The Role of Extractive Depletion in the Fungal Colonization of Western Redcedar (*Thuja plicata* Donn)

by

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B.Sc., University of Exeter, 1999

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Forestry)

The University of British Columbia

October 2006

Abstract

Western redcedar (*Thuja plicata* Donn) (WRC) is a naturally durable softwood species native to British Columbia, Canada, as well as Washington, Oregon and California in the USA. WRC wood products are valued for their durability conferred by anti-microbial extractive compounds. However, such products are still susceptible to fungal colonization that can result in decay and discoloration. The main objective of this thesis was to provide information on the relationship between durability, the change in extractives, and micro-organisms on WRC products in-service in order to help the industry to develop strategies to improve product service life.

We first developed protocols to extract, separate and quantify extractives by ultra-sonication and reverse phase high performance liquid chromatography. We developed techniques to screen a range of fungi commonly isolated from WRC in service products for extractive-tolerance *in vitro.* Results indicated that the Basidiomycete *Pachnocybe ferruginea* exhibited the highest extractive-tolerance of the range of fungi tested. The next section of this thesis focuses on black stain of WRC siding by *Aureobasidium pullulans* and the role of weathering. We characterized the effect of weathering on extractives at the surface and correlated this with ability of *A. pullulans* to colonize. UV plus water spray treatments substantially reduced extractives but did not promote fungal colonization. In contrast, UV-only treatments reduced extractive contents less but stimulated fungal colonization. *A. pullulans* exhibited high tolerance to the tropolone β-thujaplicin *in vitro*; thus loss of tropolone content may not be required for colonization. In the final part of this thesis we investigated the relationship between extractive depletion caused by leaching and how this influenced decay. Leaching resulted in an 80% reduction of extractives,

which generally resulted in a greater degree of decay by six commonly isolated fungal species. Fungi which exhibited low tolerance to WRC leachate *in vitro* were able to decay leached WRC blocks more readily than non-leached WRC blocks. Extractive-tolerant species did not require leaching of extractives for decay to occur. The Basidiomycetes *P. ferruginea*, and to a lesser extent *Acanthophysium lividocaeruleum* and *Heterobasidion annosum* consistently exhibited high extractive tolerance and could decay non-leached WRC to a similar extent as leached. Such species are candidate 'pioneer' species that may detoxify extractives in wood products, paving the way for decay by less specialized fungi to occur.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

°C	degrees Celsius
µg	microgram
µL	microliter
µm	micrometer
cm	centimetres
DW	dry weight
FD	freeze-dried
FS	feeder strips
g	grams
h	hour
HPLC	high performance liquid chromatograpghy
IS	internal standard
ITS	internal transcribed spacer
Kg	kilogram
kHz	kilohertz
Kj/m^2	kilojoules per square meter
L	litre
LSU	large subunit
MEA	malt extract agar
mins	minutes
mL	millilitre

mm	millimeter
nm	nanometers
PCR	polymerase chain reaction
UV	ultraviolet
WRC	Western redcedar
WS	water spray

Acknowledgements

I would like to thank, first and foremost, my graduate supervisor, Dr. Colette Breuil, for her guidance and support throughout this work. I would also like to thank members of my graduate committee, Dr. John F. Kadla and Dr. Paul I. Morris, for their advice and help with this project. Thanks also to Dr. Rod Stirling and Dr. Bob Daniels of the Forintek Canada Corporation for their contributions to- and assistance with this research. Thank you to Dr. Batia Bar-Nir for her assistance with NMR analysis.

I would like to thank my family, friends and colleagues for their years of support.

This work was funded by the Natural Sciences and Engineering Research Council of Canada

Co-authorship Statement

I hereby declare that I undertook the following contributions to the work reported in this thesis:

Chapter 2 Extracting and Quantifying Western Redcedar (*Thuja plicata* Donn) Heartwood Extractives Using Ultrasonication and Reverse Phase HPLC.

Authors: Russell J. Chedgy, C. R. Daniels, John F. Kadla and Colette Breuil.

Submitted to the journal of Holzforschung, submission # HOLZ-D-06-00099.

For this chapter I was the primary contributor, and took full writing responsibilities. Dr C. R. Daniels contributed by providing a method for the separation of WRC extractives using reverse phase HPLC that he had previously developed. Dr John F. Kadla and Dr Colette Breuil had supervisory roles.

Chapter 3 Isolating and Testing Fungi Tolerant to Western redcedar (*Thuja plicata* Donn) Extractives.

Authors: Young Woon Lim, Russell J. Chedgy, Sabarish Amirthalingam, and Colette Breuil.

Submitted to the journal of Holzforschung, submission # HOLZ-D-06-00100.

For this work Dr. Young Woon Lim and I were the main contributors. I conducted all duties related to the chemical analysis of WRC extractives. Specifically, I conducted HPLC analysis and quantification of WRC extractives that had leached from WRC-FSs that were inoculated on media. I tested individual extractive compounds for fungicidal activity on selected fungal strains. In addition I took approximately half of the writing responsibilities. Mr. Sabarish Amirthalingam

participated by conducting screening of fungal strains on media inoculated with WRC-FSs. This work was supervised by Dr. Colette Breuil.

Chapter 4 Black Stain of Western Redcedar (*Thuja plicata* Donn) by *Aureobasidium pullulans*: the Role of Weathering.

Authors: Russell J. Chedgy, Paul I. Morris, Young Woon Lim and Colette Breuil.

Submitted to the journal of Wood and Fiber Science, currently under review.

For this chapter I was the primary contributor, and took full writing responsibilities. Dr. Young Woon Lim provided assistance with fungal isolation and identification. Dr. Paul I. Morris and Dr. Colette Breuil had supervisory roles.

Chapter 5 The Effect of Leaching on the Decay of Second-growth Western Redcedar (*Thuja plicata* Donn).

Authors: Russell J. Chedgy, Young Woon Lim, and Colette Breuil.

For this work I was the primary contributor, and took full writing responsibilities. Dr. Young Woon Lim provided assistance with fungal isolation and identification and Dr. Colette Breuil had supervisory roles.

Appendix I Fungal Diversity from Western Redcedar Fences and Their Resistance to β-Thujaplicin.

Authors: Young Woon Lim, Jae-Jin Kim, Russell J. Chedgy, Paul I. Morris, and Colette Breuil.

For this work Dr. Young Woon Lim was the primary contributor. Dr. Lim and Dr. Jae-Jin Kim under took the majority of the fungal isolation in this work and shared writing responsibilities.

My role was to screen isolates for β-thujaplicin resistance, as well as an editorial role during manuscript preparation. Dr. Paul Morris and Dr. Colette Breuil had supervisory roles.

