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Biotransformation of organic wood preservatives by micro-organisms

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BIOTRANSFORMATION OF ORGANIC WOOD PRESERVATIVES BY MICRO-ORGANISMS

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LIST OF ACRONYMS AND ABBREVIATIONS

ABT	Amino-benzotriazole
AWPA	American Wood preservers Association
CCA	Chromated copper arsenate
EDTA	Ethylenedinitrilotetraacetic acid
HPLC	High pressure liquid chromatography
IR	Infra red analysis
IRG	International Research group on Wood Protection
MBT	Methylene-bis –thiocyanate
MIC	Minimum inhibitory concentration
P-450	Cytochrome P-450
PB	Piperonyl butoxide
TEMED	N,N,N,N tetra methylethylenediamine

ABSTRACT

Wood products are treated with biocides to prevent biodegradation by bacteria, fungi, and insects. Much attention is being directed towards testing of metal-free organic preservative systems. The major disadvantage of organics is that they are biotransformed by micro-organisms in soil and wood.

This study explored the biotransformation of the fungicide tebuconazole by a bacteria species (*Pseudomonas fluorescens*), the mold (*Trichoderma harzianum*), the white rot fungus (*Phanerochaete chrysosporium*) and the brown rot (*Meruliporia incrassata*). After incubation of cultures spiked with tebuconazole, samples were analyzed for chemical remaining and metabolites. *M. incrassata, T. harzianum*, and the bacterium all cleaved the 1, 2, 4 triazole ring on tebuconazole and performed oxidation reactions forming the alcohol and carboxylic acid oxidation products of the tert butyl moiety on tebuconazole. *P. chrysosporium* which exhibited the lowest minimum inhibitory concentration (highest tebuconazole efficacy), did not degrade tebuconazole to measurable amounts. *T. harzianum,* with the highest MIC (lowest efficacy), degraded tebuconazole to the largest extent and tolerated it at concentrations below 200ppm. These suggested that the ability of a fungus to degrade a biocide contributes to the efficacy.

The oxidation of tebuconazole was reduced when P450 inhibitors were added to the cultures leading to the conclusion that enzymes involved in the oxidation are cytochrome P450 dependent. Furthermore, the microsomal extract from *T. harzianum* exhibited a maximum peak at 440-460nm when CO was bubbled into $Na_2S_2O_4$ treated samples. Testing metal chelators EDTA and TEMED as synergistic additives to tebuconazole showed that EDTA reduced the magnitude of oxidation most likely by binding ions important in the enzymatic system. TEMED had no significant effect while the P450 inhibitors PB and ABT gave the best performance in terms of reducing tebuconazole depletion. *T. harzianum* was able to germinate and grow in the presence of tebuconazole and all additives used leading to the conclusion that in addition to

biotransformation, there are other mechanisms which this species uses to tolerate tebuconazole. While molds and bacteria species are not responsible for decay, they may metabolize a biocide into a less potent derivative making the environment more suitable to wood degrading basidiomycetes and insects.

CHAPTER 1. INTRODUCTION

Solid and composite wood products are treated with biocides to prevent biodegradation by wood attacking organisms: bacteria, fungi, and insects. Decay fungi have been responsible for annual losses of over $1 billion in the United States to in-service wood. The combined damage from Eastern and formosan subterranean termites exceeds $2 billion annually while mold claims, including pre- and post-construction, exceeded $2.8 billion in 2002 (Clausen and Yang, 2004). Heavy metals such as arsenic and chromium have undergone close environmental scrutiny spurring efforts to replace or reduce their use in preservative water borne systems. Quats containing more copper than traditional wood preservatives do not have the broad spectrum activity needed for ground contact exposure (Barnes, 1993). Critical examination of chemical systems used by the wood preservation industry and the now permanent trend towards the use of environmentally sound materials has increased the interest in wood preservation based on boron and purely organic systems. Much attention is currently being directed towards the formulation and testing of metal-free preservative systems. The major components of such preservatives are biocides originally developed for use in the agricultural sector. Organic biocides will play an increasing role in all use classes in the future. At some point, totally organic or low metallics will be mandated in the United States (Schultz and Nicholas, 2006).

1.1 Organic Preservatives- the Problem

Several problems exist with developing a totally organic wood preservative system. Most are about 10-30 fold more expensive per pound than the inorganics (Schultz et al., 2004). The newer organic agrochemicals are extremely effective at much lower doses than copper (II), but a critical minimal retention is necessary since leaching occurs for both metallics and organics. Currently, their major advantage is also their major disadvantage: they are biodegradable or transformed by wood degrading and non-degrading organisms in soil and wood. Other missing elements in the organic systems are photo protection and hydrophobicity (Evans, 2003). Chemical, and/or photo

degradation reactions, or volatilization reduce the biocide's concentration in wood over time. Consequently, at the present time in North America, no organic preservative can economically compete with the current residential 2nd generation copper-rich systems, nor is any organic system currently standardized for residential outdoor applications by the American Wood Protection Association (AWPA) (Schultz et al., 2004).

The preservative tolerance of many economically important wood-destroying agents has been studied. Tolerance is defined as the relative capacity of an organism to grow or thrive when subjected to an unfavorable environment (Green III and Clausen, 2001). However, not much is known of the factors involved in a specific tolerance. Several studies indicate inactivation of compounds used in preservation of wood. These chemicals include copper, organotin compounds, alkylammmonium compounds, and purely organic systems (Lyr, 1963; Duncan and Deverall, 1964; Belford and Dickinson, 1985; Briscoe et al., 1990; Dubois and Ruddick, 1998; Green III and Clausen, 2001; Schultz et al., 2004; Wallace and Dickinson, 2006). The question of how commonly the tolerance of a preservative by a fungus may be attributed to degradation of the preservative, through the metabolism of the wood-attacking organism or its associate, is of particular significance to the effect of a preservative in wood upon such a synergistic fungus association.

In an attempt to address the problems of organics, low cost and benign non-biocidal additives may be added to enhance the efficacy and/or reduce the biodegradation of organic biocides. Additives include antioxidants, metal chelators and water repellants (Schultz et al., 2001; Schultz and Nicholas, 2004). A combination of various organic biocides with low-cost non-biocidal antioxidants gives enhanced (synergistic) efficacy against wood-decaying fungi (Binbuga et al., 2005). It has been proposed that metal chelators reduce the degradation of organic biocides caused by molds, stains, bacteria, and decay fungi. An example of such an additive is propyl gallate, which is both an antioxidant and a metal chelator. Combination

systems relying on synergism will be common place in the next century (Barnes, 1993). The successful organic preservative is likely to be a mixture of two to three biocides to counteract specific tolerances within the broad range of organisms threatening wood, to enhance synergies, and to reduce costs when some individual components are expensive (Cookson and Yang, 2004; Schultz and Nicholas, 2006).

Microbial associations, in addition to being numerous and varied, are frequently so complex and sensitive to slight changes in environment that it is difficult to discover the primary phenomena that contribute to the end result (Duncan and Deverall, 1964). Micro-organisms can modify the environment often making an otherwise unsuitable environment suitable to another micro-organism. Species diversity can be very high as in soil, or very low as in toxic environments, such as treated wood. The highly diverse microorganisms, including non tolerant species may be capable of detoxification at a faster rate than observed in laboratory studies (Briscoe et al., 1990). Activity against the causal decay organisms and resistance to physical losses are no longer the sole performance criteria that need to be understood when developing a new wood preservative. Microbial mediated detoxification of biocides by both bacteria and fungi may contribute significantly to the failure of treated wood (Briscoe et al., 1990). If wholly organic wood preservatives are to be utilized in the long term in high hazard situations, such as soil contact, it will be necessary to fully understand the biotransformation of the constituent biocides, and attempt to control it (Wallace and Dickinson, 2006). There is a need to establish the biotransformation pathways and use this knowledge to stabilize biocides in wood. An understanding of the mechanisms involved in detoxification of fungicides or termiticides by bacteria, fungi, and termites is needed to provide a means for defeating these processes.

1.2 The Study

This study utilized bacteria and fungal species which in previous studies by other researchers have been isolated, identified and, associated with failed preservative-treated wood.

A bacteria species, a brown rot, a white rot, and a mold fungus were each separately used to access *in vitro* the biodegradation rate and products of metabolism of tebuconazole. Tebucanazole - is the generic name of the compound 1-(4-chlorphenyl)-4,4-dimethyl-3-(1H-1,2,4-triazole-1-methyl)amyl-3-alcohol. It is a broad spectrum fungicide and a component of copper azole a commercial preservative. This dissertation is composed of six interrelated chapters addressing the biotransformation of tebuconazole by different classes of micro-organisms.

Chapter 1 (this chapter) is an overall introduction to the dissertation. In Chapter 2, the resistance of wood inhabiting ascomycetes to triazole based wood preservatives is studied and discussed. *Trichoderma harzianum* is known to be tolerant to antisapstain compounds like methylene-bis –thiocyanate (MBT) and was used to study biotransformation mechanisms involved using tebuconazole as the test chemical. The alteration of the compounds by a sapstain or mold may detoxify it to a level where wood –rotting basiodiomycetes could grow and breakdown wood.

Chapter 3 presents the study of different classes of wood inhabiting fungi and their capacity to biotransform tebuconazole. Species used to study different classes of fungi were: (i) *Phanerochaete chrysosporium*, a white-rot fungus recognized for its ability to metabolize a large diversity of compounds (Mendoza-Cantu et al., 2000), (ii) *Meruliporia incrassata,* a brown rot fungus reported to tolerate chromated copper arsenate (CCA) and cause substantial weight loss in pine blocks treated with CCA or copper sulphate (Illman and Highley, 1996), (iii) *T. harzianum* a mold and (iv) a bacteria strain (*Pseudomonas fluorescens*). The results show that the initial mechanism(s) involved in detoxifying tebuconazole by the bacteria, *T. harzianum* and *M. incrassata* were the same. The initial metabolites were the same.

Chapter 4 describes the study of the proteineous extracellular extracts responsible for biotransformation of tebuconazole by bacteria and wood inhabiting fungi. Each organism

produces proteineous extracts when subjected to media containing tebuconazole. The results showed that although different enzymes may be produced, the cytochrome P450 family of enzymes is involved in the initial oxidation of tebuconazole by *T. harzianum* and *M. incrassata*. Phase I reactions result in (more) polar metabolites of tebuconazole. Phase II reactions (conjugation reactions) were observed in cultures of *T. harzianum* only.

Chapter 5 addresses the issue of additives that may to slow down the degradation of tebuconazole by micro-organisms. Additives studied were two metal chelating agents and two enzyme inhibitors. Results in cultures containing only tebuconazole and a combination of tebuconazole and additive were compared and presented. The chapter also presents the results of the use of a standard mold test in the efficacy and synergies observed when tebuconazole is used in combination with the additives. Chapter 6 provides overall conclusions for the dissertation. Conclusions on mechanisms involved in biotransformation by the four different microorganisms are discussed. Conclusions on methods of intervention using additives to slow down the process of transformation are also presented.

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CHAPTER 2. RESISTANCE OF SAPSTAIN *Trichoderma harzianum* TO TRIAZOLES-POSSIBLE MECHANISMS INVOLVED

2.1 Introduction

Triazoles are a broad class of organic fungicides with low mammalian toxicity. Increasingly being used in agriculture, triazoles inhibit the synthesis of sterols required for cell growth in fungi. They inhibit the fungal cytochrome P-450 3-A dependent enzyme 14-alpha demethylase, thereby interrupting the synthesis of ergosterol. Inhibition of this critical enzyme in the ergosterol synthesis pathway leads to the depletion of ergosterol in the cell membrane and accumulation of toxic intermediate sterols, causing increased membrane permeability and inhibition of fungal growth (Lamb et al., 1999). Whereas mammalian cell membranes contain primarily cholesterol, ergosterol is the predominant sterol in many pathogenic fungi. The triazole tebuconazole is a broad spectrum second generation fungicide. It is a component of copper azole a commercial wood preservative.

Tebuconazole and propaconazole act synergistically and are standardized by the American wood preservers association (AWPA). Tebuconazole is stable, resistant to hydrolysis at pH 4-9, can be formulated in solvent as well as water borne formulations, and is compatible with insecticides (Grundlingerv and Exner, 1990). Efficacy data indicates efficacy of tebuconazole against basiodiomycetes. A retention of <0.54 kg/m^3 is required for white rot *Coriolus versicolar* and 0.026-0.03 kg/m^3 for brown rot *Gloeophyllum trabeum.* The dry rot *Serpula lacrymans* requires a higher retention of 0.8-1.28 kg/m^3 (Buschhaus, 1995). While efficacy data for tebuconazole suggests activity against a wide range of wood degrading fungi, triazoles appear to be less effective against ascomycetes (staining fungi and molds). *Trichoderma* spp. possesses innate resistance to most chemicals, including fungicides, although individual strains differ in their resistance. The tolerant fungus *Trichoderma harzianum* causes more than 75% reduction in the concentration of preventol A4S (N,N-dimethyl-N'-phenyl-N'-

fluorodichloromethylthio sulphamide and preventol A5 (tolylfluanid) in test solutions (Briscoe et al., 1990). It is known to be tolerant to the antisapstain compound methylene-bis –thiocyanate (MBT) at concentrations ranging from 4ppm to 34ppm. Considerable increases in both threshold and toxic limits have been observed when wood treated with alkylammonium compounds is first subjected to *T. harzianum* and then basidiomycetes in a second step. *Trichoderma* spp. are present in nearly all soils and other diverse habitats. In soil, they frequently are the most prevalent culturable fungi. Because of tolerance of ascomycetes to triazole chemicals, manufacturers of triazole containing formulations do not recommend them for treatment against ascomycetes. Not much is known about the actual mechanisms used by these microorganisms with regards to tolerance. Table 1 shows minimum inhibitory concentrations of tebuconazole to various ascomycetes.

Table 2.1. Tebuconazole minimum inhibitory concentrations (MICs) in nutrient agar for ascomycetes

Species	MIC (ppm)
Altenaria alternata	200
Aspergillus niger ATCC 10575	10
Aureobasidium pullulans ATCC 9348	35
Chaetomium globusum ATCC 6205	15
Gliocladium virens ATCC 9645	75
Penicillium glaucum	15
Phialophora fastigiata Wfpl 234 a	50
Scherophoma pityophila	5
Trichoderma viride	>1000

Source (Grundlingerv and Exner, 1990).

Biocide detoxification may be brought about if the biocide is rendered unavailable by adsorption on the surface of the microbial cells. Such adsorption processes are most likely in the cases of quaternary ammonium compounds and metal ions which dissociate to form ions in solution. Microorganisms may detoxify a biocide by assimilation into the cell where the active ingredients of the biocide become immobilized (e.g., in the uptake of copper from external media by a fungus followed by the formation of insoluble copper oxalate within and around he fungal

hyphae). The biocide may be inactivated extracellularly by becoming bound to microbial metabolites or it is possible that metabolites produced by microorganisms may induce the breakdown of the biocide to less toxic products (Briscoe et al., 1990). Microbial mediated detoxification of biocides by both bacteria and fungi may contribute significantly to the failure of treated wood. Bacteria and actinomycetes are probably the most common wood-inhabiting microorganisms and certainly are the most adaptable in terms of environmental influences. They can modify the environment often making an otherwise unsuitable environment suitable to another microorganism such as decay fungi (Wallace and Dickinson, 2004). Wood-inhabiting fungi, not necessarily responsible for major decay, have been shown to be capable of degrading a toxic compound or metabolizing it into a less potent form or derivative, thus rendering it less effective in protecting wood from less tolerant basidiomycetous wood-destroyers and insects (Duncan and Deverall, 1964; Ruddick, 1984). This study uses tebuconazole as a model triazole for the investigation of biotransformation pathways and identification of metabolites produced by *T. harzianum.* The results will broaden knowledge on mechanisms used by the ascomycetes in tolerating triazoles. Understanding the mechanisms involved in detoxification of biocides is important to provide a means for stabilizing them in wood.

2.2 Materials and Methods

2.2.1 Test Chemicals and Fungi

Ninety five percent tebuconazole (preventol A 8) was provided by Lanxess Corporation (Pittsburgh, PA). The tebuconazole standard was obtained from Sigma Aldrich (St. Louis, MO). Original strains of *T. harzianum* were provided by the US Forest Products Laboratory, Department of Agriculture Madison (WI). Cultures were grown out and maintained on media containing 1.5% agar, 2% malt extract, and 0.001% yeast extract.

2.2.2 Determination of MICs

Preliminary work involved determination of the minimal inhibition concentrations for tebuconazole against *T. harzianum.* Using the same media above, plates of nutrient agar containing tebuconazole in concentrations ranging from 0-200ppm were prepared in triplicate. Plates were kept at room 25^{o}C for 7 days. The MIC (the lowest concentration at which no visible growth of mycelium was observed) was determined. Subsequent biodeterioration studies were performed at levels lower than the MIC.

2.2.3 B3 Media Suitability

The study utilized nutrient limited B3 media. Because B3 media was studied and developed specificaly for *Phanerochaete chrysosporium*'s extracellular peroxidase production, preliminary studies were necessary to ascertain its suitability for growing *T. harzianum* in subsequent degradation studies. B3 media for N-limitation represents the situation on wood which has low amounts of nitrogen. In this preliminary study, one circular plug was obtained from one week old cultures on petri dishes and used to inoculate 100ml of sterilized "B3"in Erlenmeyer flasks. Twenty eight flasks were inoculated, covered with parafilm, and kept at 25^{o}C for two weeks on a shaker at 100rpm. Growth was monitored by weighing the dry fungal cell mass harvested by filtering the liquids through Whatman #2 filter paper at time intervals of 0-14 days. Two flasks were harvested every two days. The cell mass retained on filter paper was rinsed twice with deionized water and air dried at 30°C to constant weight. The composition of B3 media per liter of distilled water was as follows: 1.73 t-aconitic acid adjusted to pH to 4.5 with KOH, 2g KH_2PO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.1g $CaCl_2 \cdot 2H_2O$, 0.2g ammonium tartrate, 10g glucose, 0.001g thiamine, and 10mls mineral elixir. The mineral elixir contained the following per liter: 1.5 g nitrilotriacetic acid (pH adjusted to 6.5 with KOH, 3.0g $MgSO_4 \cdot 7H_20$, 0.5g $MnSO_4 \cdot H_2O$, 1.0g NaCl, 0.1g $FeSO_4 \cdot 7H_2O$, 0.1g $CoSO_4$, 0.1g $CaCl_2$, 0.1g $ZnSO_4 \cdot 7H_2O$, 0.01g $CuSO_4$, 0.01g

$AlK(SO_4)_2 \cdot 12H_2O$, 0.01g H_3BO_3, and 0.01g $NaMoO_4 \cdot 2H_2O$. Separate flasks were grown using the same procedure but using malt extract broth as the growth media. Malt extract broth contained 17g/l of mycological peptone and 3 g/l of malt extract. Growth rates for the two types of media were compared at the end of 14 days.

2.2.4 Biotransformation Studies

Erlenmeyer flasks containing 100ml of B3 media were supplemented with 0.1ml of 0.2% tebuconazole to give a final concentration of 20 ppm of each flask. Flasks were inoculated with an agar plug from actively growing colonies. Cultures were incubated at 25^{o}C and shaken at 120rpm for a period ranging from 3-21days. The filtrate was separated by centrifugation (10 min. at 15000xg and 4^{o}C). The centrifuge used was a fixed angle rotor, induction drive Beckman model J2-21M. Three replicates were used for each time interval. A sample of 5mls was removed from each flask for further analysis. Two controls were run simultaneously. The culture control consisted of a media blank in which *T. harzianum* was grown under identical conditions but without tebuconazole. The substrate control consisted of tebuconazole and sterile media incubated without fungal innoculum to determine whether tebuconazole could chemically decompose or transform under experimental conditions.

2.2.5 Quantitative Determination of Tebuconazole Retained in Samples

Five ml of filtered medium was extracted in solid-phase extraction (SPE) columns. The columns (Bond Elut –C18, 500mg, 3ml Varian #12102028) were washed and preconditioned with two column volumes of methanol, followed by two column volumes of milli-Q water under vacuum. Five ml of sample was loaded and pulled through at one drop per second, followed by a column rinse of 50:50 acetonitrile water. Retained compounds were eluted with 4 column volumes of methanol and collected in 15ml centrifuge tubes. Each tube was dried to below 5ml on a nitrogen evaporator and transferred to a second tube through sodium sulfate. The final volume

was adjusted to 5ml using methanol. Samples were cleaned with a 0.2 µm Teflon™ filter prior to quantitative analysis by gas chromatography –mass spectrometry (GC-MS). The GC-MS was an Agilent 6890gas chromatograph using 5973 mass selective detector. The injection port temperature was 250^{o}C, the detector temperature 280^{o}C, and the carrier gas was helium at 1 mm min^{-1}. The oven was initially held at 80^{o}C for 2 min followed by a 30^{o}C min^{-1} increase to 190^{o}C and then an 8^{o}C min^{-1} increase to a final temperature of 300^{o}C. The capillary column model No. RTX35 was 30m X 250µmX 0.25µm. Injection volume was 2.0µl and total runtime was 20.42 min. Tebuconazole eluted at 15.9 min. Running in selected ion mode (SIM), ions detected for tebuconazole were 250 as the target ion, and 125 and 252 as the qualifier ions. Calibration was done using a four point curve. Concentrations of the samples were calculated using the formula:

$(X / V_1)^* (V_2)^*$dilution factor. [1]

Where: X = value from the curve reading from the instrument.

