

Enzymatic Mechanism of Oxalate Production in the TCA and Glyoxylate Pathways using Various Isolates of *Antrodia radiculosa*

K.M. Jenkins

S.V. Diehl

Mississippi State University
Starkville, Mississippi

C.A. Clausen

F. Green, III

USDA-FS Forest Products Laboratory
Madison, Wisconsin

ABSTRACT

Brown-rot fungi produce oxalate in large amounts; however, levels of accumulation and function vary by species. Copper-tolerant fungi, like *Antrodia radiculosa*, produce and accumulate high levels of oxalate in response to copper. Oxalate biosynthesis in copper-tolerant fungi has been linked to the glyoxylate and tricarboxylic acid (TCA) cycles. Within these two cycles, it has been proposed that oxalate production relies on twelve specific enzymes. In this study, *Antrodia radiculosa* isolates causing decay of untreated and 1.2% copper citrate treated wood were used to evaluate oxalate concentration and enzyme activity in five of the twelve enzymes. The enzyme activity of fumarase, glyoxylate dehydrogenase, isocitrate lyase, malate synthase, and oxaloacetase, was determined. Future gene expression analysis will determine any variations in enzymatic activity, as well as attempt to establish a pathway involved in the direct production of oxalate.

INTRODUCTION

Oxalate is a metabolic byproduct of the decay process, and is also a major factor that leads to the tolerance of copper based preservatives (Arango et al. 2009). The role of oxalate tends to vary with the type of copper treatment and species. Most brown-rot fungi produce oxalate regularly; however, copper-tolerant fungi produce it in much higher quantities. Clausen and Green (2003) found that copper-tolerant fungi produce 2-17 times more oxalate in copper treated blocks when compared to untreated controls. They also found that at week four, copper stimulated 66-93% more oxalate production when compared to untreated controls. Because oxalate plays a huge role in the decay process of copper-tolerant fungi, a certain mechanism must be utilized for its production.

Munir et al. (2001) investigated the role of oxalate biosynthesis in the copper-tolerant fungus, *Tyromyces palustris* and linked it to both the glyoxylate (GLOX) and tricarboxylic acid (TCA) cycles. This study hypothesized that 1mol of glucose is converted to 2mol of oxalate rather than producing the traditional CO₂ from the TCA cycle. Through their work, it was discovered that there are two major oxalate producing enzymes, glyoxylate dehydrogenase and oxaloacetase. It was also proposed that isocitrate lyase is a vital enzyme involved in producing oxalate. These authors hypothesized that oxalate production was coupled with energy production and proposed the overall oxidation of 2mol of acetyl-CoA, yielding 2mol oxalate, generates a net amount of 4 NADHs and 2 ATPs. Figure 1 shows the proposed biosynthesis of oxalate from glucose in the TCA and GLOX cycles.

Using the Munir et al. study as a model, it was hypothesized that there are three unique pathways for oxalate production. One pathway produces oxalate utilizing the TCA cycle only, another produces oxalate from the GLOX cycle only, and the third switches from the TCA to the GLOX cycle. In the TCA cycle, fumarate is synthesized to malate via fumarase. Malate is then converted to oxaloacetate via malate dehydrogenase; this leads to the production of oxalate by oxaloacetase. Within the GLOX cycle, glyoxylate is converted to malate via malate synthase. Malate is then synthesized to oxaloacetate by malate dehydrogenase and that is converted to oxalate via oxaloacetase. In the third potential pathway, isocitrate is synthesized in the both the GLOX and TCA cycle and then produces glyoxylate utilized by the GLOX cycle via isocitrate lyase. Glyoxylate is then converted directly to oxalate via glyoxylate dehydrogenase.

Although many studies have been conducted on oxalate production, little is known about the exact mechanism employed by copper-tolerant fungi. The objective of this research is to gain insight into the biosynthesis of oxalate in order to understand the mechanism utilized by *Antrodia radiculosa* isolates.