Chapter 1

General Introduction and Research Objectives

1.1 Introduction

The emphasis of this project is on the depletion of extractives from western redcedar (*Thuja plicata* Donn) (WRC) wood products caused by weathering and how this affects the ability of fungi to colonize and cause discoloration or decay. This research is relevant to product service life. This chapter begins by assessing the chemistry and occurrence of WRC extractives and their biological activity. Also discussed are the modern day applications of several key extractive compounds that have roles in the wood protective coating, pharmaceutical, and cosmetic industries. Then the effect of weathering on WRC extractives is explored, followed by the fungal colonization and decay of WRC wood products. Finally, I have included a brief review on extractive biosynthesis that touches on their biological and ecological roles in members of the Cupressaceae family. Chapter 1 focuses on extractive compounds that confer natural durability to WRC wood products due to anti-microbial activity. Chapter 2 describes the development of protocols to extract, separate and accurately quantify extractives. These protocols were then utilized for the work described in Chapter 3 that describes a novel method to screen fungi isolated from WRC products for tolerance to specific WRC extractive constituents. Chapter 4 investigates the effect of various types of weathering on extractives and how this influences the colonization by black staining fungi of WRC siding products. This chapter also summarizes data on the resistance of several species of black staining fungi to WRC extractives. The relationship between extractive depletion by leaching and decay are examined in Chapter 5. The growth and decay ability of six frequently isolated fungi from WRC products are assessed on leached versus

non-leached WRC heartwood. Finally, the implications of the work conducted in this thesis as well as future research are discussed in Chapter 6.

1.2 The Importance of WRC

WRC is a naturally durable softwood species native to British Columbia, Canada, as well as Washington, Oregon, and California in the USA. The heartwood of this species contains an array of antimicrobial extractive compounds which act as a natural chemical defense shield against pathogen invasion in standing trees (Rennerfelt, 1948; Barton & MacDonald, 1971; Van der Kamp, 1986; Johnson & Croteau, 1987; Zaprometov, 1992; Belanger *et al.*, 1997; DeBell *et al.*, 1997) and confer natural durability in WRC products (Rudman, 1962; Barton & MacDonald, 1971). WRC is utilized in the manufacture of wood products with exterior residential applications which account for a significant portion of Canada's forest products industry (Gonzalez, 2004). Despite this, WRC products can still fail in-service due to fungal decay as well as discoloration by staining fungi which can lead to premature replacement of wood products. Such failure may be attributable in part to 1) colonization by extractive-tolerant micro-organisms, or 2) depletion of anti-microbial extractives by weathering, as well as 3) biodegradation of extractives by micro-organisms paving the way for colonization by less specialized wood-destroying micro-organisms.

1.3 The Chemistry of WRC Extractives

The chemical composition of WRC heartwood is homologous to other softwood species such as douglas-fir (*Pseudotsuga menziesii*) and black spruce (*Picea mariana*) in terms of the relative abundance of major structural constituents such as alpha-cellulose, hemicelluloses and lignin (Lewis, 1950) (Table 1.1). Unlike other North American softwood species like true firs (*Abies*

Table 1.1 Chemical composition of western redcedar, western hemlock and douglas-fir heartwood (Lewis, 1950)

Species	Alpha cellulose	Hemi-celluloses	Lignin	Total extractives	Ash
Western redcedar	47.5 (52.8)	13.2 (14.7)	29.3 (32.6)	10.2	0.2
Western hemlock	48.8 (51.6)	14.7 (15.5)	28.8 (30.4)	5.3	0.5
Douglas-fir	53.8 (57.2)	13.3 (14.1)	26.7 (28.4)	5.9	0.3

Note: numbers represent the percentage content on a moisture-free basis; figures in parenthesis represent percentage composition on a moisture-free extractive-free basis.

spp.), western larch (*Larix occidentalis*), colorado blue spruce (*Picea pungerns*), douglas-fir and *Pinus* species, WRC produces only trace amounts of oleoresin (Penhallow, 1907; Fahn, 1979; Johnson and Croteau, 1987; Lewinsohin *et al.*, 1991, 1994) as a chemical deterrent and a physical barrier against pathogen attack (Johnson and Croteau, 1987; Zaprometov, 1992). However, WRC has an unusually high proportion of extractives that are aromatic and polyphenolic in nature (Barton and MacDonald, 1971). Several of these compounds exhibit antimicrobial and insecticidal activities, (Inamori *et al.*, 1999, 2000; Arima *et al.*, 2003; Morita *et al.*, 2004a, 2004b) as well as herbicidal properties (Sakagami *et al.*, 2000), with efficacy comparable to commercial pest control agents (See Section 1.4). Such extractives also contribute to many of cedar's desirable qualities such as its distinctive red-brown color, pleasant odor and excellent finishing quality (Barton and MacDonald, 1971). Conversely, their presence can adversely affect wood-pulping processes, leading to increased production costs (Wethern, 1959).

The concentration of extractives is subject to wide variation throughout the tree. They increase radially, reaching a maximum at the heartwood-sapwood border before rapidly declining in the sapwood region; and decrease longitudinally from the tree base to the crown (MacLean and Gardner, 1956; Nault, 1988; DeBell *et al.*, 1999). WRC extractives are a mixture of tropolone and lignan compounds. Tropolones are 2-hydroxy-2,4,6-cycloheptatrien-1-one molecules and their derivatives which possess special characteristic properties due to the 1,2 arrangement of the carbonyl and hydroxyl groups on an unsaturated seven-membered carbon ring (Dewar, 1945). Lignans are nonstructural, dimeric, phenolic metabolites, many of which are complex molecules of unusual structure (Gang *et al.*, 1998). To date, at least eight tropolone and eleven lignan compounds have been identified and characterized in WRC. Many of these compounds occur in species throughout the Cupressaceae family (Zavarin *et al.*, 1967). Most research has been

centered on tropolones extracted from WRC of North America and a species native to Japan: *Thujopsis dolabrata* (Sieb. *et* Zucc. *var*. hondai Makino), also know as 'aomori hiba', and the 'dwarf hiba cedar'.

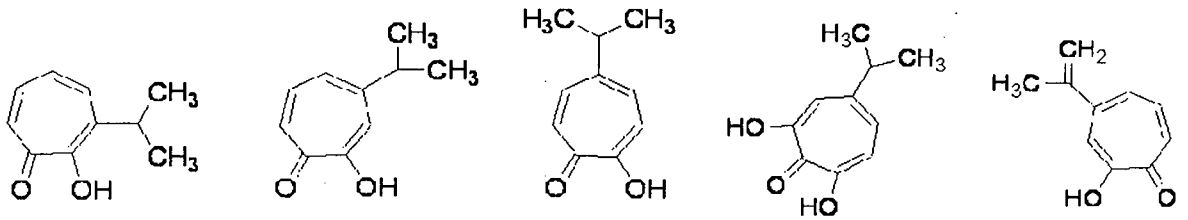
The tropolone content of WRC heartwood is estimated at 1-1.5% (w/w) while the lignan portion comprises 5-15% (w/w) (Barton and Macdonald, 1971). Within the group of tropolone compounds are three isomeric isopropyl-tropolones known as the 'thujaplicins'. These are specifically: (i) α-thujaplicin, (ii) β-thujaplicin, and (iii) γ-thujaplicin. Table 1.2 shows the common name, chemical description and relevant literature for each of the extractive compounds cited, and Figure 1.1 displays their chemical structures. α-Thujaplicin is normally present in trace amounts only at 0-1% (w/w) (Barton and MacDonald, 1971; Jones and Falk, 2005). γ-Thujaplicin is marginally more abundant than β- thujaplicin. Analysis of the methanol extractive of WRC heartwood shows that these two compounds account for approximately 17% (w/w) of the total tropolone content (Jones and Falk, 2005). The other tropolone compounds identified are (iv) β-thujaplicinol, (v) β-dolabrin, (vi) thujic acid, (vii) methyl thujate, and finally, (viii) nezukone. β-Thujaplicinol is estimated to account for approximately one tenth of the total thujaplicin content. Thujic acid is the most abundant tropolone comprising 26% (w/w) of the total tropolone portion (Jones and Falk, 2005), while β-dolabrin, methyl thujate and nezukone are present only in trace amounts (Barton and MacDonald, 1971). The non-tropolone compound (ix) carvacrol methyl ether is also found in trace amounts (MacLean, 1970). Also noteworthy is the additional tropolone compound (x) 4-acetyltropolone which has not been reported in WRC but is known to be common in other Cupressaceous species (Zavarin *et al.*, 1967).