V_1= Initial vol. of sample extracted by SPE

V_2= final vol. of sample after SPE

2.2.6 Analysis of Tebuconazole Absorbed on Mycelia

To evaluate tebuconazole content adsorbed on the fungal mycelia, the biomass was filtered from culture media after 21 days, washed with distilled water, and frozen at -32ºC before freeze-drying. The dried mass was ground to small particle size, 100ml of methanol was added to it in a flask, and shaken at 100 rpm in an orbital shaker for 24 hrs. The filtrate was separated by centrifugation (10 min. at 15000xg and 4^{o}C). Two repeat extractions with methanol were done on triplicate samples ensure all the tebuconazole was extracted. The extract was subjected to solid phase extraction prior to quantitative analysis by GC-MS.

2.2.7 Preparation of Samples for HPLC

To isolate the major metabolites in sufficient quantities for structural elucidation, the transformation of tebuconazole by *T. harzianum* was carried out on a preparative scale. Ten

250mls Erlenmeyer flasks containing 100ml of B3 media were supplemented with 0.1ml of 0.2% tebuconazole to give a final concentration of 20 ppm. Flasks were inoculated with an agar plug from actively growing colonies, incubated at 25^{o}C, shaken at 100rpm for a period of 21 days after which they were centrifuged (10 min. at 15000xg and 4^{o}C) to separate the mycelia and media. The liquid samples were pooled into two equal composite samples which were then subjected to extraction and cleanup by the following procedure: 500ml of sample was transferred into a one liter separating funnel to which 75ml of dichloromethane was added. The sample was subjected to shaking for 1.5 min. vented every 20 seconds to release pressure, then left to stand for 3 min. The bottom layer was passed over sodium sulfate in a large funnel into a 400ml beaker. This extraction procedure was repeated twice. The sample was then placed on a water bath at 45^{o}C and evaporated until almost dry. Five 5ml. of acetonitrile was added for solvent exchange, re-dried to about 2ml, and transferred to a centrifuge tube with several rinses of acetonitrile while passing it over sodium sulfate funnel. The tube was dried to below 5ml on a nitrogen evaporator, the final volume adjusted to 5ml using acetonitrile and subjected to HPLC qualitative analysis. The culture control consisted of a blank in which *T. harzianum* was grown under identical conditions but without tebuconazole. The substrate control consisted of tebuconazole and sterile media incubated without fungi.

2.2.8 HPLC Analysis

Several reverse-phase HPLC runs were done to detect and purify any metabolites and byproducts. The HPLC system consisted of a Waters 600 pump with a Waters 2487 UV detector. The output from the UV detector was connected to a fraction collector. The column was a Waters Delta-Pak C18, 300 Angstrom, 15 micron, 8mm x 10 cm radial compression module. Eluant composition as a function of time was as follows: isocratic from 0-5 min. 70% water + 0.1% formic acid and 30% acetonitrile, linear increases of acetonitrile from 5-30 min to 85% and isocratic at 85% acetonitrile for 5 min. Flow rate was 1 ml min^{-1} and detection was at

224 nm wavelength. Tebuconazole eluted at 28 min. Fractions were evaporated in vacuum and metabolites extracted from the remaining solution using acetonitrile.

Qualitative analysis using authentic standards of metabolites was not possible as metabolites of tebuconazole could not be obtained. Tebuconazole peaks and those of metabolites were identified by comparison of elution time, protonated molecular mass and identification of functional groups of in the tebuconazole standard, media only, media containing tebuconazole before exposure to the fungi and, media containing tebuconazole after exposure to the fungi.

2.2.9 Mass Spectral Characterizations

Exact mass determination of HPLC fractions of tebuconazole and its metabolites was performed by electrospray ionization (ESI). The instrument operated in positive ESI mode was an Agilent 6210 Time of flight (TOF) LC/MS mass spectrometer.

2.2.10 IR Analysis

HPLC fractions and authentic tebuconazole were subjected to IR analysis to determine functional groups retained or changed on the metabolites. The FT-IR system was a Bruker Tensor 27 system single beam instrument connected to an optical user software (OPUS) version 4.2 data collection and analysis program. Two drops of sample were placed on a Real crystal IR KBr card and air dried before determination of spectra. Transmission spectra were obtained after 16 background and 16 sample scans.

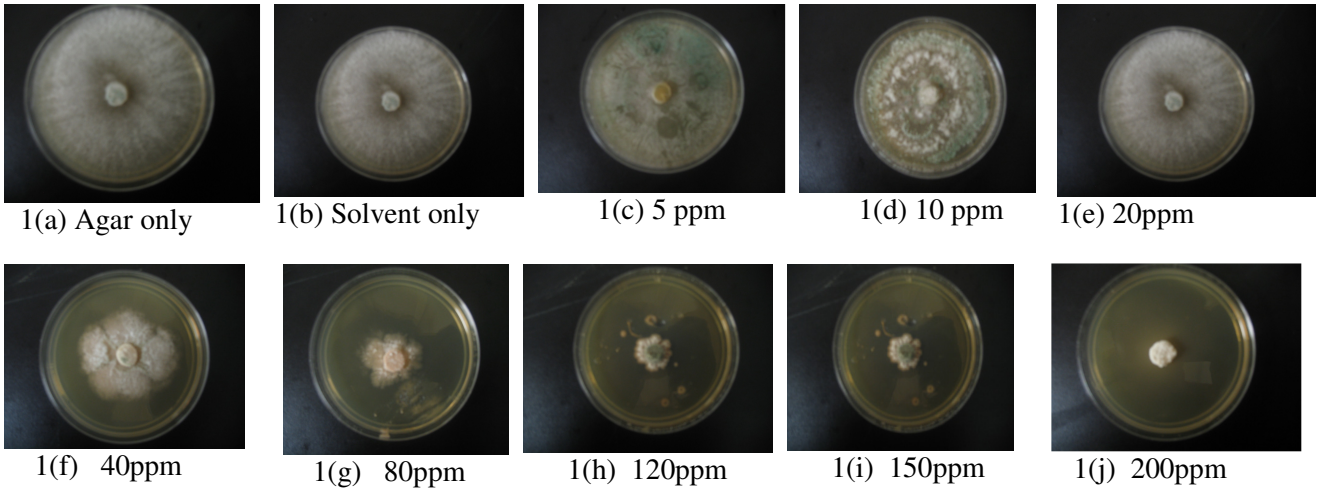
2.3 Results and Discussion

2.3.1 Minimum Inhibitory Concentrations

The lowest at which no growth was observed was 200ppm. Results are shown in Figure 2.1. The fungus was tolerant at concentrations below 200ppm. Subsequent metabolism studies were conducted at a concentration lower than this.

2.3.2 Suitability of B3 Media

After a lag phase of two days, the fungi grew at a fast rate, steadily increasing in mycelia mass until the 14th day. Growth rates are compared in Figures 2.2a and 2.2b. The mean increase in mycelia weight after 14 days for the two types of media found not to be different (p=0.1,df=13) in a paired t-test.



1(a) Agar only 1(b) Solvent only 1(c) 5 ppm 1(d) 10 ppm 1(e) 20ppm

1(f) 40ppm 1(g) 80ppm 1(h) 120ppm 1(i) 150ppm 1(j) 200ppm

Figure 2.1. MICs for tebuconazole against *T. harzianum*.

Growth in B3 did not differ from that in malt extract broth recommended for molds. B3 was appropriate for use in further tests. B3 was preferred for further experiments because with N-limitation, it represents more of the situation in wood and has no proteins which would have interfered with further work on proteineous extracts produced by the fungi.

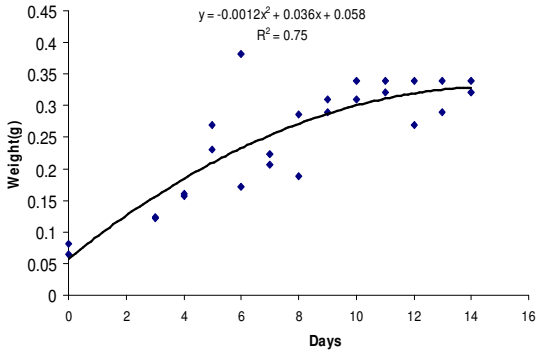


Figure 2.2a. Growth in B3 media.

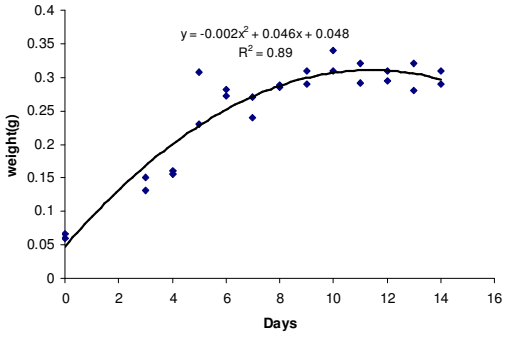


Fig 2.2b. Growth in malt extract broth.

2.3.3 Quantitative Analysis of Degradation of Tebuconazole

After 6 days, it was apparent that tebuconazole amount in media inoculated with fungi was reducing at a steady rate. After 21 days, 68.2% of the original 20ppm was depleted. The actual values retained are shown in Figure 2.3. Less than 10% was depleted in control cultures.

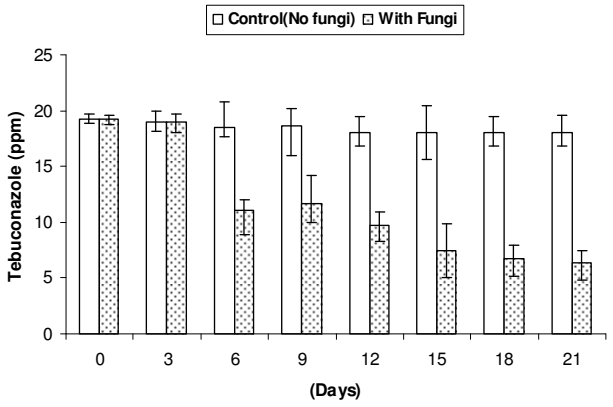


Figure 2.3. Tebuconazole retained in media after 21 days.

2.3.4 Quantitative Analysis of Tebuconazole Absorbed on Mycelia

Analysis of tebuconazole absorbed on fungal mycelia revealed that only 0.09ppm (<1%) was extracted by 100mls of methanol. This minute amount led to the conclusion that adsorption of the chemical on the surface of the microbial cells or assimilation into the cell for immobilization are not the mechanisms employed by this species in tolerating tebuconazole.

2.3.5 HPLC Analysis

Secondary metabolites produced by *T. harzianum* did not affect the analysis of tebuconazole. Chromatograms of the standard, test tebuconazole in media only (culture control), and test tebuconazole in media exposed to fungi for 21 days are compared in Figure 2.4. Parent tebuconazole eluted at 28 min. Major components of the media eluted at 5 and 21 min. Eluting compounds thought to be metabolites were those not found in the media and eluting at times different from tebuconazole. Four fractions with metabolites (M1, M2, M3, and M4) were

identified and collected. Their retention times were 3.8, 9.1, 16, and 34 min. respectively. Three metabolites were more hydrophilic than tebuconazole and eluted from the column faster than tebuconazole. One metabolite eluted after tebuconazole.

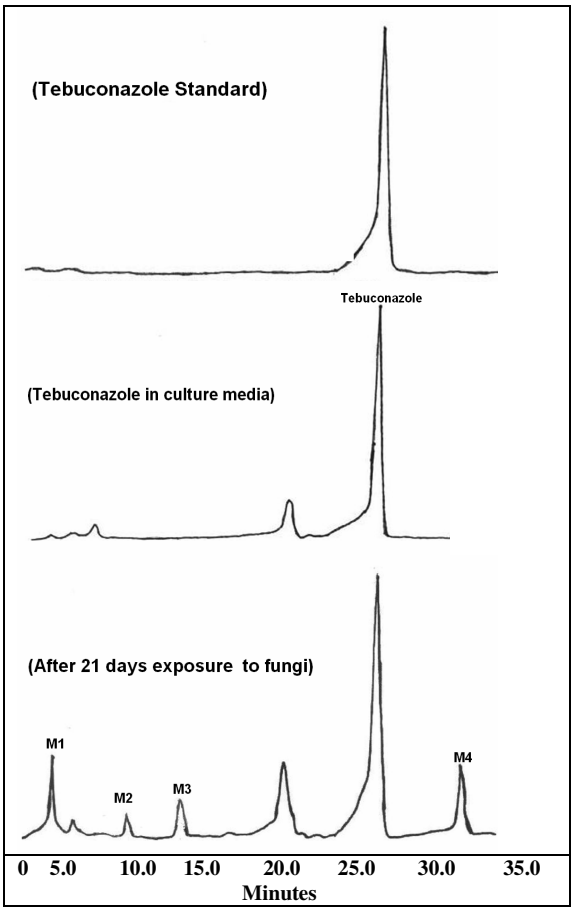


Figure 2.4. HPLC chromatograms.

2.3.6 Mass Spectral Characterizations

Intact molecular mass is not a unique identifier because different compounds may have the same mass. However, determination of an intact molecular mass was useful in this case because a large part of the molecule (tebuconazole) was already known and only a minor unknown change or changes needed to be characterized. Molecular mass was measured to within an accuracy of 5 ppm. The spectrum for parent tebuconazole showed the dominant ion at m/z 308.15, consistent with the protonated molecular ions ($M+H^+$). Figure 2.5a shows the mass spectra of the whole culture at

beginning of incubation, and fraction 3 after 5 and 21 days incubation respectively. Results of all other mass spectra are summarized in Table 2.1. Metabolites in HPLC fractions had intense peaks of protonated ion ($M+H^+$) at 338.34, 324.15, 281.17, and 366.36, for M1 to M4 respectively.

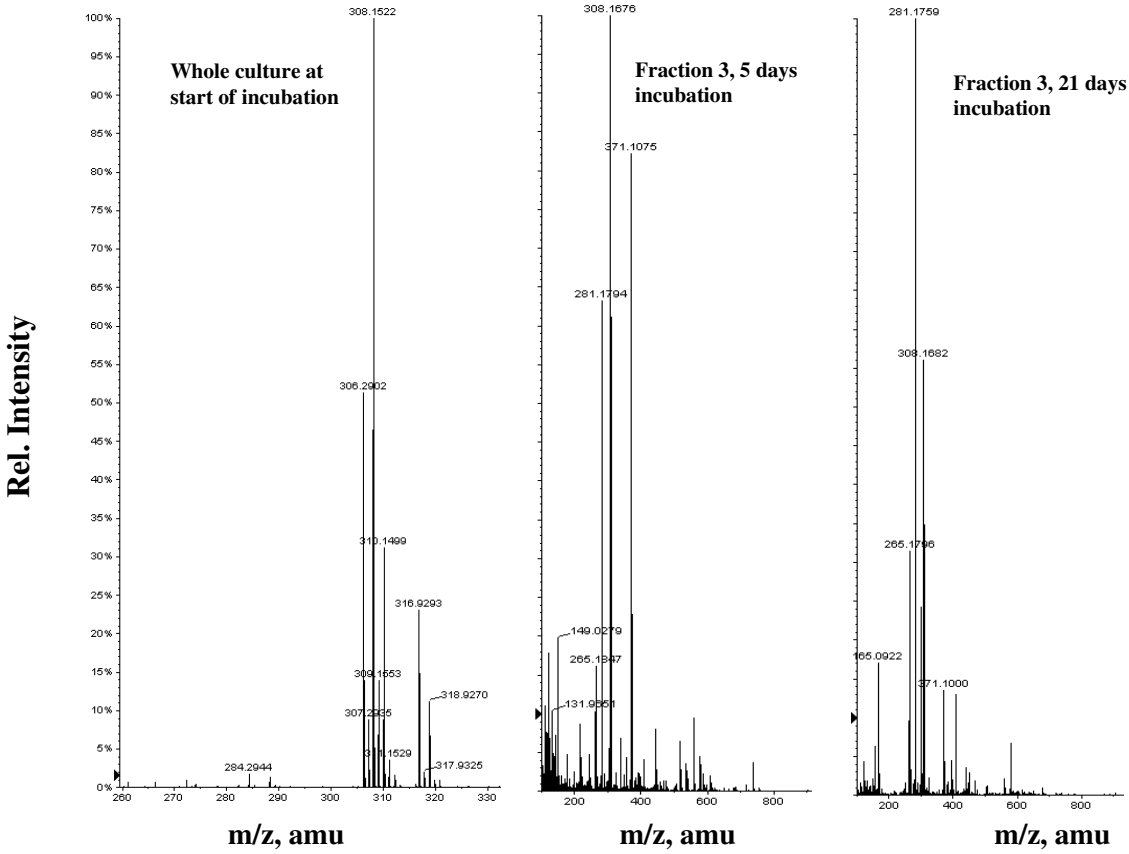


Figure 2.5. Mass spectra of whole culture and fraction 3.

Each fraction may have had more than one compound, but only the major component identified by MS is discussed. The others were in minute amounts and their structure could not be determined by the method used in this study.

The number of possible chemical formulae assigned to an accurate determined mass was reduced by comparing spectral and isotope patterns using the method by Stoll et al. (2006). The ^{35}Cl :^{37}Cl, 3:1 intensity isotope pattern distribution was apparent and was used to identify actual metabolites as opposed to those arising from the media or fungal secretions. Thus spectra were used to rate and exclude compounds not resulting from the metabolism of tebuconazole. The

intensity ratios in the isotopes are due to the natural abundance of the isotopes. Tebuconazole and all four metabolites had the 3:1 intensity isotope pattern. Figure 2.6 shows this pattern in tebuconazole and metabolite M3. There is a possibility that metabolites without Cl remained unidentified.

Table 2.2. Summary of positive ESI-MS spectra - dominant ions and intensities

Sample	Peaks (m/z, amu)	Intensity counts (%)	Most intense peaks
Tebuconazol standard	308.15	100	308.15
	309.15	10	
	310.15	25	
	371.11	82	
Fraction 1	308.15	80	338.34, 308.15
	310.15	25	
	338.34	100	
	340.36	20	
	371.11	82	
	264.13	18	
Fraction 2	308.15	100	324.15, 308.15
	310.15	25	
	324.33	80	
	326.32	21	
	312.15	14	
	371.11	75	
Fraction 3	281.17	100	281.17
	283.18	25	
	308.15	57	
	265.17	33	
	371.10	15	
Fraction 4	281.17	25	366.37
	324.15	22	
	308.17	45	
	364.36	21	
	366.37	100	
	368.35	24	
	371.11	72	
Media blank	371.21	100	371.21
	121.97	70	121.97
	131.96	35	

Based on the molecular mass and the reversed nitrogen rule since the molecular ions were generated by protonation, an even mass for tebuconazol and three of the degradation products indicated presence of an odd number of nitrogens (three). This led to the conclusion that these degradation products contained the intact triazole ring. One product (M3) with a molecular mass 281.17 indicated an even number of nitrogens. These coupled with a loss of 27 amu, led to the

conclusion that the HCN fragment was lost after cleavage of C-N and/or N-N bonds on the 1,2,3-triazole ring to form an imine. Tebuconazol, M1, M2 and M4 all have a degree of unsaturation of 7 while M3 has 6 further confirming of the cleavage of the triazole ring. Two peaks corresponding to 281.17 were observed in the TIC for M3. This corresponds to two possible products of triazole cleavage at two different places both resulting in the loss of an H-C-N fragment (Table 2.2).