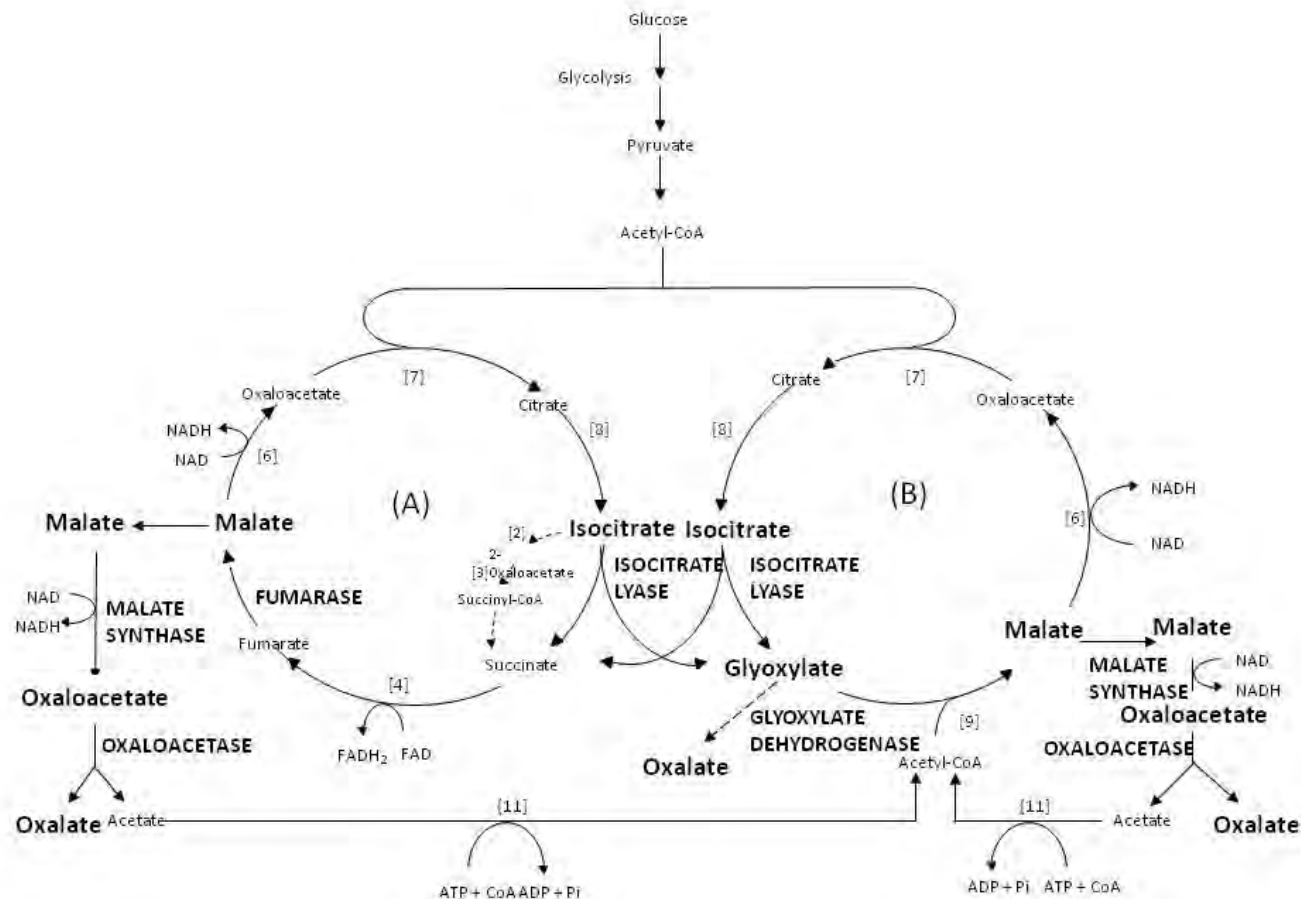


Figure 1. A proposed mechanism for oxalate biosynthesis. (A) the TCA cycle; (B) Glyoxylate cycle. The five enzymes measured for activity in this study are shown in CAPS.