Table 1.2 Common names, chemical structures and relevant literature for extractives

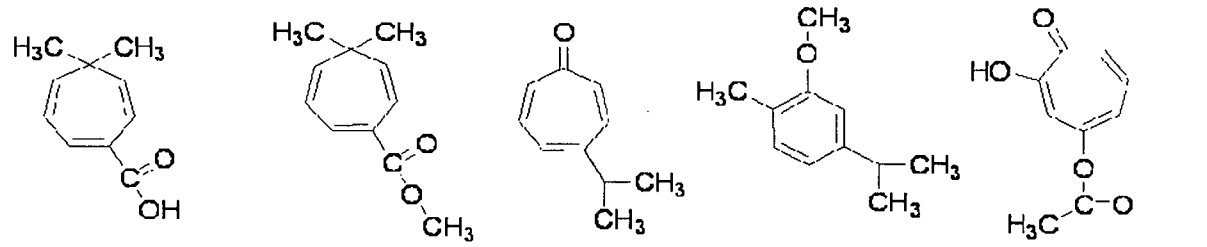
Compound Name	Chemical Structure	Relevant Literature
(i) α-Thujaplicin	2-Hydroxy-3-isopropyl-2,4,6-cycloheptatrien-1-one	Gripenberg, 1948
(ii) β-Thujaplicin	2-Hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one	Erdtman and Gripenberg, 1948
(iii) γ-Thujaplicin	2-Hydroxy-5-isopropyl-2,4,6-cycloheptatrien-1-one	Erdtman and Gripenberg, 1948
(iv) β-Thujaplicinol	2,7-Dihydroxy-4-isopropyl-2,4,6-cycloheptatrein-1-one	Gardner *et al.*, 1957
(v) β-Dolabrin	2-Hydroxy-4-isopropenyl-2,4,6-cycloheptatrien-1-one	Nozoe *et al.*, 1957; Gardner and Barton, 1958
(vi) Thujic acid	5,5-Dimethyl-1,3,6-Cycloheptatriene-1-carboxylic acid	Gripenberg, 1956; Davis and Tulinsky, 1962
(vii) Methyl thujate	7,7-Dimethyl-3-carbomethoxy-1,3,5-cycloheptatriene	Barton and Gardner, 1954
(viii) Nezukone	4-Isopropyl-2,4,6-cycloheptatrien-1-one	Hirose *et al.*, 1966, 1967, 1968
(ix) Carvacrol methyl ether	5-Isopropyl-2-methylanisole	MacLean, 1970
(x) 4-Acetyltropolone	4-Acetyl-2-hydroxy-2,4,6-cycloheptatrien-1-one	Zavarin *et al.*, 1967
(*xi*) (-)-Plicatic acid	2,3,6-Trihydroxy-7-methoxy-2-hydroxymethyl-4-(3',4'-dihydroxy-5'-methoxyphenyl)-tetralin-3-carboxylic acid	Gardner *et al.*, 1960, 1966; Swan *et al.*, 1967
(*xii*) (-)-Plicatin	Naphtho[2,3-c]furan-1(3H)-one	MacDonald and Swan, 1970
(*xiii*) (-)-Thujaplicatin methyl ether	2-(4"-Hydroxy-3",5"-dimethoxybenzyl)-3-(4'-hydroxy-3'methoxybenzyl)-butyro-lactone	MacLean and Murakami, 1966a; Nishibe *et al.*, 1974
(*xiv*) (-)-Thujaplicatin	2-(3",4"-Dihydroxy-5"-methoxybenzyl)-3-(4'-hydroxy-3'-methoxybenzyl)-butyrolactone	MacLean and Murakami, 1966a; Nishibe *et al.*, 1974
(*xv*) (-)-Hydroxythuja -plicatin methyl ether	Dihydro-3-hydroxy-3-[(4-hydroxy-3,5-dimethoxyphenyl)methyl]-4-[(4-hydroxy-3-methoxyphenyl)methyl]-2(3H)-furanone	MacLean and Murakami, 1966b.

Table 1.2 (Continued) Common names, chemical structures and relevant literature for extractives

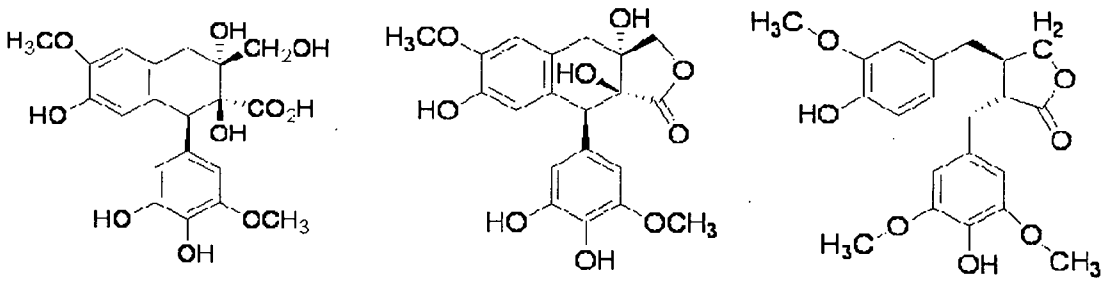
Compound Name	Chemical Structure	Relevant Literature
(*xvi*) (-)-Dihydroxy-thujaplicatin methyl ether	2,3-dihydroxy-2-(4"-hydroxy-3",5"-dimethoxybenzyl)-3-(4'-hydroxy-3'-methoxybenzyl)-butyrolactone	MacLean and Murakami, 1966c.
(*xvii*) (-)-Dihydroxy -thujaplicatin	2,3-dihydroxy-2-(3",4"-dihydroxy-5"-methoxybenzyl)-3-(4'-hydroxy-3'-methoxybenzyl)-butyrolactone	MacLean and MacDonald, 1966
(*xviii*) Plicatinaphthol	1,6-dihydroxy-2-(hydroxymethyl)-7-methoxy-4-(3',4'-dihydroxy-5'-methoxyphenyl)-3-naphthoic acid lactone	MacLean and MacDonald, 1969a
(*xix*) Plicatinaphthalene	6-hydroxy-2(hydroxyl-methyl)-7-methoxy-4-(3',4'-dihydroxy-5'-methoxyphenyl)-3-naphthoic acid lactone	MacLean and MacDonald, 1969b
(*xx*) γ-Thujaplicatene	2-(3",4"-dihydroxy-5"-methoxybenzylidene)-3-(4'-hydroxy-3'-methoxybenzyl)-butyrolactone	MacDonald and Barton, 1970
(*xxi*) β-Apoplicatitoxin	1,4-dihydronaphthalene-6-hydroxyl-2-(hydroxymethyl)-7-methoxy-4(3',4'-dihydroxy-5'-methoxyphenyl)-3-carboxylic acid lactone	MacDonald and Barton, 1973