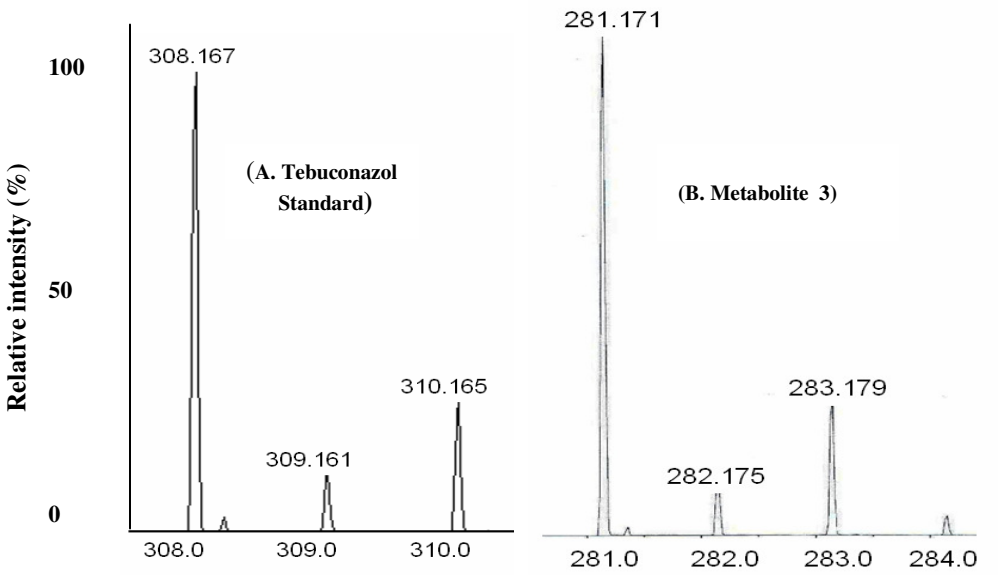


Figure 2.6. Isotope distribution pattern in tebuconazole and M3

2.3.7 IR Analysis

Discovering the identity of each metabolite involved analysis of functional groups. Figure 2.6 shows the IR spectra of the four fractions. IR analysis of functional groups (Figure 2.7) revealed no difference in relevant bands in the spectra of M3 and that of tebuconazole. Relevant bands in the IR spectra for M1 and M2 showed presence of the carboxylic acid and hydroxyl groups in the molecule. Most likely these compounds are the primary oxidation products of the methyl groups on the tert butyl moiety, i.e., the hydroxyl and the carboxyl. Table 2.3 presents a summary of relevant bands observed in IR spectra and change in molecular mass in the protonated ion.

Within 21 days, 68.2% tebuconazole was metabolized by *T. harzianum* into a form that may be less toxic thus enabling the fungi to grow. Less than 1% was absorbed on the mycelia.

Hence adsorption of the chemical on the surface of the microbial cells or assimilation into the cell for immobilization were not the mechanisms employed by this species in tolerating tebuconazole. The combination of MS and FTIR reveals that in addition to cleavage of the triazole ring, this mold performs oxidation reactions as a prelude to the detoxification of tebuconazole as is common with other biocides (Kanaly and Harayama 2000; Kelley et al. 1993).

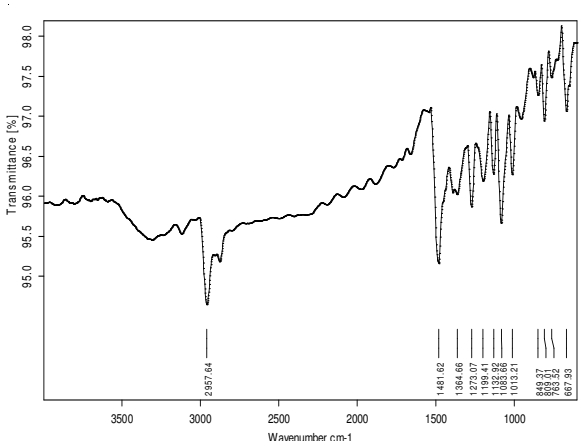
Table 2.3. Summary of change in molecular mass and relevant IR bands

Protonated ion mass $(M+H)^+$	**Change in amu**	**Unique IR band observed**	**Proposed pathway**
308.15 (Tebuconazol)	0	-	
324.15	+16	3200-3000	Oxidation
338.34	+30	3500-2500 1700-1800	1. Oxidation 2. Carboxylation
366.37	+58	1700-1800	1. Oxidation 2. Acetylation.
281.17	-27	-	Triazole ring cleavage

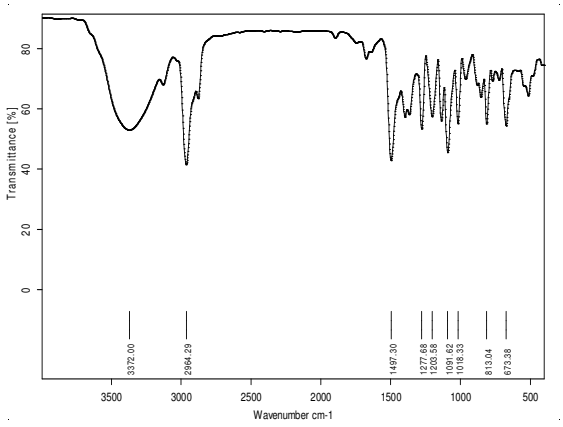
The products resulted from the oxidation of the methyl groups on the tert butyl moiety, (i.e., the alcohol and the carboxylic acid readily identified by the sharp C=O stretch between 1680 and 1725 cm^{-1}) and the O-H stretch of the carboxylic group appearing as a broad peak in the 2500-3000 cm^{-1} region. Oxidation may also have occurred on the phenyl ring, and at α –carbon. In contrast with the work of Strickland et al. (2004), there was no evidence of dechlorination or oxidative cleavage of the phenyl ring. Cleavage of the 1,2,4 triazole ring is most likely the major pathway for detoxifying tebuconazole. It is through binding of the triazole to the heme of the cytochrome P-450 enzyme sterol 14α demethylase that ergosterol synthesis is inhibited thus retarding growth of the fungi. Once the triazole ring is broken down, this mechanism is interfered with. Monooxygenase type reactions were shown to occur in agreement with the work

of Strickland et al. (2004). Oxidation metabolites identified are likely not the final products. Many fungi after initial oxidation perform phase II reactions in detoxification of xenobiotics.

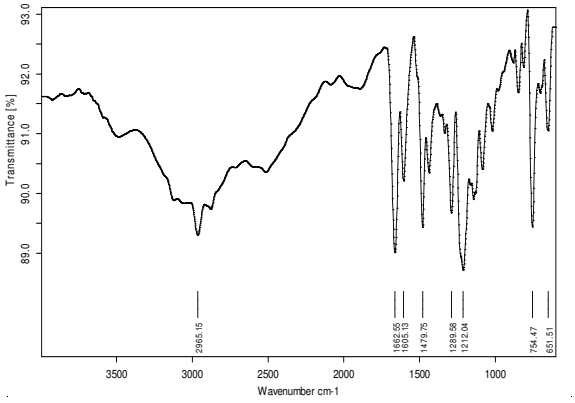
Tebuconazol



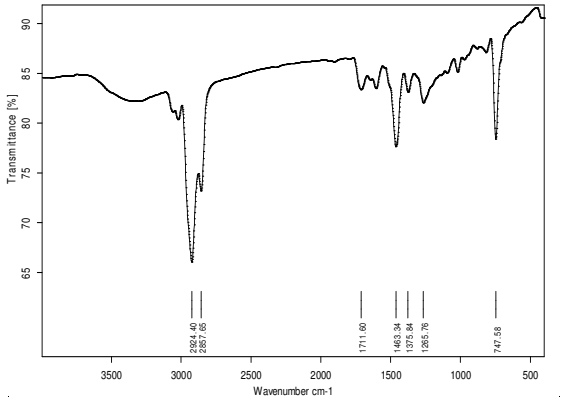
M1



M2



M3



M4

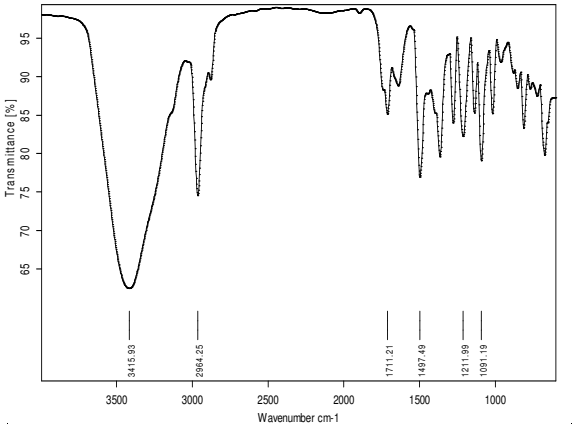


Figure 2.7. IR spectra of tebuconazole and fractions

Conjugation (methylation, glucosidation, acetylation and sulphonation) are thought to be reactions used by fungi in metabolizing chemicals to non toxic metabolites (Cerniglia et al. 1982). These results showed that *T. harzianum* further metabolizes the hydroxyl group formed on the tert butyl moiety by acetylation to form an ester corresponding to the intense strong band in the 1670-1700 cm^{-1} double bond region in M4 and a corresponding increase in molecular mass (Table 2.2). Acetylation deactivates the chemical or its phase 1 metabolite and results in a less hydrophilic product (Cerniglia et al. 1982). No evidence of glucosidation and sulphonation was observed. However, there is a possibility that they occurred. Sulphonation would be evidenced by IR absorption at 700-900cm^{-1} indicating the presence of S-OR. However this absorption is also evidence of a C-Cl bond, present in tebuconazole and all metabolites. Fungi can perform many different complex conversions of polyaromatic hydrocarbons and steroid compounds. Biochemical evidence indicates that such bioconversions are often mediated by cytochrome P450 (P450) as a part of monooxygenase enzyme systems. P450s are a family of hemoproteins acting in many organisms ranging from bacteria, fungi, to mammals. They catalyze monooxygenation of a wide variety of endogenous and exogenous compounds. The monooxygenase systems are multicomponent, consisting of P450 and an electron donating system, needed for the oxygen insertion in the substrate molecule (Makovec and Breskvar, 2000). The most common reaction catalysed by P450 is a monooxygenase reaction (i.e., insertion of one atom of oxygen into an organic substrate (RH) while the other oxygen atom is reduced to water in a phase I type reaction). Although the presence of P450 was not confirmed in this study, monooxygenase type reactions were shown to extensively occur. Based on MS, IR spectra and isotope pattern the proposed metabolic pathway for transformation of tebuconazole is shown in Figure 2.8.

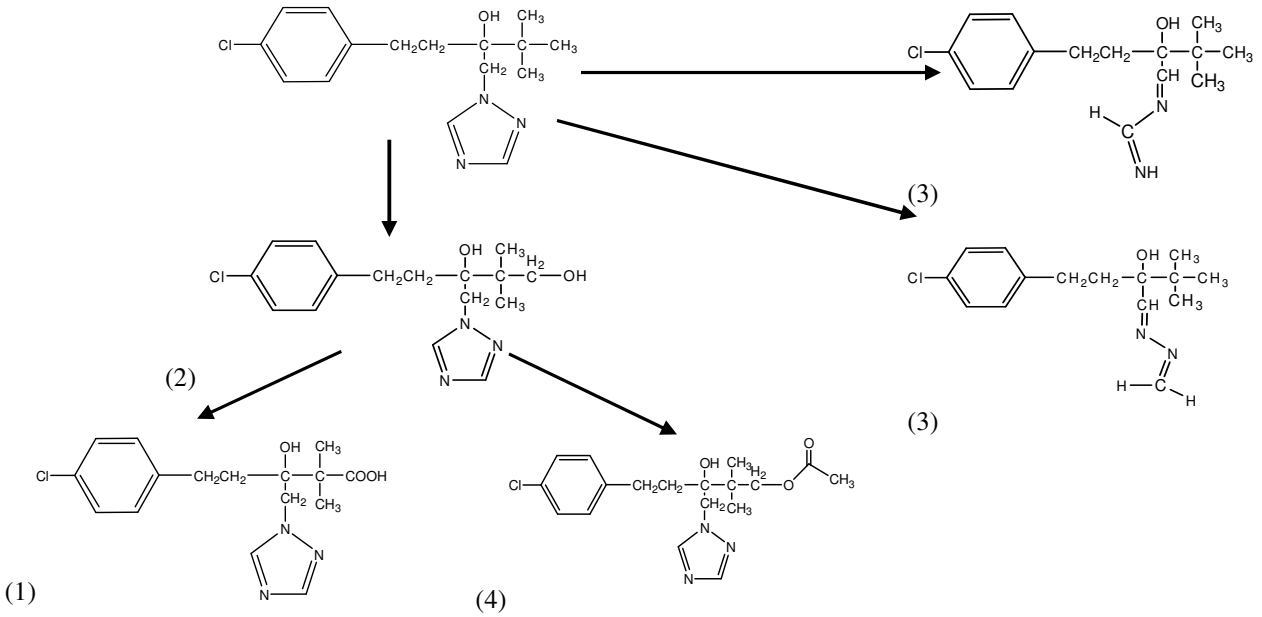


Figure 2.8. Proposed metabolic pathway of tebuconazole by *T. harzianum* (fraction numbers in parentheses)

Intervention mechanisms to stabilize or increase the durability of tebuconazole in treated timber would likely involve use of an additive that acts as an inhibitor to the initial reactions. Further work will involve identification of P450 in culture filtrates using spectral properties and occurrence of phase II type reactions which are thought to be used by the fungi in excretion or tolerance of tebuconazole. Since the initial oxidative reactions are mediated by P450, a suitable additive to tebuconazole would be one that is an inhibitor of P450. The chemical inhibitors must be specific toward cytochrome P450-mediated reactions in fungi. Several compounds that have an inhibitory effect on P-450 monooxygenases are known and may be of use. An example is piperonyl butoxide (PB). PB can act as a pesticide synergist. Although it does not have pesticidal properties, when added to a wood preservative mixture, its efficacy will be increased because the principal detoxification pathway (monooxygenation) is inhibited. Prevention or decrease in the rate of the initial oxidation will ensure the phase II reactions such as acetylation and methylation do not occur. EDTA (ethylenediamine tetraacetic acid) is an amino acid and chelating agent widely used to sequester di- and trivalent metal ions. EDTA may inhibit mono

oxygenation by chelating Fe^{3+} and Fe^{2+} ions important the P450 cycle. EDTA binds to metals via four carboxylate and two amine groups. Inhibiting the detoxification pathway allows higher unmetabolised systemic concentrations of the active insecticide to remain within the timber for a longer period.

The method used in this study was not able to identify more metabolites due to low concentrations, interference of media components and the complex pattern of IR peaks below 1500 cm^{-1} which are difficult to assign. Using radio active labeled ^{3}H of ^{14}C tebuconazole would give a more complete picture of all types of metabolites and pathways involved.

2.4 Conclusions

T. harzianum is able to tolerate tebuconazole at concentrations below 200ppm because it biotransforms the chemical mainly by cleavage of the 1, 2, 4 triazole ring and oxidation reactions to produce more hydrophilic metabolites. The tert butyl alcohol, the tert butyl carboxylic acid, and are the initial products. These initial oxidation products undergo acetylation or in a second step resulting in less hydrophilic metabolites. Mold fungi are able to tolerate tebuconazole by this mechanism. However, biotransformation may not be the only mechanism employed by *T. harzianum* in tolerating tebuconazole.

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CHAPTER 3. BIOTRANSFORMATION OF TEBUCONAZOLE BY BACTERIA AND FUNGI: A COMMON MECHANISM?

3.1. Introduction

The trend towards the use of environmentally sound building materials has increased the interest in wood preservation based on boron and purely organic systems. Much attention is being directed towards the formulation and testing of metal-free preservative systems. Organic biocides will play an increasing role in all user classes in the future (Schultz and Nicholas, 2006). Currently, the major advantage of organic systems is also their major disadvantage: their biotransformation by wood degrading and wood inhabiting but non-degrading microorganisms. Other missing elements in the organic systems are photoprotection and hydrophobicity (Evans, 2003). Microbial mediated detoxification of biocides may contribute significantly to the failure of treated wood (Briscoe et al., 1990).

Intricate groups of highly diverse microorganisms living in close association may be found in wood that has been exposed to the atmosphere or the soil for a long period of time. Any of the microorganisms present, including non-wood destroying organisms may be capable of biotransformation of chemicals at a faster rate than observed in laboratory studies (Briscoe et al., 1990). One species may modify the environment often making an otherwise unsuitable environment suitable to another micro-organism. Wood-inhabiting fungi, not necessarily responsible for major decay, have been shown to be capable of degrading a toxic compound or metabolizing it into a less potent form or derivative, thus, rendering it less effective in protecting wood from decay by typical wood-destroying fungi, which are less-tolerant basidiomycete wood-destroyers (Duncan and Deverall, 1964; Ruddick, 1984). A study of the biodetoxification of 3-Benzo[b]thien-2-yl-5, 6-dihydro-1, 4, 2-oxathiazine-4-oxide) isolated 11 species of proteobacteria that showed the ability to degrade the biocide (Wallace and Dickinson, 2004). Bacteria cause reductions in biocide concentrations ranging from 14% for 2-thiocyanomethylthio

benzothiazole (TCMTB) to nearly 65% in MBT (Briscoe et al., 1990). Through HPLC and a bioassay, Wallace and Dickinson (2006) demonstrated that tebuconazole, chlorothalonil, and IPBC are detoxified when exposed to the *Rastolia* strain of bacteria. No degradation products were isolated. Wood-destroying basidiomycetes that secrete the enzyme laccase into the culture medium are able to overcome the toxic effects of pentachlorophenol (PCP) (Lyr, 1963). Increases in both threshold and toxic limits have been observed when wood treated with alkylammonium compounds is first subjected to staining fungi and then basiodiomycetes in a second step (Ruddick, 1984). *Gliocladium roseum* is a mold that is capable of transforming significant amounts of DDAC (didecyldimethylammonium chloride). In one study nearly 50% of the DDAC in sawdust was degraded over 11 weeks (Dubois and Ruddick, 1998). It was hypothesized that the alteration of the quat compounds by the mold may detoxify them to a level where wood rotting basidiomycetes could grow. The metabolite was identified as a hydroxylated quaternary ammonium compound (QAC). *Phanerochaete chrysosporium* is recognized for its ability to metabolize a large diversity of compounds, including PCP, by the lignin degrading system (Mendoza-Cantu et al., 2000). *M. incrassata* has been reported to tolerate chromated copper arsenate (CCA) and cause substantial weight loss in pine blocks treated with CCA or copper sulphate (Illman and Highley, 1996). *Trichoderma harzianum* is known to be tolerant to the anti-sapstain methylene-bis –thiocyanate (MBT) at concentrations ranging from 4ppm to 34ppm and most fungicides. Considerable increases in threshold and toxic limits have been observed when wood treated with alkylammonium compounds is first subjected to *T. harzianum* and then basidiomycetes in a second step. *Trichoderma* spp. are present in nearly all soils and other diverse habitats.

The effects of bacteria have long been recognized as the ability to cause pronounced increase in permeability and more recently their ability to degrade preservative treated wood and highly durable timbers containing high extractive levels (e.g., *Eusideroxylon zwageri*) and

timbers high lignin contents (e.g., *Alstonia scholaris*) (Daniel, 2003). The role of bacteria in the degradation of organic preservatives is important when considering the long term performance expected from treated wood in service. Activity against the causal decay organisms and resistance to physical losses are no longer the sole performance criteria that need to be understood when developing new wood preservatives. Despite the role played by microorganisms in wood degradation or preservative detoxification, they are not normally considered in biocide evaluation. As the wood preservative industry becomes more reliant on wholly organic fungicides, there is a need to establish biotransformation pathways of biocides and use this knowledge to attempt to stabilize them in wood by disruption of the detoxification process. An understanding of mechanisms involved in detoxification by bacteria, fungi, and termites is needed to provide means for defeating these processes (Wallace and Dickinson, 2006).

In the current study one bacteria strain (*Pseudomonas fluorescens*), a mold fungus (*T. harzianum*), a white rot fungus (*P. chrysosporium*) and a brown rot fungus (*M. incrassata*) were used to access the extend of biodegradation and the initial products of metabolism of tebuconazole. This study proposes the mechanism(s) for the metabolism and explores the possibility of a common biotransformation mechanism for all species studied. If it proves possible to disrupt the mechanism of degradation, this could herald a new generation of environmentally friendly wood-preservatives.

3.2 Materials and Methods

3.2.1 Test Chemicals and Fungi

Ninety five percent tebuconazole (Preventol A8) was provided by Lanxess Corporation (Pittsburgh, PA) and the standard (99%) was obtained from Sigma Aldrich (St Louis, MO). An original freeze-dried strain of the bacteria species (ATCC No. 11150) was obtained from the American Type Culture Collection (Manassas, VA). All fungal species were provided by the

US. Forest Products Laboratory (Madison, WI). Fungal cultures were grown out and maintained on media containing 1.5% agar, 2% malt extract, and 0.001% yeast extract.

3.2.2 Determination of MICs

Preliminary work involved determination of the minimal inhibition concentrations (MIC) for tebuconazole against *P. chrysosporium* and *M. incrassata.* The MIC of *T. harzianum* had been determined in a prior study (Chapter 2). Plates of nutrient agar containing tebuconazole in concentrations ranging from zero (control) to 200ppm were prepared in triplicate. Plates were kept at 25^{o}C for 14 days. MIC the lowest concentration at which no visible growth of mycelium was observed was determined.