METHODS

Antrodia Isolates

For this study, *Antrodia radiculosa* isolates, FP-90848-T, L-11695-SP, TFFH 294, and L-9414-SP, were obtained from the USDA-FS Forest Products Laboratory (Madison, WI). These four were selected from fifteen potential isolates based on their varying production of oxalate determined from an earlier study. Untreated and 1.2% copper citrate treated Southern Yellow Pine (SYP) wafers (70mm x 22mm x 4mm) were inoculated with the four isolates listed above in a modified soil block test incubated for an 8-week period.

Sample Preparation

Untreated and 1.2% copper citrate treated SYP wafers exposed to the four isolates were harvested at weeks 2, 4, 6, and 8. Blocks were rasped into sawdust-like material and placed into 2mL tubes. The 2mL tubes were dropped in liquid nitrogen to flash freeze any enzymatic activity the samples contained at the time and stored at -80°C until processed. To determine oxalate concentration or enzymatic activity, 1.5mL of buffer (specific to the assay in question) were added and the sample was homogenized; then, the crude homogenate was used to measure oxalate concentration and enzymatic activity of the sample.

Oxalate Analysis and Enzyme Analysis

Oxalate concentration was analyzed spectrophotometrically by the Biotek Epoch 96-well plate reader (Biotek, Winooski, VT) at 560nm using oxalate reagents from Trinity Biotech (Wicklow, Ireland). Enzymatic activity of fumarase (Sigma-Aldrich 1998), glyoxylate dehydrogenase (Tokimatsu et al. 1998), isocitrate lyase (Sigma-Aldrich 1996a), malate synthase (Sigma-Aldrich 1996b), and oxaloacetase (Akamatsu et al. 1992) was analyzed spectrophotometrically. Assay protocols were modified for 96-well plate volumes. To determine activity, absorbance values were measured every 15 seconds for a 2 minute period and the change in absorbance/minute ($\Delta A/\text{min}$) was calculated from these values. Varying amounts of enzyme filtrate (crude homogenate) were used for each sample.

RESULTS AND DISCUSSION

Oxalate Analysis

In general, Figure 2 shows that oxalate concentration (mg/mL) was highest at week 6 for most of the isolates when exposed to 1.2% copper citrate with a few exceptions. L-11659-SP had highest production at week 4 and FP-90848-T had highest production at week 8. There was no observable difference in oxalate production between the untreated and treated samples until week 8.