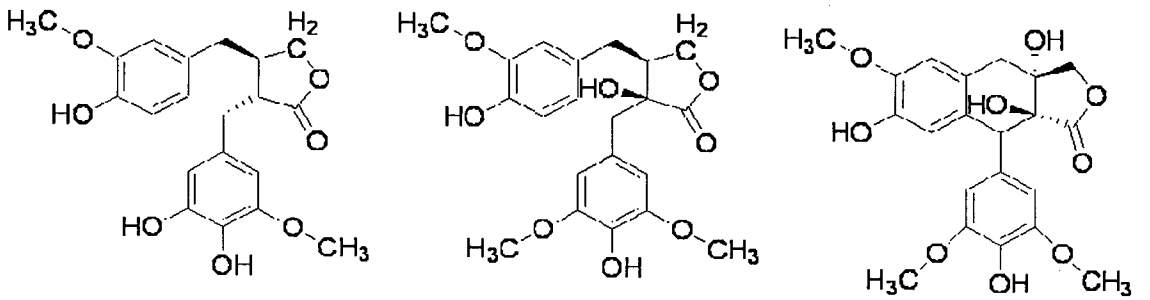
(i) α-Thujaplicin (ii) β-Thujaplicin (iii) γ-Thujaplicin (iv) β-Thujaplicinol (v) β-Dolabrin



(vi) Thujic Acid (vii) Methyl Thujate (viii) Nezukone (ix) Carvacrol Methyl Ether (x) 4-Acetyltropolone*



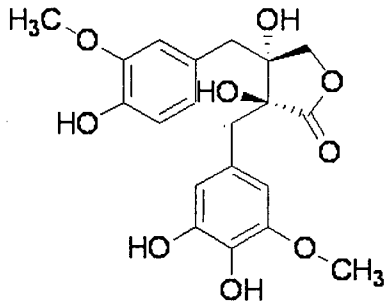
(xi) (-)-Plicatic Acid (xii) (-)-Plicatin (xiii) (-)-Thujaplicatin Methyl Ether



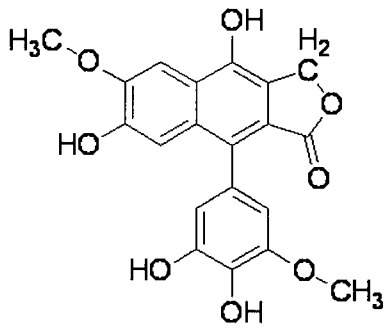
(xiv) (-)-Thujaplicatin (xv) (-)-Hydroxythujaplicatin Methyl Ether (xvi) (-)-Dihydroxy-thujaplicatin Methyl Ether

* Not present in *Thuja plicata*

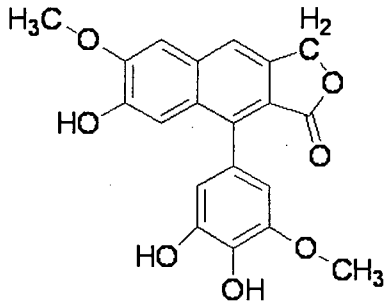
Figure 1.1 Common extractive compounds of the Cupressaceae family (Barton and MacDonald, 1971)



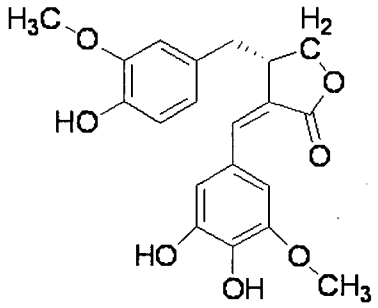
(xvii) (-)-Dihydroxythujaplicatin



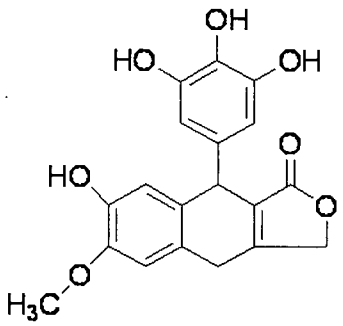
(xviii) Plicatinaphthol



(ixx) Plicatinaphthalene



(xx) γ-Thujaplicatene



(xxi) β-Apoplicatitoxin

Figure 1.1 (Continued) Common extractive compounds of the Cupressaceae family (Barton and MacDonald, 1971)

Of the lignan extractive compounds, (xi) (-)-plicatic acid is the most abundant, accounting for 40-50% of this portion of extractives (MacDonald and Swan, 1970; Barton and MacDonald, 1971; Jones and Falk, 2005). It is a reactive, polyoxyphenolic, amorphous acid, which is optically active as well as heat and light sensitive. It is a very strong acid because of its highly hydroxylated side chain. Kai and Swan (1990) suggested that lignans such as plicatic acid are responsible for the red-brown color of WRC heartwood. Further to this, Johansson *et al.* (2000) isolated an insoluble polymeric fraction that accounted for 34% by weight of the methanol extractive but 72% of the color. Analysis suggested that these polymers were less related to lignin and more closely related to the highly phenolic lignans such as plicatic acid and plicatin. Plicatic acid has been reported to cause occupational asthma – an issue amongst forestry workers exposed to cedar dust particles on a regular basis (Chan-Yeung *et al.*, 1980; Frew *et al.*, 1993, 1998; Chan-Yeung, 1994).

The second lignan in the series is (xii) (-)-plicatin which is a plicatic acid lactone. Other lignans include (xiii) (-)-thujaplicatin methyl ether, the first syringyl derivatives found in softwood and the first lignan known to possess both guaiacyl and syringyl rings, and (xiv) (-)-thujaplicatin. Together, plicatin, thujaplicatin methyl ether, and thujaplicatin make up approximately 30-40% of the total lignan content (Barton and MacDonald, 1971). The remaining 10% of the lignan portion comprises of seven other lignan compounds: (xv) (-)-hydroxythujaplicatin methyl ether, (xvi) (-)-dihydroxy-thujaplicatin methyl ether, (xvii) (-)-dihydroxythujaplicatin, (xviii) plicatinaphthol, (ixx) plicatinaphthalene, (xx) γ-thujaplicatene, and finally (xxi) β-apoplicatitoxin (see Table 1.2 for appropriate references).