3.2.3 B3 Media Suitability

The study utilized nutrient limited B3 medium for N-limitation. B3 represents a more applicable situation for wood which has low amounts of nitrogen. B3 media per liter of distilled water contained: 1.73 t-aconitic acid adjusted to pH to 4.5 with KOH, 2g KH_2PO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.1g $CaCl_2 \cdot 2H_2O$, 0.2g ammonium tartrate, 10g glucose, 0.001g thiamine and 10mls mineral elixir. Mineral elixir contained the following per liter: 1.5 g nitrilotriacetic acid (pH adjusted to 6.5 with KOH, 3.0g $MgSO_4 \cdot 7H_20$, 0.5g $MnSO_4 \cdot H_2O$, 1.0g NaCl, 0.1g $FeSO_4 \cdot 7H_2O$, 0.1g $CoSO_4$, 0.1g $CaCl_2$, 0.1g $ZnSO_4 \cdot 7H_2O$, 0.01g $CuSO_4$, 0.01g $AlK(SO_4)_2 \cdot 12H_2O$, 0.01g H_3BO_3, and 0.01g $NaMoO_4 \cdot 2H_2O$. Because B3 media was studied and developed specifically for *P. chrysosporium*'s extracellular peroxidase production, it was necessary to ascertain its suitability for growing *M. incrassata* in subsequent degradation studies. One circular plug obtained from 8-day old cultures of *M. incrassata* and 12-day cultures of *P. chrysosporium* was used to inoculate 100mls of sterilized B3 in Erlenmeyer flasks. Twenty eight flasks were inoculated for each species, covered with parafilm, and kept at 25^{o}C for two weeks on a shaker at 100rpm. Growth was monitored by weighing the dry fungal cell mass harvested

by filtering the liquids through Whatman #2 filter paper at time intervals of 0-14 days. Mycelia were rinsed twice with deionized water and air dried to constant weight. Separate flasks were grown using malt extract broth as growth media. The broth contained 17g/l of mycological peptone and 3 g/l of malt extract. Growth rates for the two media were compared after 14 days.

3.2.4 Biotransformation Studies

Two hundred and fifty ml. Erlenmeyer flasks containing 100ml B3 media were supplemented with 0.1ml of 0.02% tebuconazole to give a final concentration of 2 ppm for each flask. Flasks were inoculated with an agar plug from actively growing colonies. Cultures were incubated at 25^{o}C and shaken at 120rpm for a period ranging from 3-21days. Three replicates were used for each time interval. For the bacteria strain, 250 ml flasks containing 50 ml Luria Bertani (LB) media were supplemented with 0.1ml of 0.2% tebuconazole to make 20ppm of each solution. An overnight *P. fluorescens* culture (100µl) was added and flasks were incubated at 30^{o}C and shaken at 120rpm for a total of 21 days. LB Media contained: 10.0 g/l tryptone, 5.0 g/l yeast extract, and 5.0 g/l NaCl. All samples were centrifuged (10 min. at 15000xg and 4^{o}C). The centrifuge was a fixed angle rotor, induction drive Beckman model J2-21M. The culture controls consisted of media blanks in which microorganisms were grown under identical conditions in media but without tebuconazole. The substrate control consisted of tebuconazole and sterile media incubated without innoculum to determine whether tebuconazole could chemically decompose or transform under experimental conditions.

3.2.5 Quantitative Determination of Tebuconazole Retained in Samples

Five ml of filtered medium was extracted in solid-phase extraction (SPE) columns. The columns (Bond Elut –C18, 500mg, 3ml Varian #12102028) were preconditioned with two column volumes of methanol, followed by two column volumes of milli-Q water. Five ml of sample was loaded and pulled through at one drop per second, followed by a column rinse of 50:50 acetonitrile water. Retained compounds were eluted with 4 column volumes of methanol

collecting in 15ml centrifuge tubes. Each tube was dried to below 5ml on a nitrogen evaporator and transferred to a second tube while passing over sodium sulfate. The final volume was adjusted to 5ml. Samples were cleaned with a 0.2 µm Teflon™ filter prior to quantitative analysis by gas chromatography (GC-MS). The GC-MS was an Agilent 6890 gas chromatograph using a 5973 mass selective detector. The injection port temperature was 250°C, the detector temperature 280°C, and the carrier gas was helium at 1 mm min^{-1}. The oven was initially held at 80°C for 2 min followed by a 30°C min^{-1} increase to 190°C and then an 8°C min^{-1} increase to a final temperature of 300°C. The capillary column model No. RTX35 was 30m X 250µmX 0.25µm. Injection volume was 2.0µl, and total run time was 20.42 minutes. Tebuconazole eluted at 15.9 min. Running in selected ion mode (SIM), ions detected for tebuconazole were 250 as the target ion, and 125 and 252 as the qualifier ions. Calibration was done using a four point curve. Concentrations of the samples were calculated using the formula:

$(X / V_1)^* (V_2)^*$dilution factor [1]

Where: X - off the curve reading from the instrument.

V_1- Initial vol. of sample extracted by SPE.

V_2- final vol. of sample after SPE.

3.2.6 Preparation of Samples for HPLC

To isolate the major metabolites in sufficient quantities for structural elucidation, the transformation of tebuconazole was carried out on a preparative scale. For each fungal species, ten 250ml Erlenmeyer flasks containing 100ml of B3 media were supplemented with 0.1ml of 0.02% tebuconazole to give a final concentration of 2 ppm. Flasks were inoculated with an agar plug from an actively growing colony, incubated at 25°C, shaken at 100rpm for 21 days, and centrifuged (10 min. at 15000xg and 4°C). The liquid samples were pooled into two equal composite samples. For the bacteria, ten 250 ml flasks containing 100 ml. Luria Bertani (LB) media were supplemeted with 0.1ml. of 0.2% tebucanazole to make 20ppm of each solution. An

overnight *P. fluorescens* culture (100µl) was added and flasks were incubated at 30°C while shaking at 120rpm for 21 days. Samples were then centrifuged (10 min. at 15000xg and 4°C) and filtered and pooled into two equal composite samples.

Each sample was subjected to extraction and cleanup by the following procedure: The sample was transferred into a 1L separating funnel to which 75ml of dichloromethane was added. The sample was subjected to shaking for 1.5 min. vented every 20 seconds to release pressure, then left to stand for 3 min. The bottom layer was passed over sodium sulfate into a 400ml beaker. Extraction was repeated twice. The sample was then placed on a water bath at 45°C and evaporated until almost dry. Five ml of acetonitrile was added for solvent exchange, re-dried to about 2ml, and transferred to a centrifuge tube with several rinses of acetonitrile while passing over sodium sulfate. The tube was dried to below 5ml. on a nitrogen evaporator, the final volume adjusted to 5ml before qualitative analysis by high pressure liquid chromatography (HPLC). The culture controls consisted of blanks in which microorganisms were grown without tebuconazole. The substrate control consisted of tebuconazole and sterile media without fungi.

3.2.7 HPLC Analysis

Several reverse-phase HPLC runs were necessary to detect and purify metabolites. The system consisted of a Waters 600 pump with a Waters 2487 UV detector. The column was a Waters Delta-Pak C18, 300 Å, 15 micron, 8mm x 10 cm radial compression module. Eluant composition as a function of time was: isocratic from 0-5 min. 70% water+ 0.1% formic acid 30% acetonitrile, linear increases of acetonitrile from 5-30 min. to 85%, and isocratic at 85% acetonitrile for 5 min. Flow rate was 1 ml $min.^{-1}$ and detection was at 224 nm wavelength. Tebuconazole eluted at 28 min. Qualitative analysis using authentic standards of metabolites was not possible as metabolites of tebuconazole could not be obtained. Tebuconazole peaks and those of metabolites were identified by comparison of elution time, protonated molecular mass, isotopic pattern distribution, and identification of functional groups in the tebuconazole standard,

media containing tebuconazole before exposure to microorganisms and in media containing tebuconazole after exposure to microorganisms

3.2.8 Mass Spectral Characterizations and IR Analysis

Exact mass determination of HPLC fractions of tebuconazole and its metabolites was performed by electrospray ionization (ESI). The instrument operated in positive ESI mode was an Agilent 6210 Time of flight (TOF) LC/MS mass spectrometer. HPLC fractions and authentic tebuconazole were subjected to IR analysis to determine functional groups retained or changed on the metabolites. The FTIR system was a Bruker Tensor 27 system single beam instrument connected to an optical user software (OPUS) version 4.2 data collection and analysis program. Two drops of sample were placed on a Real crystal IR KBr card and air dried to dryness. Spectra were determined after 16 background and sample scans.

3.3 Results and Discussion

All results of this study were analyzed and compared to those for *T. harzianum* carried out in the previous study (Chapter 2).

3.3.1 Minimum Inhibitory Concentrations

Of the concentrations tested, the lowest at which no growth was observed was 20ppm for *M. incrassata* and 5ppm for *P. chrysosporium.* Mycelia diameter increases on Petri dishes and MICs are shown in Table 3.1.

3.3.2 Suitability of B3 media for *M. incrassata.*

After a lag phase of 7 days, *M. incrassata* grew at a fast rate, steadily increasing in mycelia mass until the 21st day. The mean increase in weight of fungal mycelia after 21 days for the two types of media were compared in a paired t-test and found not to be different ($p=0.12$, $df=13$).

Table 3.1 Diameter increase* (mm) and MICs of the fungi tested.

Tebuconazole (ppm)	*M. incrassata*	*P. chrysosporium*	*T. harzianum*
Agar only (control)	76	76	76
0 (solvent- acetone)	76	76	76
0.5	76	68	76
1	66	10	76
1.5	44	5	76
2	38	4	76
2.5	36	2	76
5	24	0	76
10	18	0	76
15	6		70
20	0		70
25	0		64
50			42
80			38
100			38
150			10
180			5
200			0
MIC	20	2.5	200

* Diameter increase = [(Diameter of mycelia –Diameter of the original plug (10mm)]

Growth in B3 media did not differ from growth in malt extract broth. Hence B3 media was appropriate for use in further tests. B3, media was preferred for further experiments because it does not contain proteins that would have interfered with further work on proteineous extracts produced by the fungi. Figure 3.1 shows growth of the fungi in the two types of media.

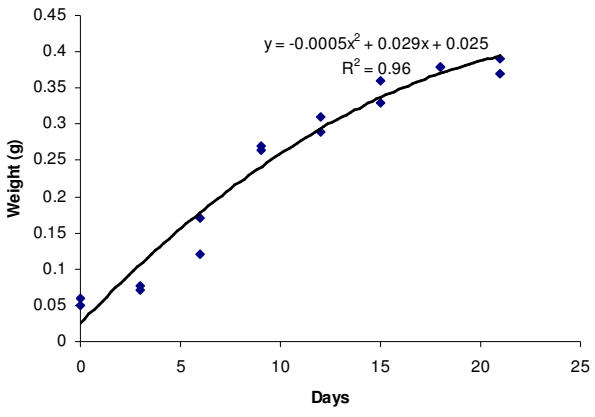


Figure 3.1a. Growth of *M. incrassata* in B3 media

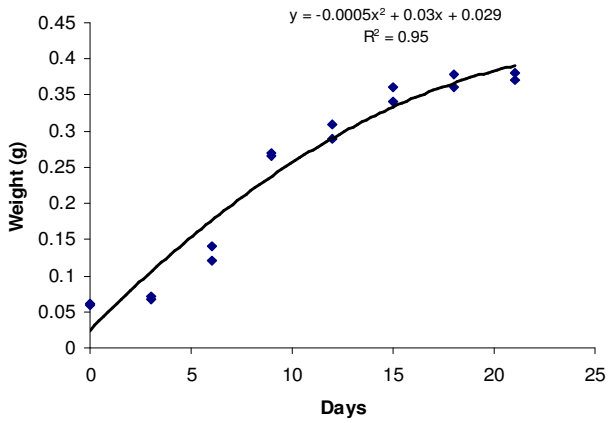
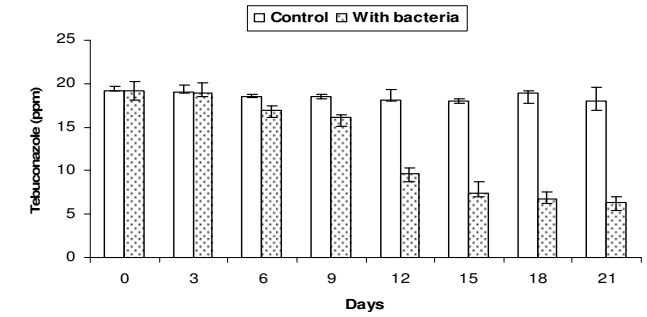


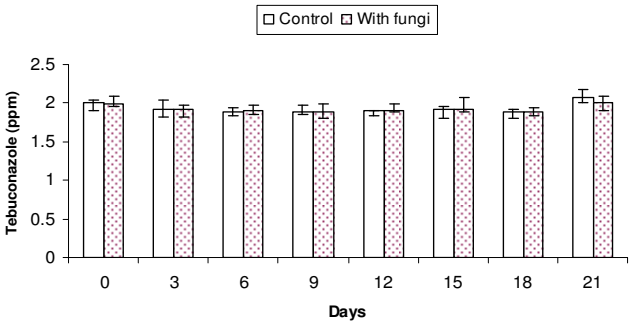
Fig 3.1b. Growth of *M. incrassata* in malt extract

3.3.3 Quantitative Analysis of Degradation of Tebuconazole

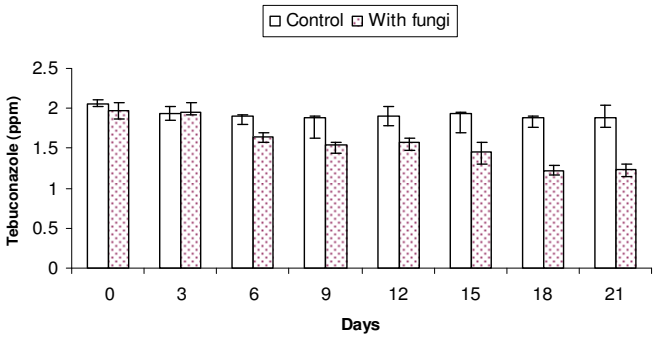
After 12 days, it was apparent that the amount of tebuconazole in media inoculated with bacteria and *M. incrassata* was reducing at a steady rate. After 21 days, 70.2% was depleted by bacteria from the LB media. In B3 media, 40.4% was depleted by *M. incrassata* while 4.1% was depleted by *P. chrysosporium.* Actual values retained are shown in Figure 3.2. The control cultures had less than 5% of the tebuconazole depleted. The average amount depleted in control cultures and in *P. chrysosporium* cultures were not significantly different (P=0.1, df =5). Hence tebuconazole degraded over 21 days in *P. chrysosporium* cultures was insignificant.



Bacteria
(*P. fluorescens*)



White rot
(*P. chrysosporium*)



Brown rot
(*M. incrassata*)

Figure 3.2. Tebuconazole retained in media after 21 days.

3.3.4 HPLC Analysis

Secondary metabolites produced by microorganisms did not affect the analysis of tebuconazole. Two major metabolites were eluted and isolated in cultures of *M. incrassata* and two in cultures of *P. fluorescens* in addition to tebuconazole. These metabolites were not found in control cultures. Chromatograms of *P. chrysosporium* cultures contained mainly tebuconazole. Chromatograms of the standard, substrate control and culture control, and test tebuconazole in media exposed to fungi or bacteria for 21 days were obtained and compared. Chromatograms are shown in Figure 3.3 and 3.4. Parent tebuconazole eluted at 28 min. Major components of B3 media eluted at 2 and 21 min. Major components of Luria Bertani media eluted at 5 and 21 min. Compounds thought to be metabolites were those not found in the media and eluting at times different from that of tebuconazole. Metabolites of *M. incrassata* had retention times of 15 and 17 min. and those for *Pseudomonas fluorescens* had retention times 15 and 11 min., respectively. All the metabolites were more hydrophilic and eluted from the column faster than tebuconazole.

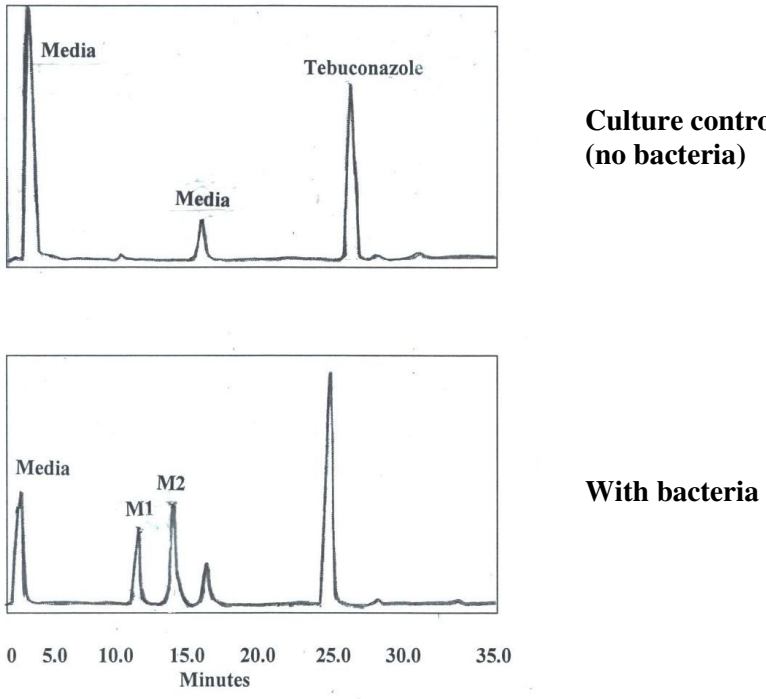


Figure 3.3. HPLC Chromatograms after exposure to bacteria.

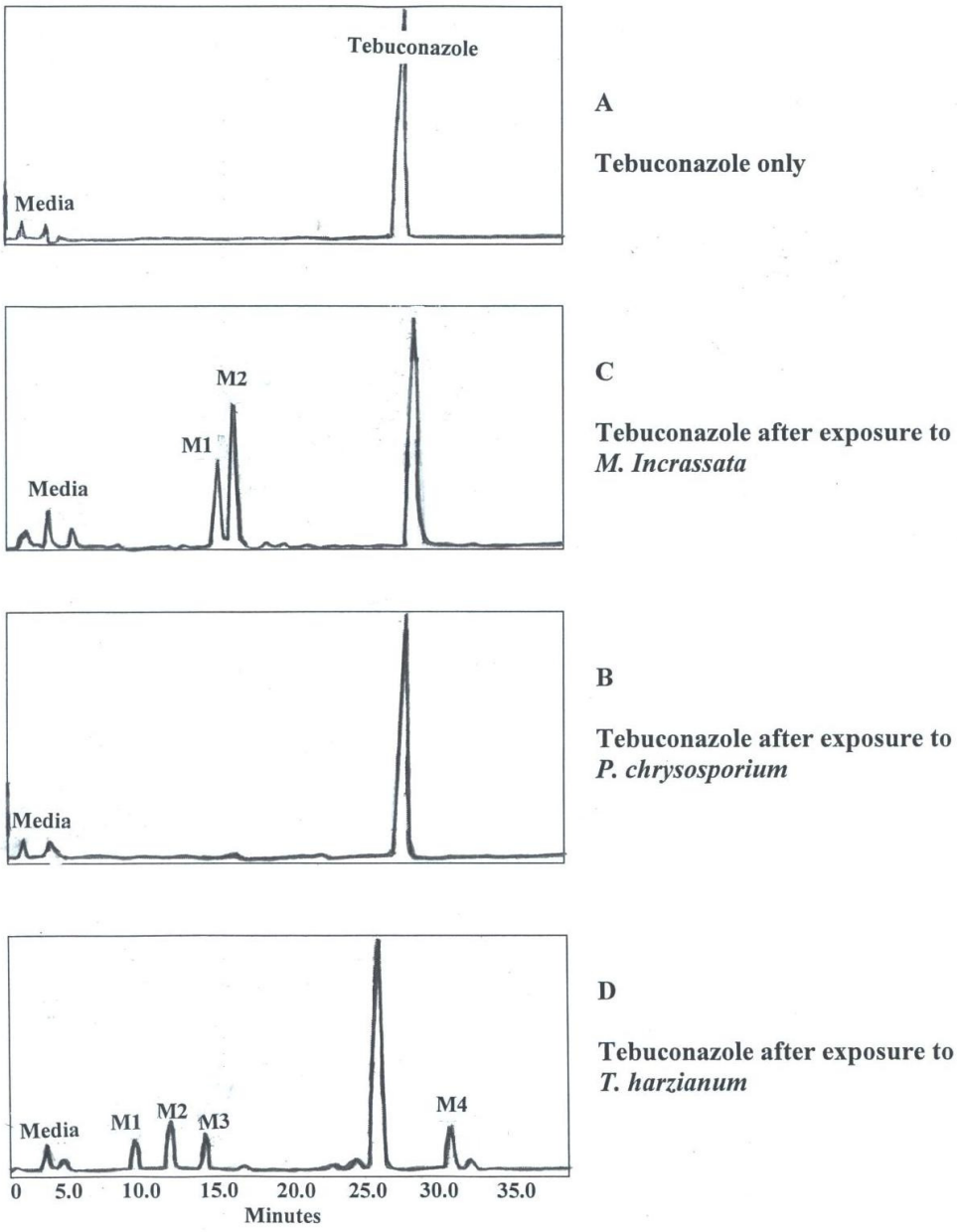


Figure 3.4. HPLC chromatograms after exposure to fungi.