Oxalate Concentration

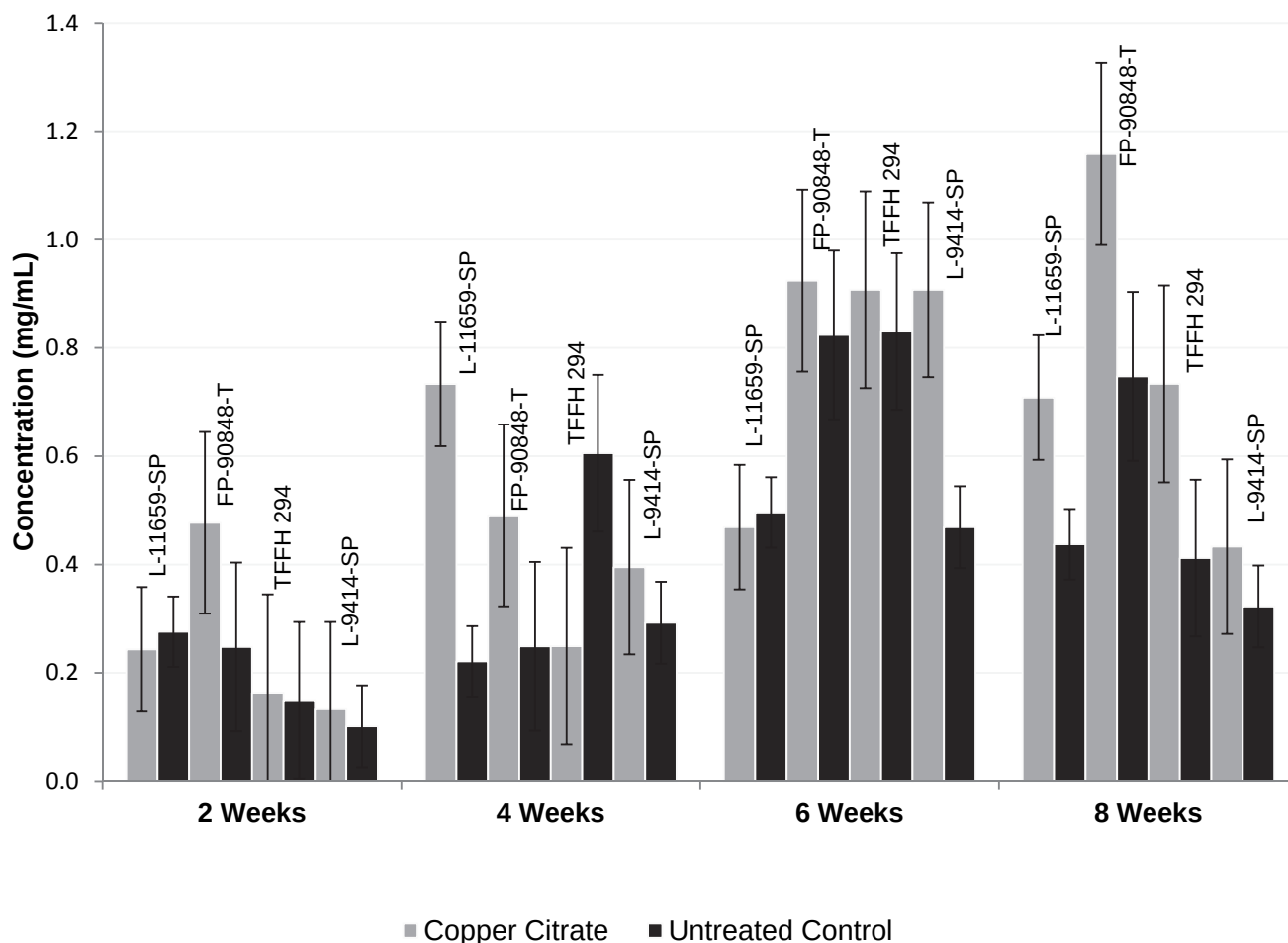


Figure 2. Oxalate concentration shown in mg/mL at weeks 2, 4, 6 and 8. Gray bars exposed to 1.2% copper citrate treatment; Black bars exposed to untreated controls. Results for each isolate are represented by a pair of bars (one gray and one black).

Enzyme Analysis

Enzymatic activity of isocitrate lyase, glyoxylate dehydrogenase, fumarase, and malate synthase for TFFH 294 can be seen in Figure 3. Enzymatic activity is shown by an increase in the $\Delta A/\text{min}$ with respect to an increasing amount of enzyme filtrate (μl) used in the assay. All isolates exposed to untreated and 1.2% copper citrate treated blocks had isocitrate lyase and glyoxylate dehydrogenase activity over the 8 week period. Isolates exposed to untreated and 1.2% copper citrate treated blocks did not have fumarase and oxaloacetase (not pictured) activity over the 8 week period. All isolates exposed to untreated blocks did not have malate synthase activity over the 8 week period. Isolates exposed to 1.2% copper citrate treated blocks had a decrease in the $\Delta A/\text{min}$ with respect to an increasing amount of enzyme filtrate (μl) used for malate synthase, which could possibly be a result of substrate inhibition caused by citrate.