1.4 Biological Activity of WRC Extractives

Of the range of extractive chemicals present, tropolones have warranted a great deal of scientific interest because of their biological activity. The tropolones α-, β-, γ-thujaplicin, β-dolabrin, 4-acetyltropolone and their derivatives have anti-fungal activity against a range wood destroying and plant pathogenic fungal species *in vitro*. Their minimum inhibitory concentrations (MICs) range from 0.2-50.0μg/ml (Raa and Goksoeyr, 1965; Inamori *et al.*, 2000; Baya *et al.*, 2001; Inamori and Morita, 2001; Morita *et al.*, 2004a, 2004b). This range is comparable to biocides such as Amphotericin B and Pentachlorophenol. In addition, several of these tropolones exhibit anti-bacterial activity against species such as *Legionella pnuemophila* (causal agent of Legionnaires' disease) with MICs in the range of 6.3-50.0μg/ml (Morita *et al.* 2004a, 2004b). The tropolone β-thujaplicin has been used as a preservative of vegetables, flowers, and mushrooms because of its strong antibacterial activity, as well as it being a plant growth stimulator. The antimicrobial activity of tropolones has been used in commercial products ranging from aqueous ink (Yatake, 2005) to cosmetics (Pillai *et al.*, 2005).

In addition to anti-microbial activity, insecticidal and acaricidal properties have also been reported for several of these tropolones. They have been demonstrated to be highly effective against species such as *Coptotermes formosanus* (formosan subterranean termite), *Reticulitermes speratus* (Japanese termite), *Dermatophagoides farinae* (house mite), and *Tyrophagus putrescentiae* (mould mite) with a 50%-leathal concentrations (LC_{50}) ranging from 0.02-0.66g/m^2 (Inamori *et al.*, 2000; Inamori and Morita, 2001; Morita *et al.*, 2004a). This is comparable to commercially available insecticides such as Chlorpyrifos and DEET (N,N-diethyl-m-toluamide). Given this, a WRC oil-containing miticide for domestic use was developed by Ishibashi *et al.* (1992). The researchers soaked porous ceramic granules (0.1-3.0

mm) in 5% WRC oil which resulted in a mite mortality rate of 95% in 24 hours. There is also some evidence that the WRC tropolone thujic acid acts as insect juvenile hormone in species such as the Yellow Mealworm beetle (*Tenebrio molitor*) (Barton, *et al*, 1972).

Tropolones also have phytogrowth-inhibitory activity. Sakagami et al. (2000) showed that γ- and β-thujaplicin, and β-dolabrin inhibited germination in seeds of field mustard (*Brassica campestris*) and sesame (*Sesamum indicum*) at concentrations as low as 10ppm. This phytogrowth-inhibitory activity was as high as the herbicide sodium 2,4-dichlorophenoxyacetate. These tropolones were also found to significantly decrease the amount of chlorophyll in cotyledons of the two plant species. This phytogrowth-inhibitory action might be a common biological activity of tropolone compounds.

From a pharmaceutical perspective, several of the tropolone compounds present in heartwood of WRC and other members of the Cupressaceae family have been shown to have cytotoxic activity against a range of cancerous cell lines *in vitro* (Matsumura *et al.*, 2001; Inamori *et al.*, 2004; Morita *et al.*, 2004a, 2004b). The tropolones α-, β-, γ-thujaplicin, β-dolabrin, and 4-acetyltropolone are effective against cell lines such as murine P388 lymphocytic leukemia, human stomach cancer KATO-III and Ehrlich's ascites carcinoma. The inhibition within 24 hours is >70-95% at concentrations ranging from 0.3-5.0µg/ml (Matsumura *et al.*, 2001; Morita *et al.*, 2001, 2002, 2004a, 2004b; Inamori *et al.*, 2003, 2004). In addition, several tropolone compounds have potential applications in the field of sunburn protection as they have an inhibitory effect on ultraviolet B-induced apoptosis in keratinocytes (also known as sunburn cells) (Baba *et al.*, 1998; Arima *et al.* 1997; Nakano *et al.* 2006). The thujaplicins compounds have strong antioxidant abilities and protect cultured cells from oxidative stress-mediated

damaged (Paschalis-Thomas *et al.*, 2005), and may have pharmaceutical applications for the prevention of UV-induced photo-damage in skin cells. Overall, the pharmaceutical utilization of tropolones may be feasible given that in preliminary trials in mice suggested that they showed low toxicity.

The ability of tropolones to chelate bi- and trivalent metal ions has been implicated in their anti-microbial and cytotoxic activity, although the exact mechanism is still subject to investigation (Miyamoto *et al.*, 1998). Tropolones are known to form stable chelates with various metals ions which include: Fe^{3+}, Fe^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} (Oka and Matsuo, 1958; Oka *et al.* 1964, 1968; Oka and Yanai, 1965; Hirai and Oka, 1970). Raa and Goksoeyr (1965) demonstrated that the β-thujaplicin-copper chelate inhibited respiration in Yeast (*Saccharomyces cerrivasae*) at concentrations $>10^{-6}$M. They also suggested that the keto-enol group of the tropolone molecule is essential for the inhibition since the methyl ether of β-thujaplicin was not toxic. Inamori *et al.* (1999) postulated that at least part of this activity is due to metal chelation between the carbonyl group at C-1 and the hydroxyl group at C-2 in the tropolone skeleton since β-thujaplicin-acetate, which was unable to chelate divalent metals ions, did not show any antimicrobial activity. Budihas *et al.*, (2005) explored the role of β-thujaplicinol as a potent and selective inhibitor of the ribonuclease H (RNase H) activity of human immunodeficiency virus-type 1 reverse transcriptase (HIV-1 RT). They concluded that the ability of thujaplicins to chelate divalent metals such as Cu^{2+} and Zn^{2+} via their β-diketone moiety (Endo *et al.*, 1988) may play a role. They suggested that metal chelation and/or altering the co-ordination geometry of the divalent metal in the RNase H catalytic center, which is essential for catalysis, would seem a plausible mechanism of action. Based on this hypothesis of inhibition by metal chelation, Wakabayashi *et al.* (1997) developed wood preservatives containing troponoid metal complexes.

An α-thujaplicin-Cu^{2+} complex at 200ppm inhibited the lignin-decomposing fungus *Coriolus versicolor*, and the cellulose-decomposing fungus *Tyromyces palustris*. It was effective at inhibiting fungal growth and was non-toxic to humans and showed good fixation to wood. Given the strong and broad-spectrum of antimicrobial activity (Sakai, 2004), and the many other biological activities of tropolone compounds, there is an increasing demand for these compounds in the industry (Sakai, 2004).

1.5 Weathering of WRC Products

WRC products are utilized heavily for exterior residential applications given their natural durability and offer an alternative perceived as more environmentally friendly than wood products treated with biocides such as CCA (chromated copper arsenate). However, exposure to weathering can cause depletion of extractives which confer durability. Several fungicidal tropolone extractive compounds are prone to photo-degradation caused by ultra-violet (UV) radiation from sunlight. Coombs and Trust (1973) observed that exposure of aqueous solutions of β-thujaplicin to UV radiation led to a measurable loss of antibacterial activity. This compound is known to have a maximum absorption peak at 244 nm with smaller absorption peaks in the near-UV region (280-380 nm). They noted that photochemical decomposition of β-thujaplicin was found to occur following shortwave irradiation (210-280 nm) and that this possibly caused a $\pi \rightarrow \pi^*$ electronic transition. A longer wavelength source (280-380 nm) possibly caused an $n \rightarrow \pi^*$ electron transition. Shibata *et al.* (2003) noted similar photochemical decomposition patterns of β-thujaplicin but also suggested that during this degradation process a reactive oxygen species was generated that also had some bactericidal properties. The possible decomposition product under laboratory light has been suggested to be isopropyl-substituted 4-oxo-cyclopentane-1-acetic acid.