3.3.5 Mass Spectral Characterizations and IR Analysis

Intact molecular mass of a compound is not a unique identifier because some compounds may have the same intact mass. However, determination of exact intact molecular mass was useful in this case because a large part of the molecule (tebuconazole) was already known and a minor unknown change or changes needed to be characterized. The Agilent 6210 Time of flight (TOF)

mass spectrometer used had high accuracy of 2 ppm mass dependent The number of possible chemical formulae assigned to an accurate determined mass was reduced by comparing spectral and isotope patterns based on mass measurement obtained using the method by Stoll et al. (2006). Thus, spectra were used to rate and exclude compounds not resulting from metabolism of tebuconazole. Parent tebuconazole showed a protonated mass $(M+H)^+$of 308.15. A summary of the spectra monitoring intense peaks of fractions and comparing them with results of *T. harzianum* from chapter 2 are shown in Table 3.2.

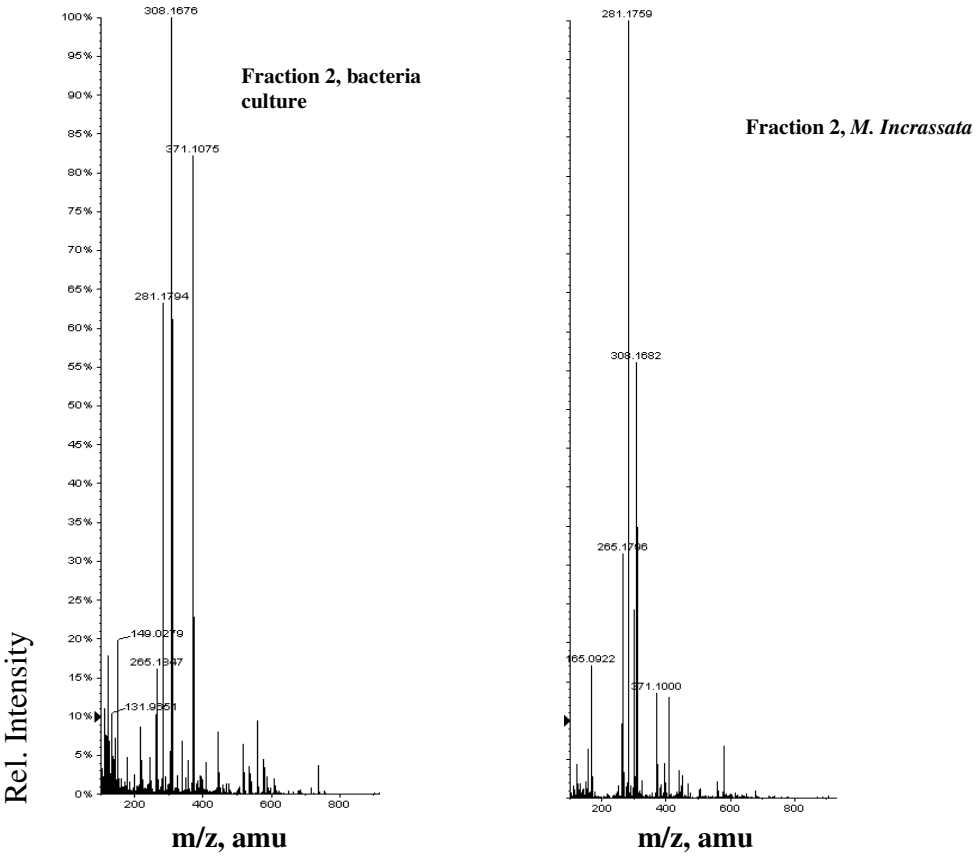


Figure 3.5. Spectra of fractions

The ^{35}Cl:^{37}Cl 3:1 intensity isotope pattern distribution shown in Figure 3.6 was apparent because of the chlorine on tebuconazole and was used to identify actual metabolites as opposed to those arising from the media or fungal secretions. Intensity ratios in the isotope are due to the natural abundance of the isotopes.

Table 3.2. Summary of positive ESI-MS m/z spectra - dominant ions and intensities

Sample		Peaks (m/z, amu)	Intensity counts (%)	Most intense peaks
Tebuconazol standard		308.15	100	308.15
		309.15	10	
		310.15	25	
		371.11	82	
P. fluorescens	Fraction 1	308.15	80	324.16, 308.15
		310.15	25	
		324.16	100	
		326.19	28	
		371.11	82	
	Fraction 2	308.15	20	281.17, 308.15
		306.35	10	
		281.17	100	
		283.19	24	
		371.11	21	
M. Incrassata	Fraction 1	308.15	20	338.34, 308.15
		324.16	20	
		338.34	100	
		340.36	23	
		371.11	82	
		264.13	18	
	Fraction 2	281.17	100	281.17
		283.18	25	
		308.15	57	
		265.17	33	
		371.10	15	
P. chrysosporium	No major fraction identified	308.15	100	308.15
		309.15	10	
		310.15	24	
		324.17	34	
		371.11	53	
T. harzianum	Fraction 1	308.15	80	338.34, 308.15
		310.15	25	
		338.34	100	
		340.36	20	
		371.11	82	
	Fraction 2	308.15	45	324.15, 308.15
		324.33	80	
		326.32	21	
		371.11	75	
	Fraction 3	281.17	100	281.17
		283.18	25	
		308.15	57	
		265.17	33	
		371.10	15	
	Fraction 4	281.17	25	366.37
		308.17	45	
		364.36	21	
		366.37	100	
		368.35	24	
		371.11	72	
Media blank		371.21	100	371.21
		121.97	70	121.97
		131.96	35	

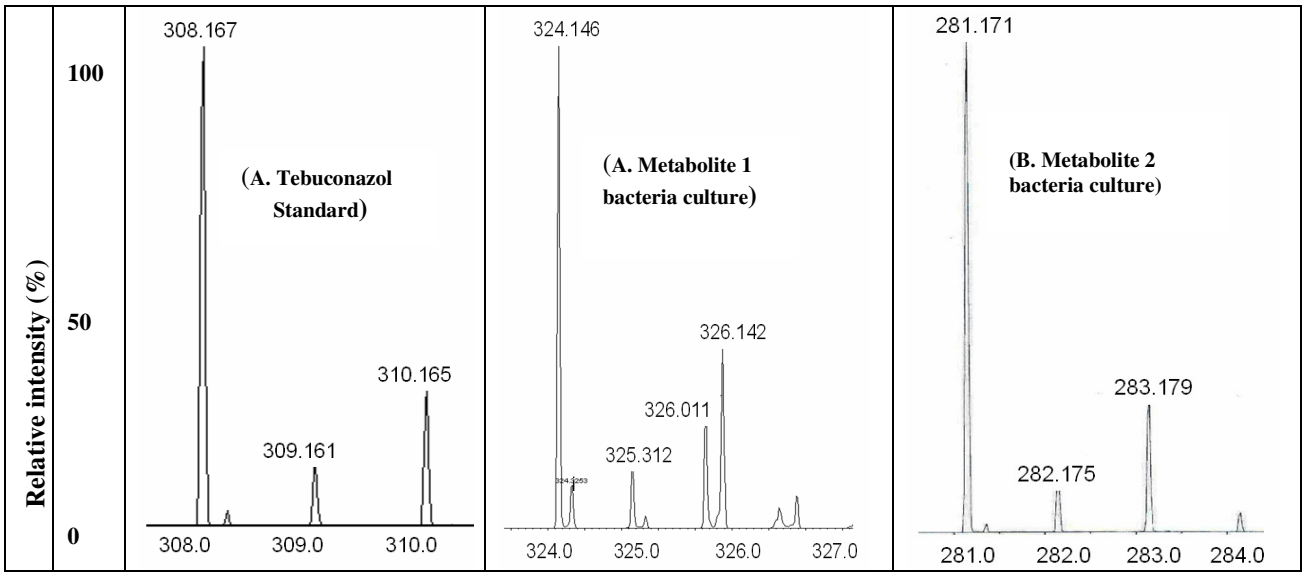


Figure 3.6. Tebuconazole and metabolite isotope distribution pattern.

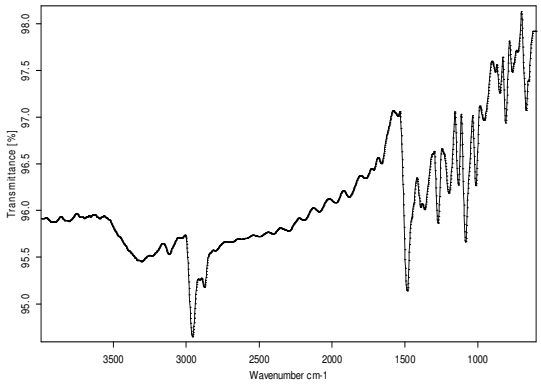
Within 21 days, 40.4% and 70.2% of tebuconazole was metabolized by *M. incrassata* and *Pseudomonas fluorescens* respectively, into a form that may be less toxic. Although *P. chrysosporium*, is recognized for its ability to metabolize a large diversity of compounds, including pentachlorophenol (PCP), by the lignin degrading system (Kullman and Matsumura, 1996; Sack et al., 1997; Mendoza-Cantu et al., 2000) it showed little or no ability to metabolize tebuconazole. Little degradation occurred over the exposure period of 24 days. This collaborates the results of Morrell and Velicheti (1994) who showed that *P. chrysosporium* was not able to degrade triazoles to a significant or measurable amount.

Establishing the identity of metabolites in the bacterial and *M. incrassata* cultures involved analysis of functional groups. Figure 3.6 shows the IR spectra of the 2 HPLC fractions from *M. incrassata* culture and the two fractions of the bacterial culture. Changes in protonated mass and IR spectra, indicated that these two species degraded tebuconazole by cleaving the triazole ring and also by oxidation reactions. Based on the reversed nitrogen rule since the molecular ions were generated by protonation, an even mass for tebuconazole and some of the degradation products indicated presence of an odd number of nitrogens (three). This led to the

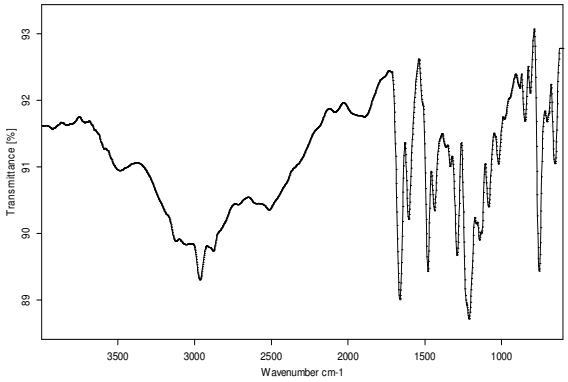
conclusion that these degradation products contained the intact triazole ring. One product in fraction 2 of both *M. incrassata* and the bacteria culture had a molecular mass 281.17 indicating an even number of nitrogens. These coupled with a loss of 27 amu, led to the conclusion that the H-C-N fragment was lost after cleavage of C-N and/or N-N bonds on the 1,2,3-triazole ring to form an imine. Tebuconazol has a degree of unsaturation of 7 while this metabolite has 6 further confirming the cleavage of the triazole ring. Two peaks corresponding to 281.17 were observed in the total ion count spectra of the fraction containing this metabolite. This indicates two possible products of the same molecular mass after cleavage of the triazole ring at two different places both resulting in the loss of an H-C-N fragment. This same metabolite was also observed in *T. harzianum* cultures in (Chapter 2). This is evidence of a common mechanism in all fungal species and the bacterium studied.

Oxidation of the tert butyl group was a major metabolic pathway in both species. The main products were the oxidation of the methyl groups on the tert butyl moiety. Oxidation may also have occurred on the phenyl ring and at the α–carbon. It was not possible to determine the exact position of the hydroxyl group by the method used in this study. Previous results (Chapter 2) showed that the mold *T. harzianum* after initial oxidation reactions performs reactions similar to phase II reactions (conjugation by acetylation) used in detoxification of xenobiotics in mammalian systems. The fungus metabolizes the hydroxyl group formed on the tert butyl moiety by acetylation to form an ester. Acetylation to form an ester was not observed in the metabolism by *M. incrassata* and *Pseudomonas fluorescens.* No evidence of glucosidation and sulphonation phase II reactions was observed. However, there is a possibility that they occured after the initial oxidation. These other phase two reactions are thought to be reactions used by the fungi in metabolizing chemicals to form non toxic metabolites such as (Cerniglia et al., 1982).

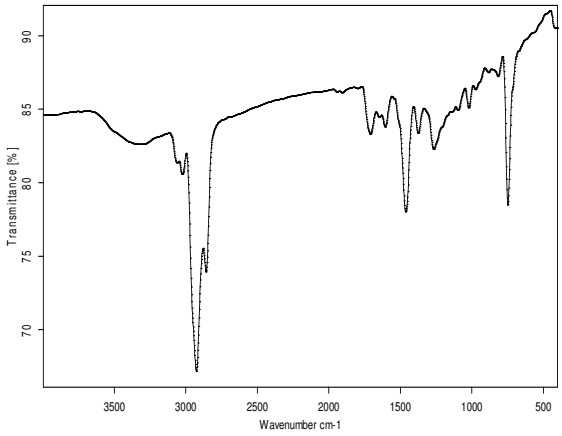
Tebuconazole



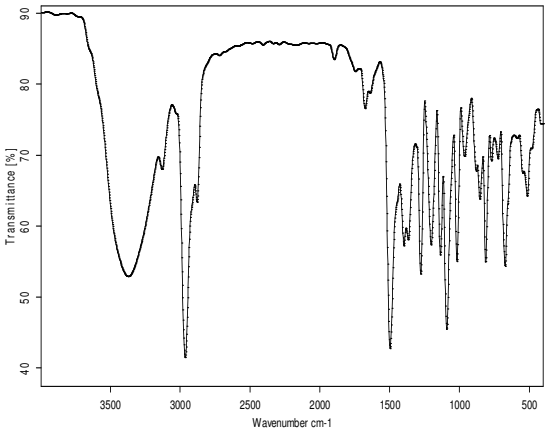
Fraction 1. *M. incrassata* culture



Fraction 2. *M. incrassata* culture



Fraction 1. *Pseudomonas fluorescens* culture



Fraction 2. *Pseudomonas fluorescens* culture

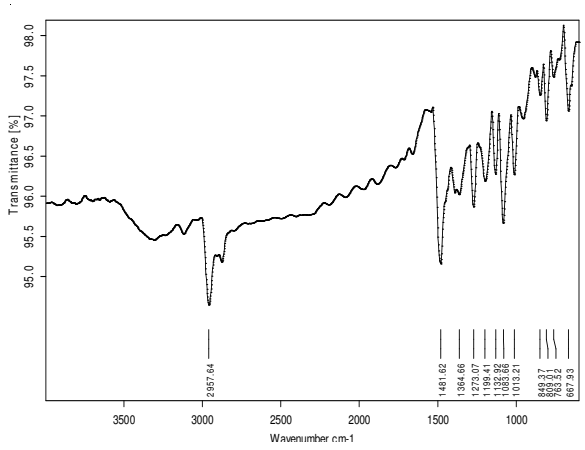


Figure 3.7. IR spectra of tebuconazole and fractions

There was no evidence of dechlorination. The chlorine atom was present in all metabolites. IR absorption at 700-800cm^{-1} and 3:1 isotope pattern in mass spectra observed in all metabolites .indicated a C-Cl bond in tebuconazole and all fractions. There was no evidence of N-dealkylation resulting in the free triazole ring or products resulting from further reaction of the triazole ring. Table 3.3 gives a summary of the changes in molecular mass and relevant IR bands identified therefore leading to the proposed structures of metabolites and pathways To detoxification

Table 3.3. Summary of change in molecular mass and relevant IR band

Protonated ion mass	Change in amu	Unique IR band observed	Proposed pathway
308.15 (Tebuconazol)	0	-	
324.15	+16	3200-3000	Oxidation
338.34	+30	3500-2500 1700-1800	1. Oxidation 2. Carboxylation
281.17	-27	-	Triazole ring cleavage

The proposed pathways for transformation of tebuconazole by *Pseudomonas fluorescens* and *T. harzianum* are shown are shown in Figures 3.8 and 3.9 respectively.

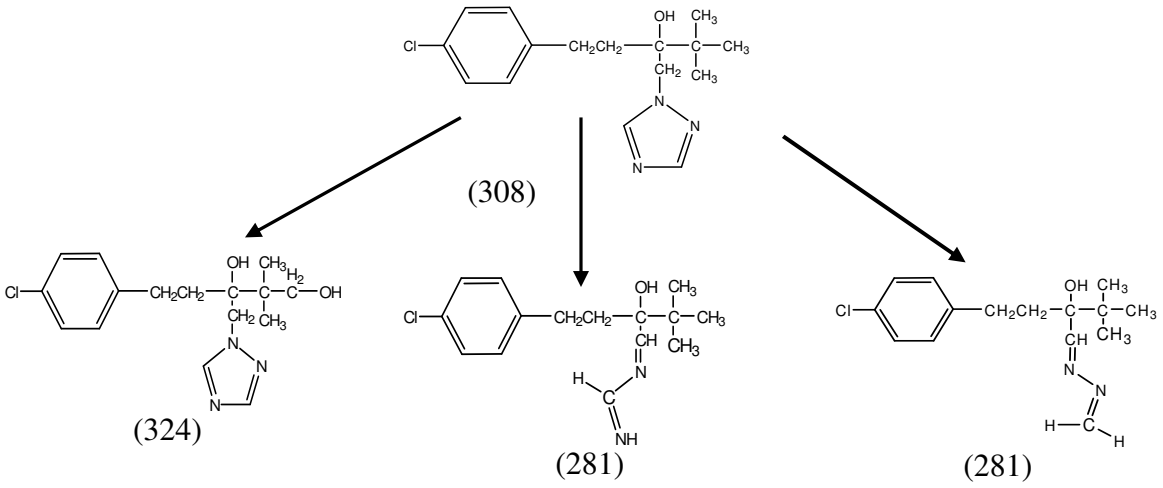


Figure 3.8. Proposed metabolic pathway by the bacteria *Pseudomonas fluorescens* (molecular weights in parentheses).

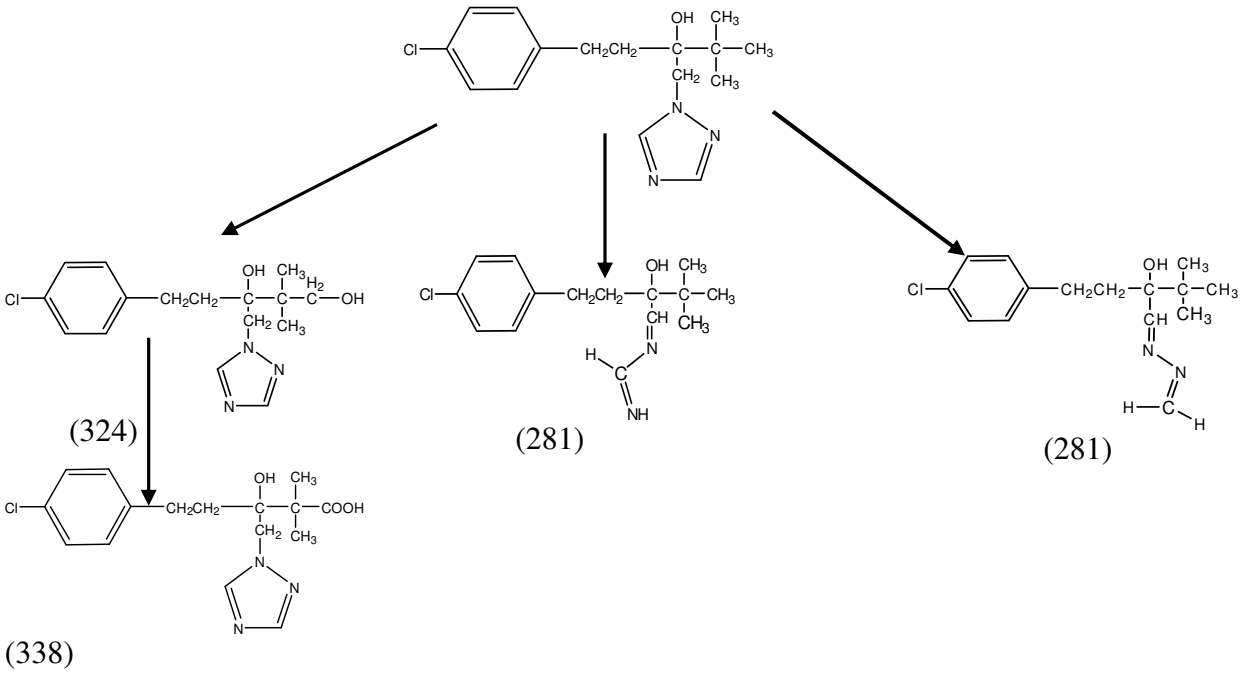


Figure 3.9. Proposed metabolic pathway by the fungi *M. incrassata* (molecular weights in parentheses)

The ability of a fungus to degrade a biocide contributes to the efficacy of the biocide. *P. chrysosporium* with the lowest MIC (highest tebuconazole efficacy) was found not able to degrade tebuconazole while *T. harzianum* with the highest MIC (lowest efficacy) degrades tebuconazole and tolerates it at concentrations less than 200ppm. While molds like *T. harzianum* are not responsible for decay, they may modify the environment by lowering biocide concentrations or metabolizing it into a less potent form or derivative making an otherwise unsuitable environment suitable to a wood degrading another micro-organism. They therefore render the biocide less effective in protecting wood from decay by typical wood-destroying fungi which are less-tolerant basidiomycetous wood-destroyers.