At this stage, further analysis needs to be conducted to quantify enzymatic activity for untreated and 1.2% copper citrate treated samples exposed to all four isolates over the 8 week period. Enzyme solutions will be evaluated for protein concentration to determine specific activity values. From this information, levels of enzymatic activity will be determined for all enzymes proposed. Gene expression analysis will be conducted as a more sensitive approach to establish any differences in enzymatic activity, as well as attempt to propose the pathway involved in the production of oxalate.

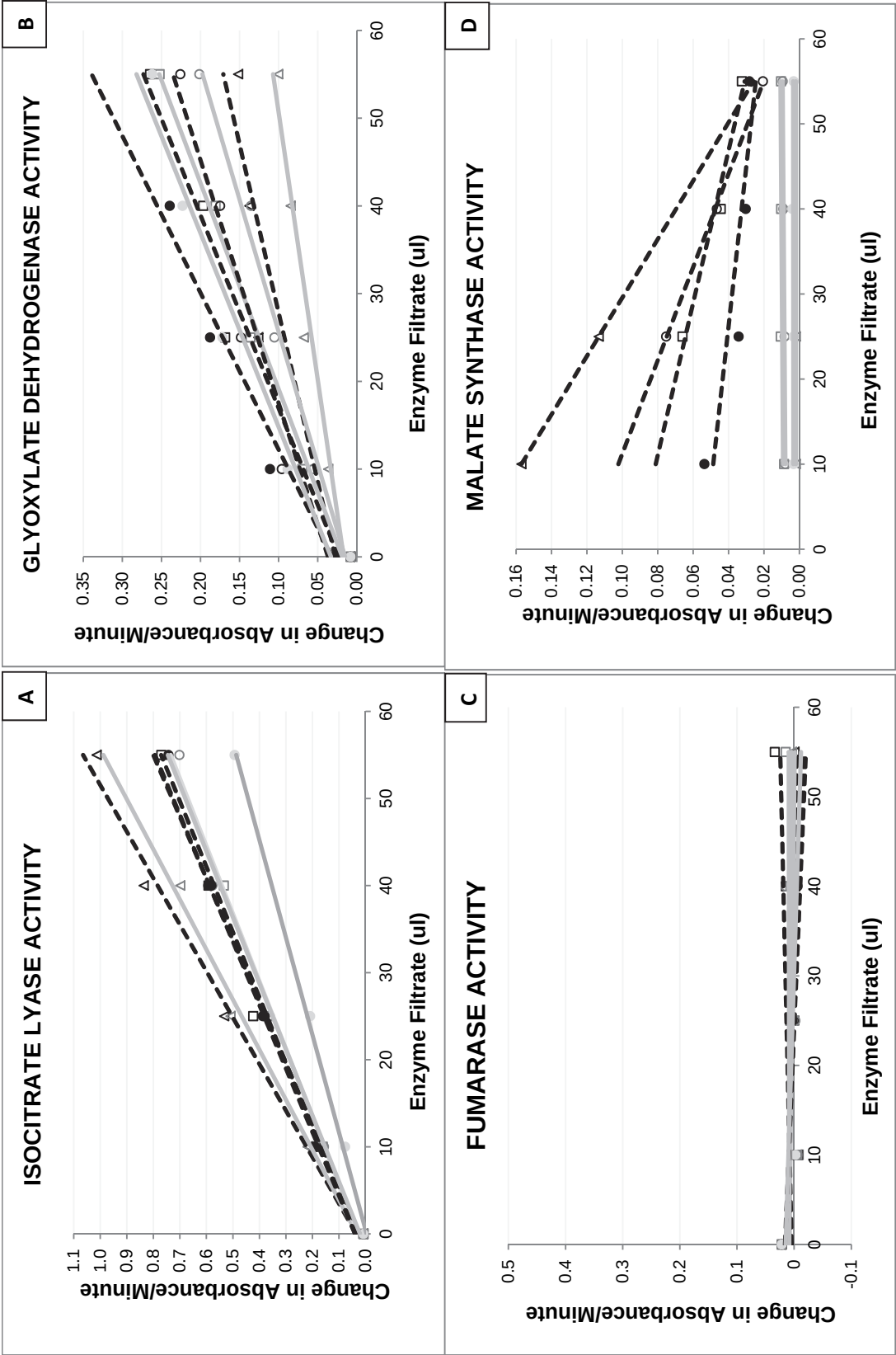


Figure 3. Representative graphs of the enzymatic activity of TFFH 294 for (A) isocitrate lyase, (B) glyoxylate dehydrogenase, (C) fumarase, and (D) malate synthase. Black dotted lines were isolates exposed to 1.2% copper citrate. Gray solid lines were isolates exposed to untreated controls. Two week results are designated by Δ ; four week results, $\square$; six week results, $\bullet$. **Note: All isolates followed this same trend over the 8 week period.**

AMERICAN WOOD PROTECTION ASSOCIATION

ACKNOWLEDGEMENTS

Special thanks should be given to Dr. Kenneth Willeford at Mississippi State University (Department of Biochemistry and Molecular Biology) for his guidance and expertise in the field of enzymology.

REFERENCES

1. Akamatsu, Y., Takahashi, M., Shimada, M., 1992. *Cell-free extraction and assay of oxaloacetase from the brown-rot fungus Tyromyces palustris*. Journal of the Japan Wood Research Society (38): 495-500.
2. Arango, R.A., Lebow, P.K., Green III, F., 2009. *Correlation between oxalate production and tolerance of Tyromyces palustris strain TYP-6137 to N',N-naphthaloylhydroxylamine*. International Biodeterioration and Biodegradation 63: 46-51.
3. Clausen, C.A., Green, F., 2003. *Oxalic acid overproduction by copper-tolerant brown-rot basidiomycetes on southern yellow pine treated with copper-based preservatives*. International Biodeterioration and Biodegradation 51: 139-144.