In addition to the reported photo-instability of WRC tropolone extractives, lignin is also susceptible to photo-degradation whereas pure cellulose and hemicelluloses absorb little UV (Crestini, 1996; Chang, 2002). Oxidative cleavage of Cα-Cβ and β-O-4 bonds that link lignin precursor guaiacyl phenylpropanoid molecules together in softwoods is known to occur as a result of UV exposure. A progressive destruction of lignin aromaticity, demethoxylation and the formation of carboxyl groups, soluble carbohydrates and lignin fragments have also been observed following irradiation of wood, pulp and paper (Chang, 2002). This is relevant for certain black staining fungal species such as *Aureobasidium pullulans* which has been reported to colonize the surface of wood products exposed to weathering (including WRC) and utilize lignin photo-degradation products as a carbon source (Dickinson, 1972; Bourbonnais and Paice, 1987; Sharpe and Dickinson, 1992a, 1993; Schoeman and Dickinson, 1997).

However, UV radiation from sunlight only penetrates the upper 1mm of exposed surfaces (Hon, 1991) and may have a limited effect on the total extractive content of WRC products. Leaching of extractives by rain, which penetrates deep, is more likely to affect extractive concentration, especially considering that the majority of WRC extractives are water soluble (Barton and MacDonald, 1971). Johnson and Cserjesi (1980) investigated the depletion of β- and γ-thujaplicin in WRC shakes exposed to natural weather conditions in Vancouver, British Columbia, Canada. They established that a one year exposure resulted in a 25% depletion of β- and γ-thujaplicin. This increased to 90% after three years. Although this may have been due in-part to biodegradation. Chedgy *et al.* (2005) monitored the thujaplicin content of WRC siding exposed to various simulated weathering treatments using a Weather-Ometer® and reported ~47% decrease in thujaplicins following UV weathering, and a 100% loss following water spray, and water spray and UV combined. This suggested that leaching of extractives may lead to

depletion of fungicidal extractives which may leave WRC products susceptible to colonization by decay and staining fungi leading to a potentially reduced service life.

1.6 Fungal Colonization and Decay of WRC Products

Lim *et al.* (2005) investigated the fungal community inhabiting in-service WRC fence material with a focus on species colonizing wood below the surface. They reported twenty-three different fungal species which included thirteen ascomycetous and ten basidiomycetous fungi. They tested isolates for their resistance to β-thujaplicin – one of the principle fungicidal agents of WRC heartwood extractives. Generally, ascomycetous fungi exhibited greater resistance to β-thujaplicin than basidiomycetous fungi. Interestingly, three soft-rot ascomycetous species, *Oidiodendron* sp., *Phialophora fastigiata* and *Phialophora* sp. 3, and two basidiomycetous species, *Pachnocybe ferruginea* and *Acanthophysium lividocaeruleum*, were frequently isolated and had high tolerance to this compound. The researchers concluded that these species could be 'pioneer' species that invade and detoxify WRC extractives, paving the way for colonization by brown-rot and white-rot fungi.

Extractive content is often used to predict service life of WRC products although little literature focuses on the relationship between extractive content and rate of decay of WRC. Wood product manufacture increasingly relies on second growth trees that typically contain 6.2-9.8% total extractive by weight, compared with 11.4-22.8% in old growth trees (Barton and MacDonald, 1971; Nault, 1988). This may lead to potentially less durable WRC wood products given the lower extractive content in second growth WRC. This issue was explored by Freitag and Morrell (2001) who examined the durability of the changing WRC stock. In standard soil block tests

using the decay fungus *Postia placenta*, they compared the durability of WRC blocks from young and older trees. They concluded that, in fact, the durability of younger WRC material has not changed from that of older stocks. Despite these encouraging observations, a greater understanding of the chemical nature of heartwood extractives and their ability to confer natural durability is imperative in order to develop systems that may enhance product service life in the future.

1.7 Biosynthesis of WRC Extractives

The major proportion of WRC extractives are formed *in situ* at the sapwood-heartwood boundary in phloem parenchyma cells and are carried to the heartwood via ray parenchyma cells (Swan and Jiang, 1970). Cedar heartwood is red-brown in color whereas the sapwood is a pale yellow, off-white color. Krahmer and Côté (1963) conducted electron microscopy photography of WRC tracheids; they noted that heartwood tracheids were heavily encrusted with extractives, whereas those in the sapwood were devoid of extractives. Across the sapwood-heartwood transition, the concentration of most extractive compounds increase by a factor of 100 times (Swan and Jiang, 1970). Metabolites are released into the heartwood from specialized ray parenchyma cells *via* pit apertures into the lumen of adjacent, dead (lignified) cells and then diffuse into neighboring, pre-lignified cells (tracheids) (Figure 1.2). It is reported that further transformation of lignans and other extractive components continues well after their release into the heartwood, beyond the visible heartwood-sapwood

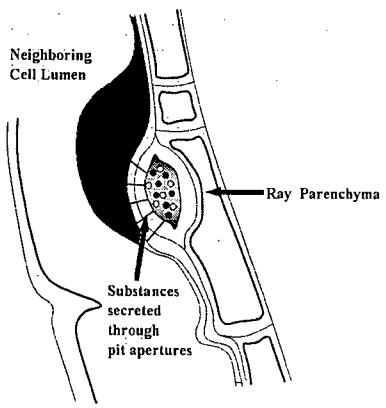


Figure 1.2 Secretion of heartwood constituents by ray parenchyma cells into the lumen of neighboring cells appears to occur through pit apertures (Krahmer and Côté (1963).

boundary, and that this process can continue for many years (Swan et al., 1969; Swan and Jiang, 1970).

WRC extractives are classed as terpenoids which are multicyclic structures, derived by repetitive fusion of a branched five carbon monomer termed isoprene which can polymerize further. Biosynthesis of the fundamental terpenoid precursor isopentenyl diphosphate (IPP) occurs principally via the acetate/mevalonate (MVA) pathway active in the cytosol and endoplasmic reticulum of cells, and via the 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathway operating in the plastids (Lichtenthaler, 1999). Following condensation of IPP monomers, the action of terpene synthases (TPS) leads to the formation of geranyl diphosphate (GPP) → monoterpenes (C_{10}) (volatile); IPP → farnesyl diphosphate (FPP) → sesquiterpenes (C_{15}) (volatile); and IPP → geranyl geranyl diphosphate (GGPP) → Diterpenes (C_{20}) and so on (Croteau *et al.*, 2000; Croteau and Johnson, 1985). All of these pyrophosphates are the common precursors for all monoterpenes and their derivatives. These precursor pools and various terpenoid products markedly increase in plant response to biotic or abiotic stresses (Bohlmann *et al.*, 1998).