Fungi can perform many different complex conversions of polyaromatic hydrocarbons and steroid compounds. Biochemical evidence indicates that monooxygenation bioconversions are often mediated by cytochrome P450 (P450) as a part of monooxygenase enzyme systems

(Makovec and Breskvar, 2000). The presence of P450 was not confirmed in this study although monooxygenase type reactions were shown to extensively occur in the transformation of tebuconazole by *T. harzianum, M. incrassata* and the bacterium *Pseudomonas fluorescens*.

Because the initial detoxification pathway is the same in all species i.e., cleavage of triazole ring and oxidation, intervention mechanisms to stabilize or increase the durability of tebuconazole in treated timber would be the same. This would most likely involve use of an additive that acts as an inhibitor to the initial oxidation reactions. The additive must be specific toward cytochrome P450-mediated reactions. Several compounds that have an inhibitory effect on P-450 monooxygenases are known and may be of use. Examples are piperonyl butoxide (PB) and EDTA (ethylenediamine tetraacetic acid). PB is a pesticide synergist. Although it does not have pesticidal properties, when added to a wood preservative mixture, its efficacy may be increased because the principal detoxification pathway (monooxygenation) is inhibited. Prevention or slow down in the rate of the initial oxidation will ensure the phase II reactions such as acetylation and methylation do not occur. EDTA is an amino acid and chelating agent widely used to sequester di- and trivalent metal ions. EDTA may inhibit mono oxygenation by chelating Fe^{3+} and Fe^{2+} ions important in the P450 cycle.

More probable metabolites could not be separated completely with this method due to low concentrations and interference of media components. Using radio active labeled ^{3}H or ^{14}C tebuconazole would give a more complete picture of types of metabolites and pathways involved.

3.4 Conclusions

Although *P. chrysosporium*, is recognized for its ability to metabolize a large diversity of compounds, it showed little or no ability to metabolize tebuconazole. Only parent tebuconazole eluted after an HPLC run of a 21 day culture. *M. incrassata* (brown rot), the mold *T. harzianum,* and bacteria *Pseudomonas fluorescens* all cleaved the triazole ring and performed mainly

oxidation processes in the initial degradation of tebuconazole. The main initial products are the imines resulting from triazole cleavage and oxidation products of the methyl groups on the tert butyl moiety (i.e., the alcohol and the carboxylic acid). *T. harzianum* was able to perform phase II type reactions by acetylation. *M. incrassata* and the *P. fluorescens* bacteria did not metabolize the chemical by acetylation. The ability of a fungus to degrade a biocide contributes to the efficacy of the biocide against that species. *P. chrysosporium* with the lowest MIC (highest efficacy) was found not able to degrade tebuconazole while *T. harzianum* with the highest MIC (lowest efficacy) degrades tebuconazole and tolerates it at concentrations less than 200ppm. Molds such as *T. harzianum* though not responsible for decay, may lower biocide concentrations or metabolize it into a less potent derivative making the environment suitable to wood degrading microorganisms.

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CHAPTER 4. PROTEINEOUS EXTRACELLULAR EXTRACTS INVOLVED IN BIOTRANSFORMATION OF TEBUCONAZOLE BY WOOD INHABITING MOLDS AND DECAY FUNGI

4.1 Introduction

Activity against the causal decay organisms and resistance to physical losses are no longer the sole performance criteria that need to be understood when developing a new wood preservative. Microbial mediated detoxification of biocides by bacteria and fungi may contribute significantly to the failure of treated wood (Briscoe et al., 1990). Though not responsible for decay, molds, sapstains and bacteria, may lower biocide concentrations or metabolize it into a less potent derivative making the environment more suitable to wood degrading microorganisms.

In Chapter 2 and 3 it was shown that the mold *Trichoderma harzianum,* brown rot *Meruliporia incrassata,* and the bacterial species *Pseudomonas fluorescens* metabolize tebuconazole by performing oxidation processes in the initial stages. The main products are the oxidation products of the methyl groups on the tert butyl moiety (i.e., the alcohol and the carboxylic acid). In addition the 1,2,4 triazole ring is cleaved resulting in imines. The products of the degradation of tebuconazole were identified and found to be the same for all three species Basiodiomycetes which produce lignin peroxidases and laccases to degrade wood, have been found capable of metabolizing biocides using these enzyme systems (Lyr, 1963; Mendoza-Cantu et al., 2000). Because *T. harzianum* and bacteria species do not degrade lignin and cellulose, they do not produce lignin peroxides, laccase, and manganese peroxides. It is hypothesiszed that a phase I cytochrome P 450 (P450) monooxygenase system is responsible for the initial steps in the degradation of tebuconazole. Oxidation reactions are characteristic P450 dependent. The P450 super enzyme family metabolizes a wide range of chemicals and has been characterized at the protein and gene levels in animals and higher plants and marine macroalgae (Plugmacher and Sanderman, 1998; Makovec and Breskvar, 2000). P450 enzymes are able to incorporate one of the two oxygen atoms of an O_2 molecule into a broad variety of substrates with concomitant

reduction of the other oxygen atom by two electrons to H_2O. The most common reaction catalyzed is monooxygenation, but a wide spectrum of reactions including N-oxidation, sulfoxidation, epoxidation, N-, S-, and O-dealkylation, peroxidation, deamination, desulfuration, and dehalogenation are catalysed (Bezalel et al., 1997; Hawkes et al., 2000). A few family members can catalyze intramolecular transfer of an oxygen atom. The sequences of more than 2000 members of the P450 superfamily are known. In fungi, P450s are key players in ergosterol biosynthesis, and they are involved in pathogenesis by detoxifying host plant defenses. Consequently, fungal CYP51 (lanosterol 14α-demethylase) is the primary target of antifungal triazole drugs. Bacterial P450s participate in antibiotic synthesis and catabolic reactions. P450s utilize NADPH as the electron donor of the monooxygenation reactions. Sterols are essential constituents of the plasma membrane of all eukaryotic cells, and CYP51 is the most ancient form of all eukaryotic P450s (Makovec and Breskvar, 2000).

This chapter reports on the characterization of the oxidizing enzyme of *T. harzianum* and *M. incrassata* from culture preparations. So far, studies of P450s in fungi have been few because of problems in the isolation of active enzymes including their low concentration in some species (Uzura, 2001; Plugmacher and Sanderman, 1998). The B3 fungal culture used initially contained no protein (N- limited). Therefore, increases in the amount of protein in the culture gave an indication of proteineous extracts released by the fungi leading to subsequent isolation and characterization. An understanding of enzymatic mechanisms may make it possible to arrive at intervention strategies to increases the service life of wood treated with triazoles.

4.2 Materials and Methods

4.2.1 Test Chemicals

Ninety five percent tebuconazole (Preventol A 8) was provided by Lanxess Corporation (Pittsburgh, PA) and the standard (99%) was obtained from Sigma Aldrich (St Louis, MO). All fungal species were provided by the US. Forest Products Laboratory (Madison, WI). Fungal

cultures were grown out and maintained on medium containing 1.5% agar, 2% malt extract, 0.001% yeast extract. NADPH (nicotinamide adenine dinucleotide phosphate). ABT (1-aminobenzotriazole), piperonyl butoxide, and phenobarbital were obtained from Fisher Scientific (Waltham, MA).

4.2.2 Growing Fungi and Preparation of Cell Extracts

For *T. harzianum*, 250ml Erlenmeyer flasks containing 100ml B3 media were supplemented with 0.1ml of 0.2% tebuconazole to give a final concentration of 20 ppm of each flask. Flasks were inoculated with an agar plug from actively growing colonies. Cultures were incubated at 25^{o}C and shaken at 120rpm for a period ranging from 3-21days. Three replicates were used for each time interval. For the white rot and brown rot cultures, 2ppm of tebuconazole was used. The culture controls consisted of media blanks in which microorganisms were grown under identical conditions in media but without tebuconazole. For *P. chrysosporium* and *M. incrassata,* 2ppm tebuconazole in culture was used as the final concentration.

Cell extracts were prepared by harvesting mycelia and freeze drying. The frozen mycelium was homogenized into a fine powder in a blender cup at 20 second intervals for 1 min. The fine powder was resuspended in 50mM pottasim phospahate buffer containing 10% glycerol. The homogenate was filtered and centrifuged at 105,00Xg for 90 min. at 4^{o}C. The microsomal pellet was resuspended in the same buffer. The supernatant and pellet were analyzed for enzymatic activities. The centrifuge was a fixed angle rotor, induction drive, Beckman model J2-21M.

4.2.3. Quantitative Determination of Total Proteins in Cultures

Potassium phosphate buffer was added to all cultures and solutions of the standard in B3 media. The amount of protein in the crude cultures was quantified by measuring absorbance using ultraviolet-visible (UV-Vis) spectrometry at 280 λ wavelength. Interference from other compounds which absorb UV was minimized by measuring absorbances at 280 nm. The

instrument was a Beckman DU®-65 spectrophotometer. The cuvettes holding the samples were made of quartz, which does not absorb light at this wavelength. Standards of bovine serum albumin (with known amounts of protein) were prepared and used to prepare the concentration versus absorbance curve which was subsequently used to compare absorbance with that of the unknown samples to estimate protein content. The standard curve was prepared using 0.0, 0.1, 0.2, 0.5, 1, 2.5, 5, and 10g/l concentrations.

4.2.4 Ion Exchange Chromatography

To separate proteins in the culture media, 200ml of fungal culture containing tebuconazole by species were centrifuged and the supernatant concentrated before subjection to ion exchange chromatography to separate the proteins based on their net charge. The instrument used was a Biorad Biologic LD ion exchanger. DEAE cellulose was used as the stationery column. The eluting solvents were buffer A: 20mM tris Hcl (low salt) and buffer B: 20mM tris Hcl buffer + 500mM Nacl (high salt). To make the stationary phase, 10g of DEAE cellulose was dissolved in tris buffer, and the slurry was allowed to settle for 10 min. The supernatant containing fines was decanted. This process was repeated twice to ensure the supernatant was clear. The column was then packed with the DEAE cellulose at atmospheric pressure. The sample (100ml.) was loaded on to the colomn. The flow through, wash, and fractions were all collected. A gradient was run from 100% buffer A: 0% B up to 0% A and 100% B.

4.2.5 Testing Active Fractions

4.2.5.1 Protein Determination and Assay of Active Fractions

The flow through, wash, and fractions were dried to 10 ml each and the amount of protein in the crude cultures was quantified by measuring absorbance using ultraviolet-visible (UV-Vis) spectrometry at 280 λ wavelength. Each fraction (20ml.) was tested against 2ppm of the test chemical (tebuconazole) to determine which one of them contained the active enzymes that oxidize it. Because P450s utilize NADPH as the electron donor of the monooxygenation

reactions, 10µl per ml of fraction of NADPH was added. The reaction mixture was incubated for two days at room temperature. The reaction was stopped by addition of 6N Hcl.

4.2.5.2 Quantitative Determination of Tebuconazole Retained in Fractions

Five ml. of the mixture was extracted in SPE columns. The columns (Bond Elut –C18, 500mg, 3ml Varian #12102028) were preconditioned with two column volumes of methanol, followed by two column volumes of milli-Q water. The sample was loaded and pulled through at one drop per second, followed by a column rinse of 50:50 acetonitrile water. Retained compounds were eluted with 4 column volumes of methanol collecting in 15ml centrifuge tubes. Each tube was dried to below 5ml on a nitrogen evaporator and transferred to a second tube while passing over sodium sulfate. The final volume was adjusted to 5ml. Samples were cleaned with a 0.2 µm Teflon™ filter prior to quantitative analysis by GC-MS. The GC-MS was an Agilent 6890 gas chromatograph using a 5973 mass selective detector. The injection port temperature was 250^{o}C, detector temperature 280^{o}C, and the carrier gas was helium at 1 mm min^{-1}. The capillary column model No. RTX35 was 30m X 250µmX 0.25µm. Injection volume was 2.0µl and total runtime was 20.42 min. Tebuconazole eluted at 15.9 min. Running in selected ion mode (SIM), ions detected for tebuconazole were 250 as the target ion, and 125 and 252 as the qualifier ions. Calibration was done using a four point curve. Concentrations of the samples were calculated using the formula: $(X / V_1)^* (V_2)^*$dilution factor.

Where: X = off the curve reading from the instrument.

V_1= initial vol. of sample extracted by SPE

V_2= final vol. of sample after SPE

4.2.6 Induction and Inhibition Tests

Because *Phanerochaete chrysosporium* showed little ability to degrade tebuconazole, the rest of the tests were carried out only for *T. harzianum* and *M. incrassata.* Separate flasks each with 100ml culture and tebuconazole were grown after supplementing the media with known P450

inhibitors ABT and piperonyl butoxide. Similarly separate flasks were supplemented with Phenobarbital, a known P450 inducer. The level of protein produced in the culture was determined using the procedure in section 4.2.2.

4.2.7 Carbon Monoxide Difference Spectra

To further confirm the production of P450s, cultures containing tebuconazole alone, tebuconazole with P450 inhibitors and those with phenobarbital the inducer were separately centrifuged, concentrated, and subjected to determination of reduced CO difference spectra according to the method of Omura and Sato (1964). In this method, an intense peak in the blue wavelength region of the visible spectrum corresponding to a wavelength of maximum absorption in the reduced form of heme-containing moieties, such as P450 is observed at 450nm when saturated with carbon monoxide. Microsomal preparations and fractions found active in section 4.2.3 were placed in sample cuvettes and a base line reading was obtained. Reduction of samples was effected using 0.01g $Na_2S_2O_4$. The content was then treated with carbon monoxide (CO), and the spectral difference thereby induced mearsured. CO was carefully bubbled through the sample for 20s to sufficiently saturate it before determination of spectra.

4.3 Results and Discussion

4.3.1 Protein in Culture Media

B3 media contained no proteins and was used to calibrate the spectrophotometer before determination of protein content in each sample. Figure 4.1 shows the calibration curve obtained using the standard. Figure 4.2 shows the total protein content in each culture. Tebuconazole did not significantly affect the total amount of protein generated by each fungal species in the culture media (P=0.13, d.f 3). The total amount of protein therefore did not give an indication on the effect of tebuconazole on amount of protein produced. The important factor lies in the type of protein / enzyme produced.

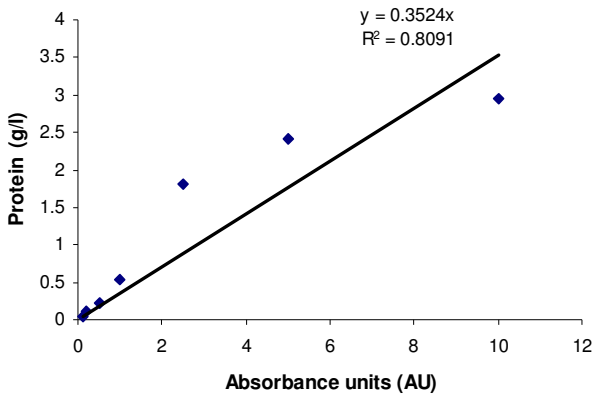

Figure 4.1. Standard curve for protein quantity.

4.3.2 Ion Exchange Chromatography

The chart recording of absorbance and time monitoring elution of proteins is shown in Figure 4.3. Absorbance gave an indication of eluting ions or proteins. From the chart recorders, all target proteins for further analysis eluted from the column in the flow through and column wash (i.e., before 250 min. and 10% buffer B) with the low salt buffer as the eluting buffer.

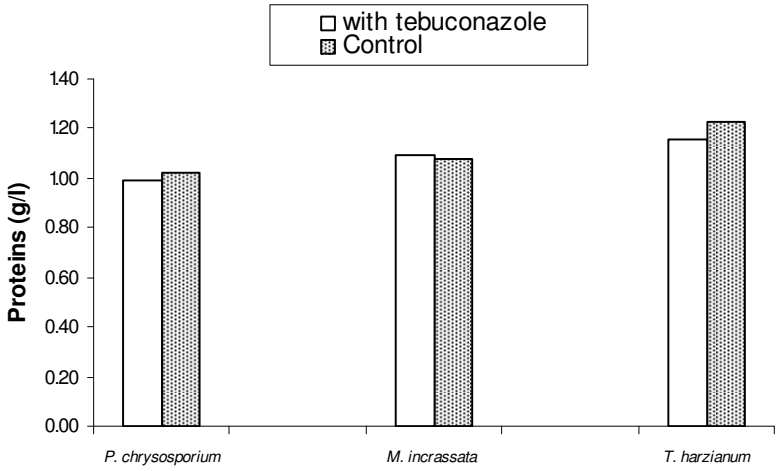

Figure 4.2. Protein quantity in cultures.

The compounds eluting in the collected fractions with high salt as buffer contained more of the media components as shown in the blank. Fractions eluting after start of the gradient were consolidated into three consecutive fractions based on the order of elution.

4.3.3 Testing Active Ion Exchange Fractions

The wash, flow through, and fractions for each culture were concentrated by evaporation and tested for ability to degrade tebuconazole. In the presence of the co-enzyme NADPH, the flow through and wash in *M. incrassata* and *T. harzianum* were able to degrade tebuconazole. This was not observed in the absence of NADPH. All fractions from *P. chrysosporium* showed no activity. Amounts of tebuconazole remaining in the test solutions is shown in Figure 4.5. 38% tebuconazole was degraded in the flow through of *M. incrassata* and 39% in the column wash. In *T. harzianum* culture, 38% was degraded in the flow through and 50.1 % in the column wash.