4. Munir, E., Yoon, J.J., Tokimatsu, T., Hattori, T., Shimada, M., 2001. *A physiological role for oxalate biosynthesis in the wood-rotting basidiomycete Fomitopsis palustris*. PNAS 98(20): 11126-11130.
5. Sigma-Aldrich Technical Document, 1998. *Enzymatic Assay of Fumarase (EC 4.2.1.2)*. <http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/General_Information/2/fumarase.Par.0001.File.tmp/fumarase.pdf>.
6. Sigma-Aldrich Technical Document, 1996a. *Enzymatic Assay of Isocitrate Lyase (EC 4.1.3.1)*. <http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/isocitratelase.Par.0001.File.tmp/isocitratelase.pdf>.
7. Sigma-Aldrich Technical Document, 1996b. *Enzymatic Assay of Malate Synthase (EC 4.1.3.2)*. <http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Enzyme_Assay/malatesynthase.Par.0001.File.tmp/malatesynthase.pdf>.
8. Tokimatsu, T., Nagai, Y., Hattori, T., Shimada, M., 1998. *Purification and characteristics of a novel cytochrome c dependent glyoxylate dehydrogenase from a wood-destroying fungus Tyromyces palustris*. FEBS Letters 437: 117-121.

PROCEEDINGS

One Hundred Seventh Annual Meeting

of the

**AMERICAN
WOOD PROTECTION
ASSOCIATION**

Marriott Harbor Beach Hotel
Fort Lauderdale, Florida
May 15-17, 2011

VOLUME 107

AMERICAN WOOD PROTECTION ASSOCIATION
P O BOX 361784 • BIRMINGHAM, ALABAMA 35236-1784 • USA

ISSN 0066-1198

COPYRIGHT, 2011

BY

AMERICAN WOOD PROTECTION ASSOCIATION
P.O. BOX 361784
BIRMINGHAM, ALABAMA 35236-1784
USA

Rights to republish papers and reports published herein, in whole or in part, or by reference are granted to all persons, provided that reference to the authors and to the AWPA Proceedings are made.

Statements made or opinions expressed in this publication shall not be the responsibility of the American Wood Protection Association.

Colin McCown, *Editor*
Beth Williams, *Assistant Editor*