1.7.1 Tropolone Biosynthesis

Among the range of secondary metabolites present in WRC are tropolones such as β-thujaplicin which are major phytoalexins - metabolites produced by a plant in response to infection by a fungus or other pathogens, or by abiotic factors such as wounding. Several lines of evidence show that biosynthesis is regulated by wounding, microorganism elicitor molecules, by methyl jasmonate (MeJA) and oxidative stress (Yin *et al.*, 1997; Steele *et al.*, 1998; Mandujano-Chavez *et al.*, 2000; Martin *et al.* 2002; Zhao *et al.* 2004a, 2004b, 2005a, 2005b). Cell cultures from

several Cupressaceae trees such as mexican cypress (*Cupressus lusitanica*), Florin (*Calocedrus formosana*) and white cedar (*Thuja occidentalis*) can produce β-thujaplicin and a number of monoterpenes (Witte *et al.*, 1983; Ono *et al.*, 1998; Matsunga *et al.*, 2003). They have been used to explore the possible biosynthetic pathways involved in tropolone production because they are easy to manipulate in the laboratory environment. Sakai *et al.* (1997) demonstrated that β-thujaplicin is synthesized via the MVA pathway using C^{14} radioactive β-thujaplicin precursors as well as by selective inhibition of one of the key regulatory enzymes in the mevalonate pathway, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. This enzyme inhibition resulted in significant suppression of β-thujaplicin synthesis. Alternatively, Fujita *et al.* (2000), also working with *Cupressus lusitanica*, demonstrated in tracer experiments that geraniol and glucose were efficiently incorporated into β-thujaplicin via the MEP pathway through an intermediate with menthane-type skeleton. This suggests that MEP pathway, rather than MVA pathway, acts as a main synthesis pathways for β-thujaplicin, although a MVA pathways also contributes a minor portion of β-thujaplicin biosynthesis (Yamaguchi *et al.*, 1997). To date, these pathways are speculative, and enzymes involved in the biosynthesis of tropolones have yet to be characterized.

A biosynthetic pathway for tropolones in the Cupressaceae family was proposed by Lai (1971). These pathways are illustrated in Figure 1.3. The pathways shown are only those relevant to WRC. The initial step in the pathway is the condensation of acetyl CoA (*A*) and α-ketoglutaric acid (*B*) to produce homocitric acid (*C*). Both of these starting materials are available from the metabolic pool and the compound acetyl CoA is a common precursor of the MVA pathway. Homocitric acid is then converted into β-ketoadipic acid (*E*) by subsequent decarboxylation and oxidation. Further isopentenylation of β-ketoadipic acid produces the intermediate (*F*) which

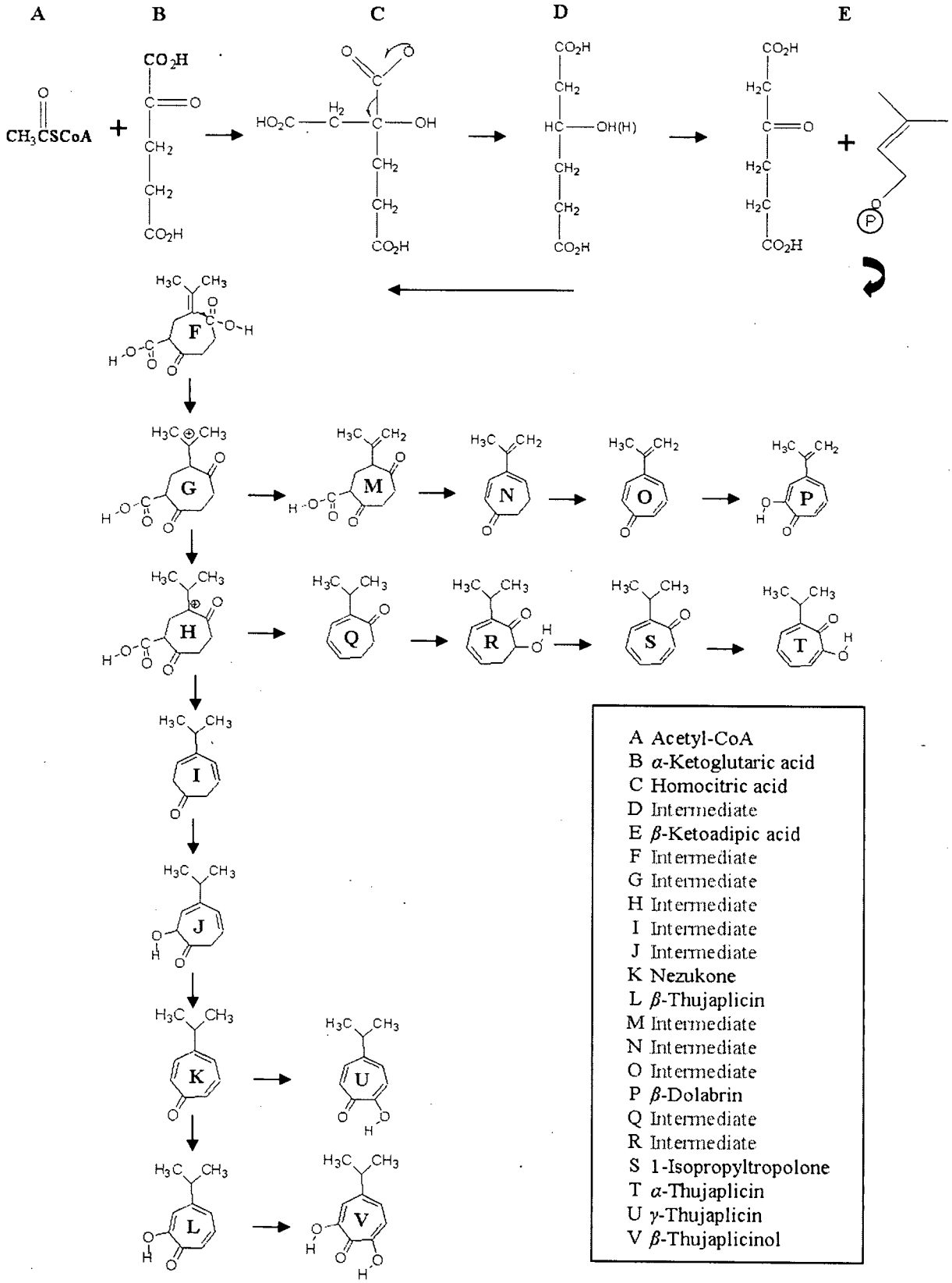


Figure 1.3 The proposed biosynthetic pathway of tropolones in WRC (Lai, 1971)

undergoes several reactions that include cyclization followed by carbonium ion rearrangement, and elimination of a hydrogen ion, reduction, dehydration, oxidative hydroxylation and finally, dehydration reactions to form the compound nezukone (*K*). Synthesis of the tropolone α-thujaplicin (*T*) is initiated from an intermediate compound (*H*), which is present in the pathways leading up to the formation of nezukone. This compound also undergoes cyclization followed by similar reactions as described above to form 1-isopropyltropolone (*S*). Compound (*S*) then undergoes a simple hydroxylation to form α-thujaplicin (*T*). The remaining thujaplicins can be synthesized from nezukone (*K*). Nezukone undergoes hydroxylation to form γ-thujaplicin (*U*), and β-thujaplicin (*L*), a hypothesis also supported by Swan *et al.* (1969). The tropolone β-thujaplicinol (*V*) is derived from β-thujaplicin by further hydroxylation, isopentenylation, hydration, and methylation processes. Lai (1971) also postulated that β-dolabrin (*P*), which is a tropolone with an unsaturated side chain, is synthesized from intermediate compound (*G*) shown in the diagram. This can undergo a direct elimination of a hydrogen ion and a series of reactions as described above to produce β-dolabrin. Again, the enzymes involved in the biosynthetic pathway have yet to be established, and these are speculative pathways.