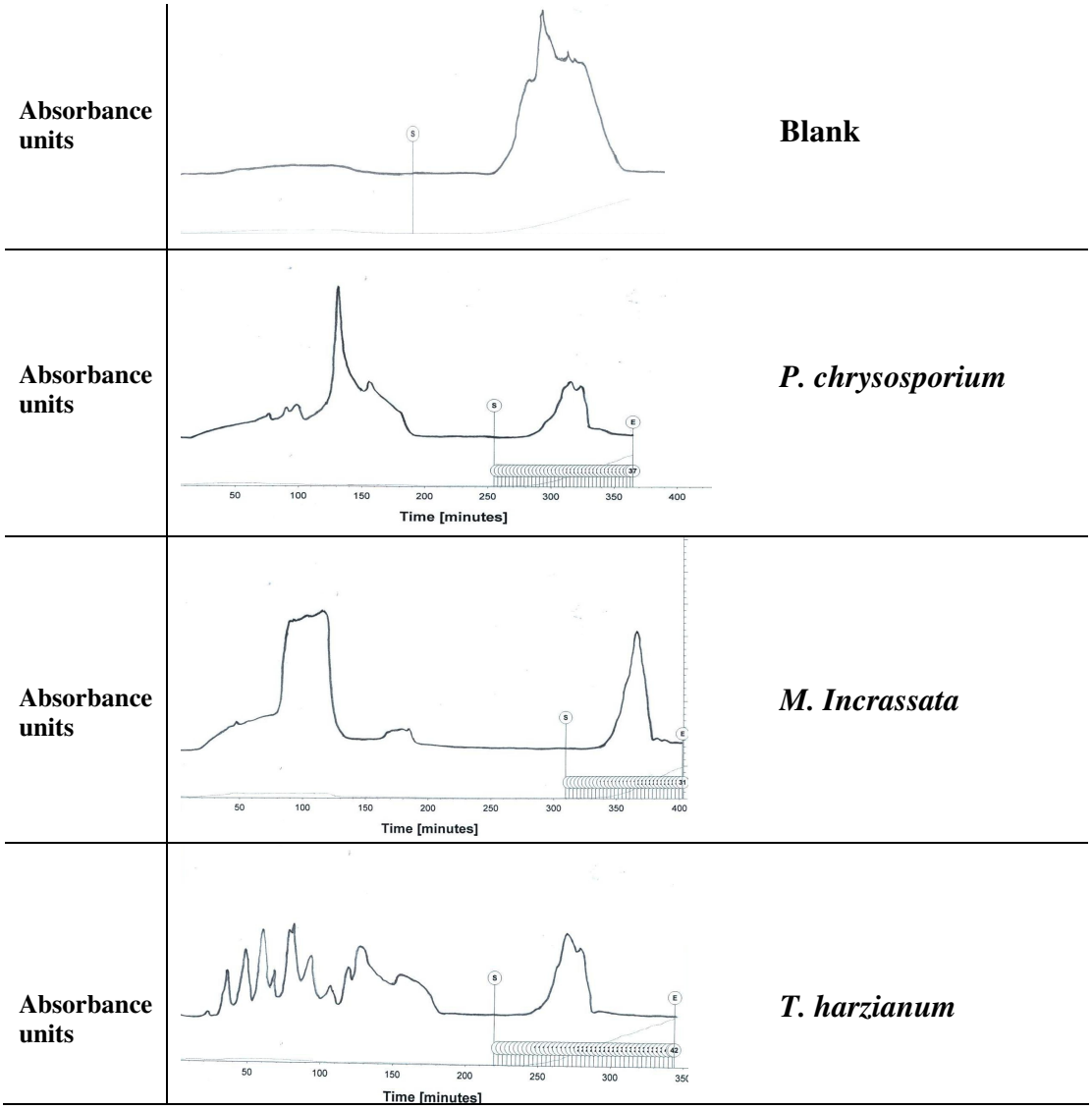

Figure 4.3. Chart recording of absorbance and time

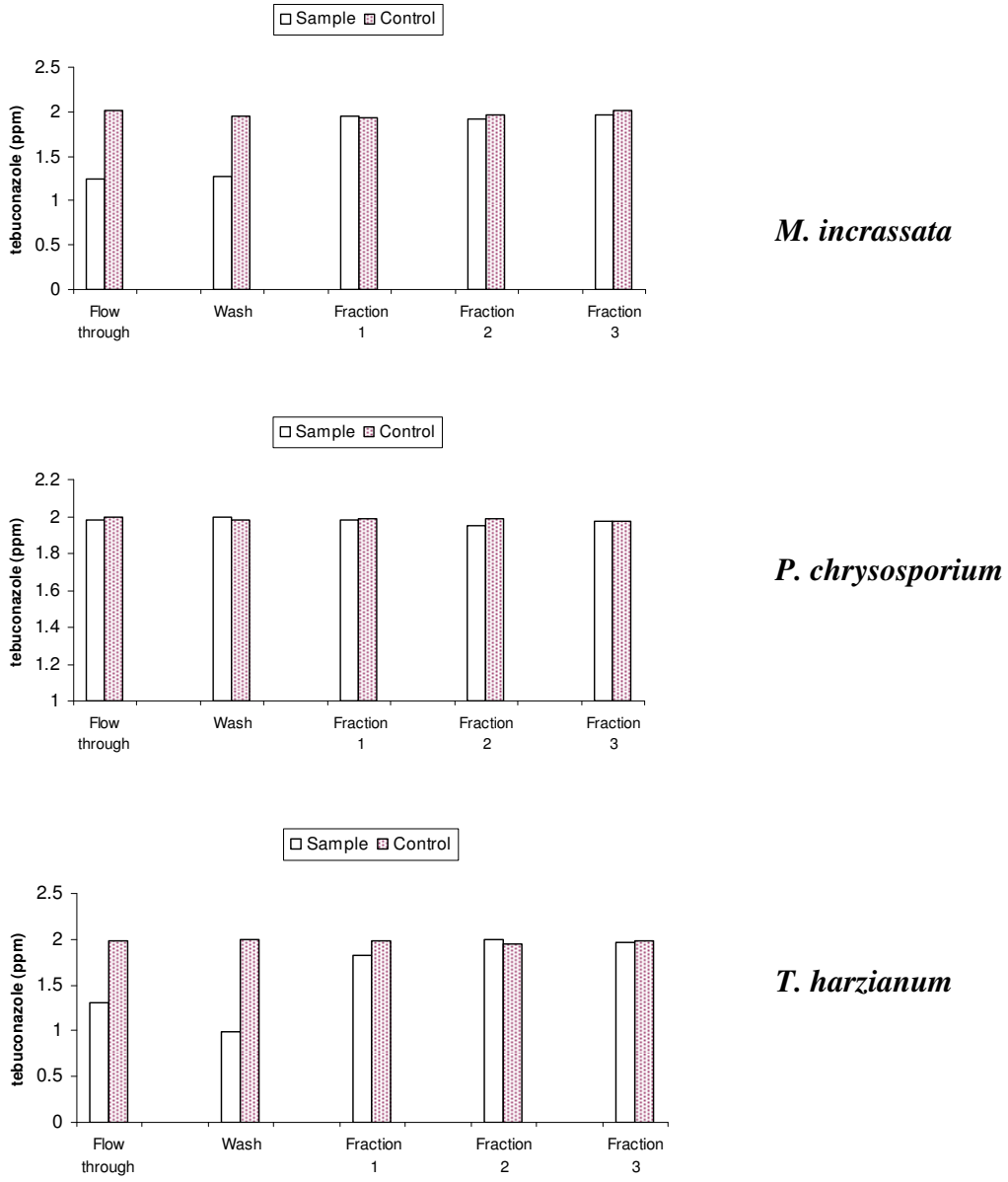

Figure 4.4. Tebuconazole remaining in the test solutions of fractions

Proteins were produced in all cultures however, proteineous extracts produced in *P. chrysosporium* cultures could not degrade tebuconazole while those in *T. harzianum* cultures had the highest capacity to degrade tebuconazole.

4.3.4 Induction and Inhibition Tests

Inhibitor tests showed that cultures with P450 inhibitors ABT and piperonyl butoxide (PB) had a reduced amount of protein produced and had less degradation of tebuconazole. The inhibitors

ABT and PB both reduced the amount of tebuconazole depleted from cultures in cultures of both *T. harzianum* and *M. incrassata.* PB inhibited degradation of tebuconazole to a lesser extent than ABT. There was a significant difference in the amount of tebuconazole depleted in cultures containing tebuconazole alone and those containing the inhibitors in addition to tebuconazole (For ABT $p=0.001$ and $p=0.03$, df =5 in *T. harzianum* and *M. Incrassata* cultures respectively). The culture with the inducer phenobarbital did not show a significant difference with that containing only tebuconazole. Also, no effect on protein level in solution was observed with phenobarbital. Phenobarbital did not induce or increase degradation of tebuconazole as expected.

Table 4.1. Effect of P450 inhibitors and inducers on tebuconazole degradation and protein production.

Species		Protein content (g/l)	Tebuconazole retained in ppm (% degraded)
M. incrassata	Control	2.03	1.99 (0.5)
	Tebuconazole only	1.89	1.75 (12.5)
	ABT	1.69	1.93 (3.5)
	Piperonyl butoxide	1.76	1.87 (6.5)
	Phenobarbital	1.92	1.95 (2.5)
T. harzianum	Control	1.8	20.12 (4.4)
	Tebuconazole only	1.35	8.02 (59.9)
	ABT	1.02	18.92 (5.4)
	Piperonyl butoxide	1.54	16.82 (15.9
	Phenobarbital	1.76	19.23 (3.85)

4.3.5 Carbon Monoxide Difference Spectra

A difference spectrum with a maximum peak at 440-460nm was observed when CO was bubbled into the $Na_2S_2O_4$ treated concentrated samples of *T. harzianum* culture extracts. The peak was not apparent in *M. incrassata* samples most likely due to low concentrations of the enzymes. The active fractions (the flow through and wash from the ion exchange column) also did not

exhibit the spectra pattern typical to cytochrome P450 most likely due to low concentrations of the enzymes.

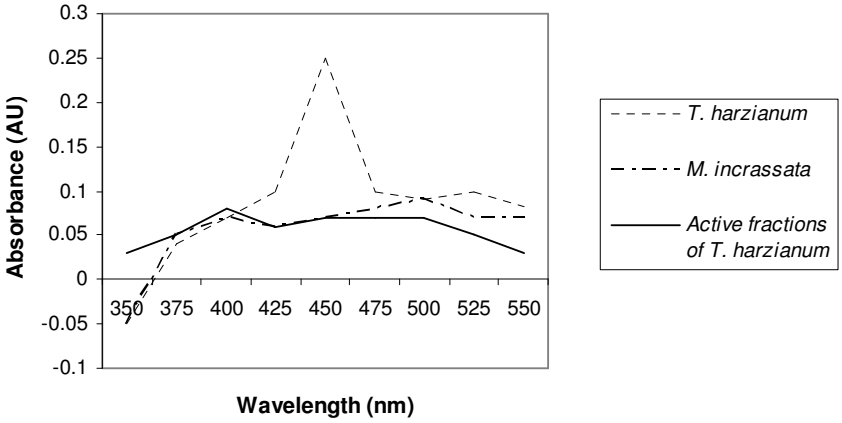

Figure 4.5. CO difference spectra of culture extracts and fractions

The maximum absorbance at 450 mn is a confirmation that cytochrome P450 family of enzymes are involved in the phase I metabolism of tebuconazole in *T. harzianum.* This spectral confirmation was not observed for *M. incrassata* cultures.

4.4 Conclusions

It had previously been shown that *T. harzianum* and *M. incrassata* both metabolize tebuconazole by initial oxidation reactions. This led to the hypothesis that monooxygenation is catalyzed by enzymes from the family of the cytochrome P450s. The quantity of proteineous extracts produced by cultures was not affected by the presence of tebuconazole. The oxidation of tebuconazole by *T. harzianum* and *M. incrassata* was reduced when PB and ABT, both known P450 inhibitors, were added to the culture than in cultures containing tebuconazole alone. This suggested that the enzymes involved are from the P450 family. Furthermore, the microsomal extract from *T. harzianum* exhibited a maximum peak at 440 - 460nm when CO was bubbled into the $Na_2S_2O_4$ treated concentrated samples. This indicates that initial oxidation reactions leading to the metabolism of tebuconazole by the two species are cytochrome P450 dependent.

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CHAPTER 5. THE EFFECT OF ADDITIVES ON BIOTRANSFORMATION AND EFFICACY OF TEBUCONAZOLE EFFICACY AGAINST MOLDS

5.1 Introduction

Several problems exist with totally organic wood preservative systems. Most are about 10-30 fold more expensive per pound than the inorganics (Schultz et al., 2004) and may not be used in ground contact on their own. Currently, their major advantage is also their major disadvantage: biotransformation by wood degrading and non degrading organisms in soil and wood. In Chapter 2 and 3 it was shown that the mold Trichoderma harzianum, brown rot fungus Meruliporia incrassata and the bacterial species Pseudomonas fluorescens all can metabolize tebuconazole.

The primary metabolism of toxicants in plants often results in metabolites with reduced or modified toxicity, increased polarity, and predisposition of the parent molecule to serve as substrate for conjugation. The specific functional groups or linkages (e.g., hydroxyl, amino, nitro, amide, halogen, carboxyl, and nitrile) found in the molecules of xenobiotics or endogenous compounds are susceptible to chemical, physical, or enzymatic transformations. Enzymatic transformation is by far the major means of detoxification and toxicants may be altered through oxidative, reductive, or hydrolytic reactions or be activated to serve as reactive intermediates for the subsequent conjugation of the parent molecules with endogenous substances such as sugars, glutathione, or amino acids (Hatzios, 2005). Most of the reactions of the primary metabolism of toxicants in plants are oxidative, catalysed by cytochrome P450 monooxygenases or esterases. Some reductive reactions are known to occur, but they are not considered very important in the primary metabolism of toxicants in plants.

Non-biocidal additives in combination with organic biocides may enhance efficacy and lower the cost of organic wood preservative systems. Possible non-biocidal additives which could be mixed with organic biocides include water repellents, antioxidants, and chelators for

specific elements including metals required by wood decaying fungi such as iron and manganese. It may be possible to tie up essential minerals, elements, or compounds such as amino acids needed by fungi from obtaining essential elements such as N, P, and Ca (Green III and Schultz, 2003). The calcium chelating compound Na- *N'* N-napthaloylhydroxamine (NHA, sodium salt) has shown positive results as a co- biocide. Antioxidants may act synergistically to biocides by disrupting the initial generation of free radicals which help open up cell walls. Finally, metals such as Fe or Mn are well known to be involved in fungal degradation mechanisms, either as part of an enzymatic system or as a free metal. Thus, addition of appropriate metal chelators might prevent the metals from being available to the fungi (Schultz and Nicholas, 2001; Green III and Schultz, 2003). Propyl gallate has dual antioxidant and Fe^{2+} metal chelating properties and gives synergistic performance when combined with various biocides by interference with fungal redox reactions (Binbuga and et al., 2005). The percent strength loss of 0.01% tebuconazole treated southern yellow pine after exposure to *G. trabeum* in a soil block test was reduced from 82% to 6% after addition of 3% EDTA to the formulation. For *T. versicolar,* the improvement was from 23% to 3% (Schultz and Nicholas, 2001).

This work explores the efficacy of two P450 inhibitors piperonyl butoxide (PB) and 1-Aminobenzotriazole (ABT) and two metal chelators N,N,N',N'-tetramethylethylenediamine (TEMED) and ethylenediaminetetraacetic acid (EDTA) in preventing or reducing the effect of monooxydenase activities which result in the initial oxidation of tebuconazole. PB is a synergist used in a wide variety of insecticides: pyrethrins, pyrethroids, rotenone, and carbamates (Kakko et al., 2000). In a study to assess its effect on the toxicity of chlorpyrifos an organophosphorus insecticide against *Ceriodaphnia dubia* (waterflea) and *Xenopus laevis* (South African clawed frog), PB at 200 µg/L reduced the toxicity of chlorpyrifos to *C. dubia* by a factor of 6. At 3000 µ/L it also reduced the toxicity of CPF to *X. laevis* with respect to mortality and malformations. This led to the conclusion that *C. dubia* and *X. laevis* have the capability to metabolize

chlorpyrifos via P450 mediated reactions (El-Merhibi et al., 2004). The study of the *in-vitro* S-oxidation of fenbendazole and fenbendazole sulphoxide in rats showed that PB significantly inhibited the sulphoxidation and sulphonation of fenbendazole. It was concluded that PB inhibited the oxidative conversion of fenbendazole into inactive metabolites (Siminszky, 2006).

1-Aminobenzotriazole (ABT) is a mechanism-based inhibitor of animal cytochrome P450. It results in a loss of 60-80% of P450 activity and drug oxidations in animal studies *In-vitro* as well as *in-vivo.* An injection of 25 and 50 mg/kg body weight ABT to rats caused concentration-dependent declines (60-80%) in adrenal mitochondrial and microsomal cytochrome P450 concentrations (Omoto et al., 2003). ABT inactivates P450 via alkylation of the prosthetic heme moiety causing loss of the characteristic P450 absorption spectrum.

EDTA is a substituted diamine chelate ligand with a high affinity constant to form metal complexes. It is a powerful complexing agent of metals and is highly stable, offering a considerable versatility in industrial and household uses. Applied predominantly in an aqueous medium, it is released into the environment through wastewaters. It forms especially strong complexes with Pb, Mn(II), Cu(II), Fe(III), and Co(III) (Abumaizar and Smith, 1999; Oviedo and Rodríguez, 2003). It may inhibit mono oxygenation by chelating Fe^{3+} and Fe^{2+} ions important in the P450 cycle. EDTA binds to metals via four carboxylate and two amine groups. TEMED is a bidentate ligand widely employed as a ligand for metal ions. It forms stable complexes with metal halides (e.g., zinc chloride, copper(I) iodide), giving complexes that are soluble in organic solvents. Inhibiting the detoxification pathway allows higher unmetabolised systemic concentrations of the active insecticide to remain within the timber for a longer period.

5.2 Materials and Methods

5.2.1 Test Chemicals

Ninety five percent tebuconazole (Preventol A 8) was provided by Lanxess Corp. (Pittsburgh, PA) and the standard (99%) was obtained from Sigma Aldrich (St Louis, MO). Fungal species

were provided by the US. Forest Products Laboratory (Madison, WI). Fungal cultures were grown out and maintained on media containing 1.5% agar, 2% malt extract and 0.001% yeast extract. ABT, PB, EDTA, and TEMED were obtained from Fisher Scientific (Waltham, MA).

5.2.2 Agar Block Tests

Growth media (600ml) containing 1.5% agar, 2% malt extract, and 0.001%yeast extract was supplemented with tebuconazole (20ppm final concentration). This was divided into 5 groups (groups 2-5) each with 90mls and further treated as shown below (Table 5.1) before pouring on to agar block Petri dishes (approximately 25 ml. each). Group 1 was the control. Plates were kept at 25^{o}C. When the fungal mycelia reached the edges of the Petri dish in control cultures (full diameter), the diameter of mycelia growth on all the Petri dishes was determined.

Table 5.1. Agar block test set up.

Treatment No.	Treatment
Group 1	Agar media only
Group 2	20ppm tebuconazole only, no further treatment
Group 3	Supplemented with 10mls of 30% EDTA dissolved in distilled water (at pH adjusted to 9 using KOH)
Group 4	Supplemented with 10mls of 30% TEMED dissolved in acetonitrile
Group 5	Supplemented with 10mls of 3% PB
Group 6	Supplemented with 10mls of 3% ABT

5.2.3 Liquid Cultures and Preparation of Cell Extracts

Fifteen Erlenmeyer flasks each with 90mls B3 media were supplemented with 0.1ml of 0.2% tebuconazole. Flasks were divided into five groups of three and further treated with additives as shown in Table 5.2. Group 1 was the culture control with B3 media only. Flasks in groups 1-6 were inoculated with an agar plug from actively growing colonies. Group 7 was the substrate control with tebuconazole but without innoculum. Cultures were incubated at 25^{o}C and shaken at 120rpm for 21days. All samples were centrifuged (10 min. at 15000xg and 4^{o}C).

Table 5.2. Liquid cultures set up

Treatment No.	Treatment.
Group 1	B3 media only
Group 2	20ppm tebuconazole only, no further treatment
Group 3	Supplemented with 10mls of 30% EDTA dissolved in distilled water (at pH adjusted to 9 using KOH)
Group 4	Supplemented with 10mls of 30% TEMED dissolved in acetonitrile
Group 5	Supplemented with 10mls of 3% PB
Group 6	Supplemented with 10mls of 3% ABT
Group 7	20ppm tebuconazole only, no innoculum

The centrifuge was a fixed angle rotor, induction drive Beckman model J2-21M. The supernatant was subjected to quantitative determination of retained tebuconazole and HPLC for detection of metabolites.

5.2.4 Quantitative Determination of Tebuconazole Retained in Samples

Five ml of filtered medium was extracted in solid-phase extraction (SPE) columns. The columns (Bond Elut –C18, 500mg, 3ml Varian #12102028) were preconditioned with two column volumes of methanol, followed by two column volumes of milli-Q water. The sample was loaded and pulled through at one drop per second, followed by a column rinse of 50:50 acetonitrile water. Retained compounds were eluted with 4 column volumes of methanol and collected in 15ml. centrifuge tubes. Each tube was dried to below 5ml. on a nitrogen evaporator and transferred to a second tube while passing over sodium sulfate. The final volume was adjusted to 5ml. Samples were cleaned with a 0.2 μm Teflon™ filter prior to quantitative analysis by gas chromatography (GC-MS). The instrument was an Agilent 6890 gas chromatograph using a 5973 mass selective detector. The injection port temperature was 250^{o}C, the detector temperature 280^{o}C, and the carrier gas was helium at 1 mm min^{-1}. The oven was initially held at 80^{o}C for 2 min. followed by a 30^{o}C min.$^{-1}$ increase to 190^{o}C and then an 8^{o}C min^{-1} increase to a

final temperature of 300°C. The capillary column model No. RTX35 was 30m X 250µmX 0.25µm. Injection volume was 2.0µl, and total runtime 20.42 min. Tebuconazole eluted at 15.9 min. Running in selected ion mode (SIM), ions detected for tebuconazole were 250 as the target ion, and 125 and 252 as the qualifier ions. Calibration was done using a four point curve. Concentrations of the samples were calculated using the formula:

$(X / V_1)^* (V_2)^*$dilution factor. [1]

Where: X = value obtained from the curve.

V_1= Initial vol. of sample extracted by SPE

V_2= final vol. of sample after SPE

5.2.5 Preparation of Samples for HPLC Analysis

The remaining liquid samples were pooled into one composite sample per treatment. Each sample was subjected to extraction and cleanup by the following procedure: sample was transferred into a one liter separating funnel and 75ml. of dichloromethane added. The sample was subjected to shaking for 1.5 min. vented every 20 seconds to release pressure, then left to stand for 3 min. The bottom layer was passed over sodium sulfate into a 400ml beaker. Extraction was repeated twice. The sample was then placed on a water bath at 45°C and evaporated until almost dry. Five ml. of acetonitrile was added for solvent exchange, re-dried to about 2ml, and transferred to a centrifuge tube with several rinses of acetonitrile while passing it over sodium sulfate funnel. The tube was dried to below 5ml. on a nitrogen evaporator, and the final volume adjusted to 5ml. before HPLC qualitative analysis.

5.2.6 HPLC Analysis

Several reverse-phase HPLC runs were necessary to detect and purify any metabolites. The system consisted of a Waters 600 pump with a Waters 2487 UV detector whose output was connected to a fraction collector. The column was a Waters Delta-Pak C18, 300 Å, 15 micron, 8mm x 10 cm radial compression module. Eluant composition as a function of time was:

isocratic from 0-5 min. 70% water+ 0.1% formic acid 30% acetonitrile, linear increases of acetonitrile from 5-30 min. to 85% and isocratic at 85% acetonitrile for 5 min. Flow rate was 1 ml. min^{-1} and detection was at 224 nm wavelength. Tebuconazole eluted at 28 min. Qualitative analysis using authentic standards of metabolites was not possible as metabolites of tebuconazole could not be obtained. Tebuconazole peaks and those of metabolites were identified by comparison of elution time, intact molecular mass, isotopic pattern distribution, and identification of functional groups of (i) tebuconazole standard alone, (ii) the media only, (iii) media containing tebuconazole before exposure to microorganisms, and (iv) media containing tebuconazole after exposure to microorganisms.

