. E., and HIGHLEY, T. L. (1991): Role of oxalic acid in incipient brown-rot decay. Material u. Organismen 26: 191 - 213.

GREEN, F. III, LARSEN, M. J., HACKNEY, J. M., CLAUSEN, C. A., and HIGHLEY, T. L. (1992): Acid-mediated depolymerization of cellulose during incipient brown-rot decay by *Postia placenta*. In: M. KUWAHARA and M. SHIMADA (Eds.): Biotechnology in Pulp and Paper Industry. Tokyo, Japan (Uni Publ. Co.), 267 - 272.

GREEN, F. III, LARSEN, M. J. and HIGHLEY, T. L. (1994): Hemicellulosic induction of oxalic acid in *Postia placenta*. Internat. Res. Group on Wood Pres., Doc. No. IRG/WP/94-10060.

HIGHLEY, T. L. (1973): Influence of carbon source on cellulase activity of white- and brown-rot fungi. Wood Fiber 5: 50 - 58.

HIGHLEY, T. L., CLAUSEN, C. A., CROAN, S. C., GREEN, F. III, ILLMAN, B. L., and MICALES, J. A. (1994): Research on biodeterioration of wood, 1987 - 1992. 1. Decay mechanisms and biocontrol. U.S. Dep. Agric., For. Serv., Madison, WI, For. Prod. Lab. Res. Pap. FPL-RP-529.

KOENIGS, J. W. (1974): Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown-rot Basidiomycetes. Wood Fiber 6: 66 - 80.

KOENIGS, J. W. (1975): Hydrogen peroxide and iron: a microbial cellulolytic system? Biotechnol. Bioeng. Symp. 5: 151 - 159.

KURIAN, P. and STELZIG, D. A. (1979): Growth of and oxalic acid production by *Cristulariella pyramidalis* on selected culture media. Phytopathology 69: 712 - 714.

MARCIANO, P., MAGRO, P. and FAVARON, F. (1989): *Sclerotinia sclerotiorum* growth and oxalic acid production on selected culture media. FEMS Microbiol. Lett. 61: 57 - 60.

MAXWELL, D. P. and BATEMAN, D. F. (1968a): Influence of carbon source and pH on oxalic acid accumulation in culture filtrates of *Sclerotium rolfsii*. Phytopathology 58: 1351 - 1355.

MAXWELL, D. P. and BATEMAN, D. F. (1968b): Oxalic acid biosynthesis by *Sclerotium rolfsii*. Phytopathology 58: 1635 - 1642.

MAXWELL, D. P. and LUMSDEN, R. D. (1970): Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. Phytopathology 60: 1395 - 1398.

MICALES, J. A. Unpublished data.

MICALES, J. A. (1992): Oxalic acid metabolism of *Postia placenta*. Internat. Res. Group on Wood Pres., Doc. No. IRG/WP/1566.

MICALES, J. A. (1994): Induction of oxalic acid by carbohydrate and nitrogen sources in the brown-rot fungus *Postia placenta*. Material u. Organismen. 28: 197 - 207.

MICALES, J. A. and HIGHLEY, T. L. (1990): Factors associated with decay capacity of the brown-rot fungus *Postia placenta*. Biodeterioration Res. 3: 285 - 308.

MICALES, J. A. and HIGHLEY, T. L. (1989): Physiological characteristics of a nondegradative isolate of *Postia* (= *Poria*) *placenta*. Mycologia 81: 205 - 215.

MICALES, J. A., RICHTER, A. L. and HIGHLEY, T. L. (1990): Extracellular glucan production by *Postia* (= *Poria*) *placenta*. Material u. Organismen 24: 259 - 269.

MULLER, H. M. (1975a): Oxalate accumulation from citrate by *Aspergillus niger*. 1. Biosynthesis of oxalate from its ultimate precursor. Arch. Microbiol. 103: 185 - 189.

MULLER, H. M. (1975b): Oxalate accumulation from citrate by *Aspergillus niger*. II. Involvement of the tricarboxylic acid cycle. Arch. Microbiol. 104: 159 - 162.

PIERSON, P. E. and RHODES, L. H. (1992): Effect of culture medium on the production of oxalic acid by *Sclerotinia trifoliorum*. Mycologia 84: 467 - 469.

PUNJA, Z. K. and JENKINS, S. F. (1984): Influence of medium composition on mycelial growth and oxalic acid production in *Sclerotium rolfsii*. Mycologia 76: 947 - 950.

SCHMIDT, C. J., WHITTEN, B. K. and NICHOLAS, D. D. (1981): A proposed role for oxalic acid in nonenzymatic wood decay by brown-rot fungi. Proc. Amer. Wood-Pres. Assoc. 77: 157 - 164.

SHIMADA, M., AKAMATSU, Y., MA, D. B., and TAKAHASHI, M. (1992): New biochemical aspects of oxalic acid decomposition by wood-destroying fungi. In: M.

KUWAHARA and M. SHIMADA (Eds.): Biotechnology in Pulp and Paper Industry. Tokyo, Japan (Uni Publ. Co.) 273 - 278.

SHIMADA, M., AKAMATSU, Y., OHTA, A. and TAKAHASHI, M. (1991): Biochemical relationships between biodegradation of cellulose and formation of oxalic acid in brown-rot wood decay. Internat. Res. Group on Wood Pres., Doc. No. IRG/WP/1472.

SREBOTNIK, E. and MESSNER, K. (1991): Immunoelectron microscopical study of the porosity of brown-rot degraded pine wood. Holzforschung 45: 95 - 101.

SUTTER, H., JONES, E. B. G. and WÄLCHLI, O. (1983): The mechanism of copper tolerance in *Poria placenta* (Fr.) Cke. and *Poria vaillantii* (Pers.) Fr. Material u. Organismen 18: 241 - 262.

TAKAO, S. (1965): Organic acid production by basidiomycetes. I. Screening of acid-producing strains. Appl. Microbiol. 13: 732 - 737.

WESSELS, J. G. H. (1978): Incompatibility factors and the control of biochemical processes. In: M. N. SCHWALB and P. G. MILES (Eds.): Genetics and Morphogenesis in the Basidiomycetes. New York (Acad. Press) 81 - 104.

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