5.2.7 Mass Spectral Characterizations

Exact mass determination of HPLC fractions of tebuconazole and its metabolites was performed by electrospray ionization (ESI). The instrument operated in positive ESI mode was an Agilent 6210 Time of flight (TOF) LC/MS mass spectrometer.

5.2.8 The Mold Test

The effect of additives on the resistance of tebuconazole treated wood to the surface growth of molds was determined in a mold test. Resistance was evaluated relative to ACQ treated and untreated wood. The standard method for evaluating the resistance of wood product surfaces to mold E 24-06 by the American Wood Preservers Association (AWPA, 2007) was used. Samples were treated by vacuum and conditioned at room temperature. Treatment solutions contained 0.1% tebuconazole and additive concentrations shown in Table 5.3. Retention of tebuconazole in each sample was determined from initial weights and final weights after treatment. A total of six treatments were exposed in an environmental chamber where temperature and relative humidity were approximately 26^{o} C and 98-100 %, respectively. Sample locations in the camber were randomized. The chamber was inoculated using mold suspensions with the following mold species: *Aureobasidium pullulans, Aspergillus niger,*

Penicillium citrinum, and *Alternaria tenuissima.* The innoculum was evenly sprayed over the soil surface in the chamber. After two weeks of sporulation and equilibration, test samples were introduced and the innoculum evenly sprayed over the samples. The chamber was operated for eight weeks. After 2, 4, 6, and 8 weeks exposure, the samples were visually rated for the extent and intensity of mold growth.

Table 5.3. Set up of the mold test AWPA E 24-06.

Treatment No.	Treatment
1.	Untreated
2.	ACQ- 4.0 Kg/m^3
3.	Tebuconazole
4.	Tebuconazole + 0.1% PB
5.	Tebuconazole+ 0.1% ABT
6.	Tebuconazole + 3% EDTA

5.3 Results and Discussion

5.3.1 Agar Block Tests

Growth of mycelia was observed on all Petri dishes. *T. harzianum* is resistant to tebuconazole below 200ppm. At 20ppm, addition of the various additives did not prevent germination of spores. The performance of the tebuconazole only treatment and that of tebuconazole mixed with the metal chelators TEMED or EDTA had no significant difference on the extent of germination and the diameter of mycelia observed. The increases in diameter for these treatments were not significantly different ($p=0.1$, $df=6$). EDTA performed better than TEMED. The P450 inhibitors PB and ABT had a synergistic effect with tebuconazole; both reduced the growth in diameter significantly. ABT performed better than PB. However none of the treatments was effective in preventing germination and growth of the fungus.

Table 5.4. Diameter increase (mm) of *T. harzianum* mycelia after 7 days.
***(20ppm tebuconazole in all treatments).**

Treatment	Diameter of mycelia	Percent decrease in diameter to that of the control
Agar only (control)	76	control
Solvent only- acetonitrile)	76	0
Tebuconazole	74	2.6
Tebuconazole + EDTA	72	5.2
Tebuconazole + TEMED	75	1.3
Tebuconazole + PB	65	14.4
Tebuconazole + ABT	36	52.6

5.3.2 Quantitative Determination of Tebuconazole Retained in Samples

The effect of the mono-oxygenase inhibitors PB and ABT and metal chelators EDTA and TEMED on tebuconazole metabolism by *T. harzianum* was measured by the amount retained in the culture media after 21 days incubation. Results are shown in Figure 5.1. Statistical analysis of the five mean amounts of tebuconazole retained using ANOVA showed that the means were significantly different (α=0.05, p=0.015 df =10). In cultures containing no additive, 49% tebuconazole was depleted at the end of culture period. TEMED and EDTA had the least effect and caused a decrease in tebuconazole depleted to 46.1% and 28.8%, respectively. PB and ABT gave the best performance in terms of retaining tebuconazole in the culture by 19.7% and 14.6%, respectively. This confirms the hypothesis that P450 type of enzymes is involved in the metabolism of tebuconazole.

5.3.3 HPLC and Mass Spectrometry Analysis

Cultures with only tebuconazole had four metabolites in addition to the tebuconazole elute from the column. The culture with TEMED had two metabolites while all other cultures had only one metabolite elute in addition to tebuconazole. Further analysis of metabolites by mass spectrometry for exact mass and Cl isotope pattern revealed that metabolites were mainly the

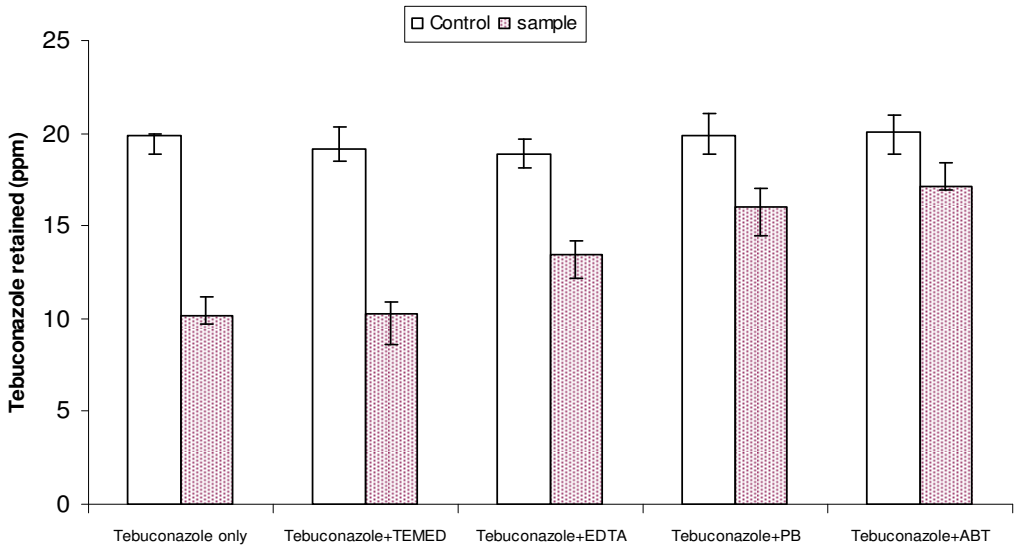

Figure 5.1. Tebuconazole retained in samples.

oxidation product. There was no evidence of phase II metabolite products in all cultures except those with tebuconazole only and those with TEMED in addition to tebuconazole. Figure 5.2 shows all the chromatograms. Fraction 2 in TEMED containing cultures was the same as fraction 4 of the tebuconazole only fraction. This indicated there was further metabolism after the initial oxidation. In all other treatments, only the initial oxidation product with molecular weight of 324.14 was identified. Table 5.5 shows the molecular ions detected in the mass spectra. Despite oxidation products being present in all treatments, the cultures with P450 Inhibitors PB and ABT had the least amount of metabolites and only the initial oxidation product with molecular weight of 324.15 was observed. This fraction had the Cl isotope pattern, confirming that apart from addition of the oxygen, no further change occurred in the structure of tebuconazole.

5.3.4 Mold Test

Relative resistance to mold growth was determined by comparing average ratings of six replicates of each treatment (Table 5.6). In the mold test all treatments with additives in the formulation showed a better performance than the treatment with tebuconazole only. The

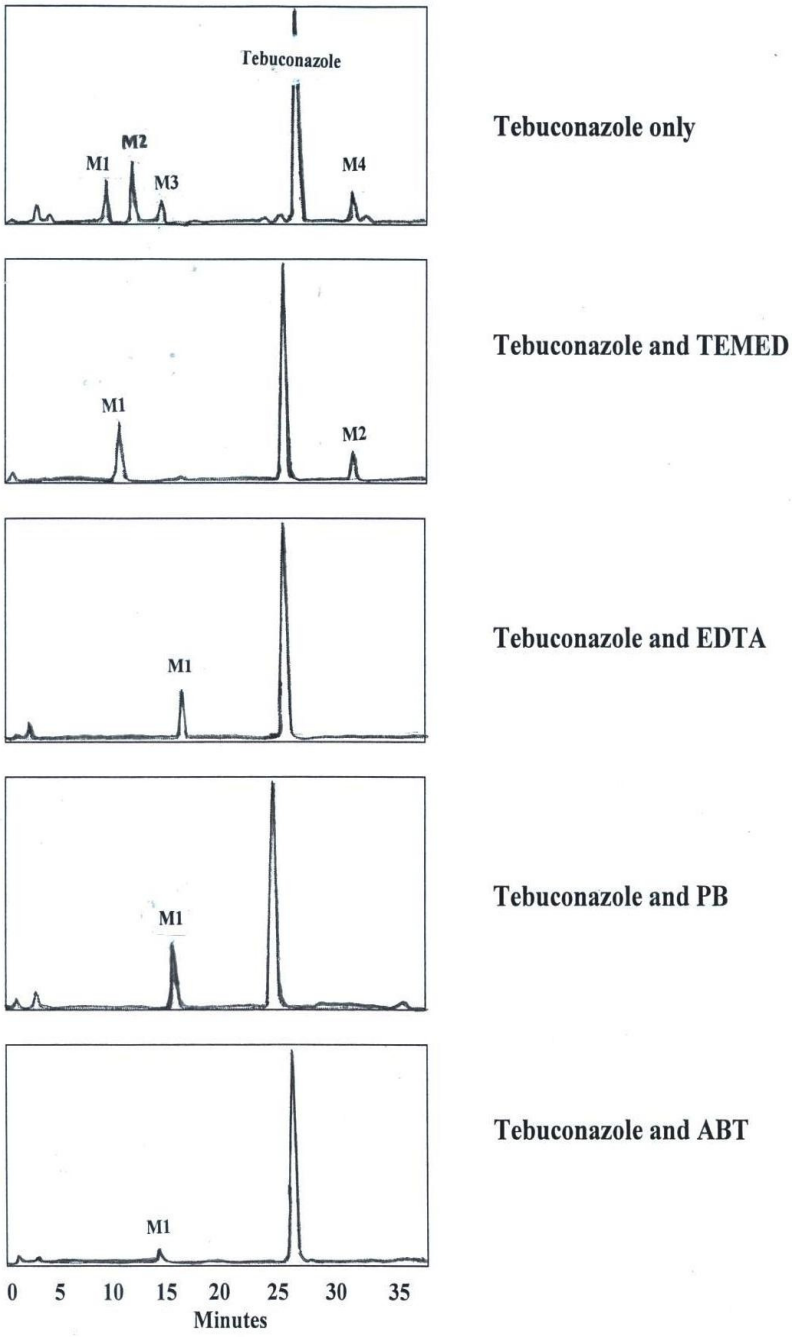

Figure 5.2. HPLC chromatograms of the treatments.

treatment with tebuconazole only and tebuconazole with TEMED did not perform as well as ACQ. All other treatments at an average retention of 0.16 kg/m^3 gave a performance similar to that of ACQ at 4.0 kg/m^3. The species used in the mold test; *Aureobasidium spp., Aspergillus spp., Penicillium sp.p and Alternaria spp.* all have much lower MICs (< 200ppm) than

Trichoderma sp (> 1000ppm) (Grundlingerv and Exner, 1990). This explains why all formulations showed a better performance in the mold test as compared to the agar block test using *T. harzianum.*

Table 5.5. Mass spectra - major peaks for each treatment.

Sample		Peaks detected (m/z, amu)	Most intense peak (% Intensity)
Tebuconazole standard		308.15, 309.16, 310.14	308.15
Culture with tebuconazole only	Fraction 1	308.15, 324.16, 338.34	338.34
	Fraction 2	306. 13, 308.15, 312.15, 338.34, 371.11, 324.15	324.14
	Fraction 3	308.15, 310.15, 338.34, 364.17 366.12	366.12
	Fraction 4	281.17, 308.15, 310.15, 338.34, 312.15	281.17
Tebuconazole + TEMED	Fraction 1	308.15, 281.14, 310.14, 324.15	324.15
	Fraction 2	308.15, 281.14, 282.15, 283.14,	281.14
Tebuconazole + EDTA	Fraction 1	308.15, 310.14, 324.15	308.15, 324.15
Tebuconazole + PB	Fraction 1	308.15, 310.15, 324.15	308.15, 324.15
Tebuconazole +ABT	No major fraction identified	308.15, 310.15, 324.15	308.15, 324.15

PB and ABT most likely work synergistically with tebuconazole in inhibiting the the fungal cytochrome P-450 3-A dependent enzyme 14-alpha demethylase, thereby interrupting the synthesis of ergosterol. EDTA had an effect on the magnitude of oxidation. As hypothesized, EDTA most likely binds metal ions like Fe^{2+} and Fe^{3+} which are important in the P450 enzymatic system, thus, interfering with tebuconazole metabolism. However, despite the positive effect of all these inhibitors an important observation was that *T. harzianum* was able to germinate and grow to some extent in the presence of tebuconazole and all additives used in this

study. This led to the conclusion that P450 inhibitors, like triazoles, cannot be effective against

Table 5.6. Retentions of tebuconazole and mold growth on wood samples.

Treatment	Tebuconazole retention Kg/m^3	Average rating			
		2 weeks	4 weeks	6 weeks	8 weeks
Untreated	-	0	4	4	4
ACQ- 4.0 Kg/m^3	-	0	0	0	0
Tebuconazole	0.16	0	0	2	2
Tebuconazole + PB	0.18	0	0	0	0
Tebuconazole+ ABT	0.152	0	0	0	0
Tebuconazole + EDTA	0.153	0	0	0	0
Tebuconazole+ TEMED	0.142	0	0	0	2

0-No visible growth.

1- Mold covering 10% of surface.

2- Mold covering 10-30% of the surface.

3- Mold covering 30-70% of the surface.

4- Mold covering more than 70% of the surface.

5- Mold covering more than 100% of the surface with intense growth obscuring greater than 70% of the surface.

resistant molds. Biotransformation of the active chemical may also not be the only mechanism used by these fungi in tolerating tebuconazole. Genetic over expression of the CYP 51- gene which leads to overproduction of the P-450 dependent enzyme 14-α demethylase may contribute to the resistance leading to germination and growth of the fungus in unfavorable environments. The preservative of choice for these organisms most likely is one whose mode of action is different from interruption of ergosterol synthesis. The spores produced may have enough sterol to germinate in the presence of these inhibitors.

5.4 Conclusion

Cytochrome P450 monooxygenases are involved in cases of resistance of fungi to fungicides. Resistance has been associated with an increase in monooxygenase activities due to cytochrome P450 activities. However, this increase does not account for all of the resistance in *T. harzianum*. Even though metabolism of tebuconazole was drastically reduced by the P450

inhibitors ABT and PB, *T. harzianum* was still able to germinate and grow at concentrations less than 200ppm tebuconazole in the presence of these additives. This led to the conclusion that biotransformation of the chemical is not the only mechanism by which this species tolerates tebuconazole. Tebuconazole does not adequately hinder P450 activities. ABT and PB increase the hindrance of P450 activities. The metal chelators also may slow down the P450 activities but do not eliminate them completely. In addition to biotransformation, there are other mechanisms by which this species tolerates tebuconazole. Tebuconazole, PB, and ABT all seem to have little effect against spore germination because spores may contain enough ergosterol for formation of germ tubes. Some spores may have enough sterol to produce infection structures hence tebuconazole may not be effective against infection of wood in service. Another reason may be genetic over expression of the CYP 51- gene which leads to overproduction of the P-450 dependent enzyme 14-α demethylase. Therefore, synthesis of ergosterol is not interfered with as expected and this leads to germination and growth of the fungus in unfavorable environments.

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CHAPTER 6. CONCLUSIONS

The objective of this work was to study the extent of biodegradation and metabolic pathways by microorganisms and the contribution it has on the tolerance of some fungal species to biocides. Currently, the major advantage of organic biocides used in wood preservation is also their major disadvantage: they are biodegradable or transformed by wood degrading and non degrading organisms in soil and wood. Tebuconazole, a triazole, was used as the test compound. One species each of a bacterial strain, a mold, a brown rot species and a white rot species were used as the test microorganisms.

The mold *T. harzianum* was found able to tolerate tebuconazole at concentrations below 200ppm because it biotransforms the chemical by cleavage of the 1,2,4 triazole ring and by performing oxidation reactions to produce more hydrophilic metabolites. The tert butyl alcohol, the tert butyl carboxylic acid, and the 3-hydroxyaryl derivatives are the initial products. These initial products undergo acetylation or methylation in a second step. Further studies using different species of fungi revealed that the ability of a fungus to degrade a biocide contributes to the efficacy of the biocide against that species. *P. chrysosporium* with the lowest minimum inhibitory concentration (MIC) was found not able to degrade tebuconazole o a significant amount, while *T. harzianum* with the highest MIC (lowest efficacy) degrades tebuconazole and tolerates it at concentrations less than 200ppm. Although *P. chrysosporium* is recognized for its ability to metabolize a large diversity of compounds, it showed little or no ability to metabolize tebuconazole. This may be a contributing factor to the high efficacy of tebuconazole towards white rot fungi. A common mechanism was involved in the initial metabolism of tebuconazole by the different types of microorganisms studied. The most prominent metabolism product in cultures of brown rot *M. incrassata,* the mold *T. harzianum,* and bacteria *Pseudomonas fluorescens* had the 1,2,4 triazole ring cleaved to form imines. Two products with the same molecular mass were identified. All species also perform oxidation processes in initial

degradation of tebuconazole. The main products are the oxidation products of the methyl groups on the tert butyl moiety (the alcohol and the carboxylic acid). T. *harzianum* is able to perform phase II type reactions by acetylation and O-methylation. Brown rot *M. incrassata* and the bacteria did not metabolize the chemical by acetylation.

Observation of mainly oxidation products led to the hypothesis that monooxygenation is catalyzed by enzymes from the family of the Cytochrome P450 monooxygenases. The oxidation of tebuconazole by *T. harzianum* and *M. incrassata* was reduced when PB and ABT, both known P450 inhibitors, were added to the culture than in cultures containing tebuconazole alone. This suggested that the enzymes involved are from the P450 family. Furthermore, the microsomal extract from *T. harzianum* exhibited a maximum peak at 440 - 460nm when CO was bubbled into $Na_2S_2O_4$ treated and concentrated samples. This indicates that initial oxidation reactions leading to the metabolism of tebuconazole by wood inhabiting fungi are cytochrome P450 dependent. Resistance is associated with an increase in monooxygenase activities and with an increase in cytochrome P450 content. However, this increase does not account for all of the resistance in *T. harzianum*. Even though metabolism of tebuconazole was drastically reduced by the P450 inhibitors ABT and PB, *T. harzianum* was still able to germinate and grow at concentrations less than 200ppm tebuconazole in the presence of these additives. This indicates that biotransformation of the chemical is not the only mechanism by which this species tolerates tebuconazole. Tebuconazole does not adequately hinder P450 activities. ABT and PB increase the hindrance of P450 activities. The metal chelators also may slow down the P450 activities but do not eliminate them completely. In addition to biotransformation, there are other mechanisms by which this species tolerates tebuconazole. Tebuconazole, PB, and ABT all seem to have little effect against spore germination because spores contain enough ergosterol for formation of germ tubes. Some spores may have enough sterol to produce infection structures hence tebuconazole may not be effective against infection of wood in service. Another reason may be genetic over

expression of the CYP 51- gene which leads to overproduction of the P-450 dependent enzyme 14-α demethylase. Therefore, synthesis of ergosterol is not interfered with as is expected and this leads to germination and growth of the fungus in unfavorable environments. The triazole group of fungicides does not adequately protect timber against molds hence suitable co-biocides may be necessary in any formulation containing triazoles. Molds like *T. harzianum* and bacteria species are not responsible for decay but may lower biocide concentrations or metabolize them into less potent derivatives making the environment suitable to wood degrading micro-organisms.

VITA

Diana N. Obanda was born in Nairobi, Kenya. She is married with children. She obtained her bachelor of science and master of philosophy degrees in wood science and technology from Moi University in Eldoret Kenya, in 1992 and 2000, respectively. She later worked for a research institute and her alma mater, Moi University, as a Lecturer. As a lecturer, she instructed undergraduate students in the field of wood preservation and bioenergy studies, prepared quizzes, administered and graded exams in three sessions of 50 students each, and participated in developing grants and proposals on agricultural policy issues in developing countries.

In 2004, she began graduate studies at Louisiana State University, Baton Rouge, Louisiana. She earned a master of science in environmental science with a concentration in environmental planning and management in August, 2007. She is completing her doctoral studies in forestry and environmental planning and management (minor) at Louisiana State University with a tentative graduation date in May, 2008.

Diana has eight years of research and writing experience on wood conservation issues and food policy issues in developing countries. As research assistant with the Louisiana Forest Products Development Center, her work included analysis of wood preservatives against insects and fungi in laboratory and field studies, testing preservative application technology, and working with industry in providing collaborative or contracted services to complement fundamental research. She is currently seeking a position in academia or a research based position in the wood preservation industry.