In WRC, nezukone is a precursor of the thujaplicins, and hydroxylation is required for this to occur. The hydroxylation mechanism is perhaps by the direct reaction of the precursor with oxygen as suggested by Frey-Wyssling and Bossard (1959) who studied the cytology of the ray cells in the sapwood and heartwood of 12 tree species. They concluded that the transition between sapwood and heartwood was characterized by a semi-anaerobic metabolism. In the heartwood, a slow oxidation and polymerization of extractives occurred by direct reaction with oxygen. Such oxidations may account for the high concentrations of thujaplicins in the outer heartwood, and may explain the decrease in extractives that is observed in mature heartwood

closer to the pith, where conditions become increasingly anaerobic. It is also probable that this observed decrease in extractives from the outer heartwood to the pith is due to lower amounts of extractives laid down in the juvenile tree and biodegradation of extractives over the long natural life of WRC trees (MacLean and Gardner, 1956; Nault, 1988).

Little is known about the enzymes and genes that are involved in β-thujaplicin biosynthesis. Recent work also suggests that both methylation and oxidation of β-thujaplicin are responsible for β-thujaplicin transformation in *C. lusitanica* cell cultures, since a certain level of β-thujaplicin is toxic to plant cells (Yamada *et al.*, 2002; Zhao and Sakai, 2003a, 2003b). All these data show that biosynthesis of β-thujaplicin in *C. lusitanica* cell culture is highly regulated and that its metabolism is also tightly controlled. Figure 1.4 illustrates a proposed biosynthetic pathway for monoterpenes including β-thujaplicin in *C. lusitanica* (taken from Zhao *et al.*, 2006).

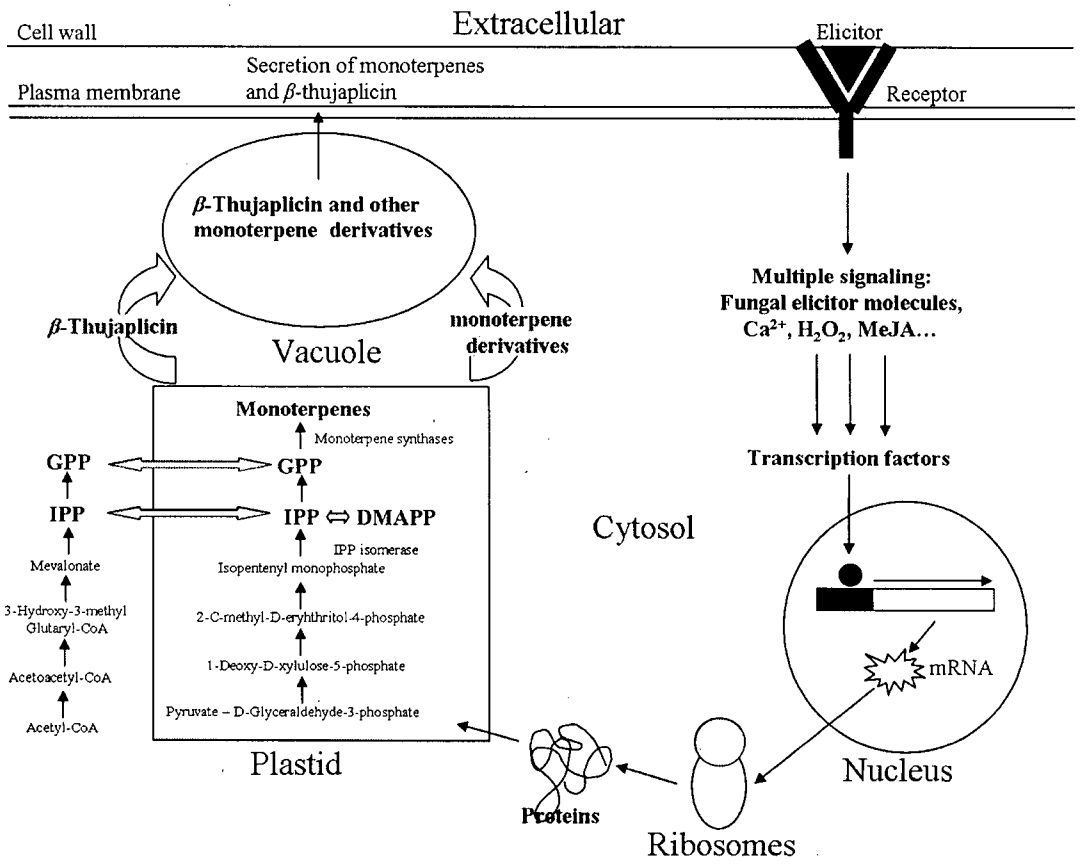


Figure 1.4 Schematic illustration of signal transduction and metabolic flux of elicitor-induced β-thujaplicin and other monoterpenes in *Cupressus lusitanica* cell cultures (Zhao *et al.* 2006).

Note: Multiple elicitor signals are incorporated into transcriptional factors, which selectively activate or inactivate metabolic gene expression and downstream metabolic pathways. β-Thujaplicin biosynthesis involves two separate pathways and compartmentation regulation by the plasmid, cytosol, and vacuole. Transport of metabolic substrates and products across through these compartments may require different transporters, which could also be regulated by elicitors. Taken from Zhao *et al.* (2006).

1.7.2 Lignan Biosynthesis

Lignan formation and accumulation differs profoundly from that leading to lignins. First, lignans are transported through specialized cells (such as ray parenchyma) and are infused into surrounding pre-lignified cells. Second, they are formed via distinct biochemical pathways. In contrast, lignification results *via* direct monomer transport from the cytoplasm of a lignifying cell into its polysaccharide-rich cell wall with subsequent polymerization. This process represents the first and final committed step of lignification, being primarily initiated and completed in maturing cell walls not far from the cambial zone.

Gang *et al.* (1998) reviewed lignan biosynthesis in several softwood species including cedar (Figure 1.5). Much of the initial work on the lignan biosynthetic pathway had been done using *Forsythia intermedia*, also known as 'Border Forsythia'. It was suggested that the same pathway must also operate in the WRC heartwood metabolite forming process, and that these precursors undergo further transformation to give lignans such as plicatic acid. In cedar, coniferyl alcohol serves as the initial precursor, being subsequently metabolized with precise regio- and stereochemical control to produce the first species-specific lignans. Two monoligols of *E*-coniferyl alcohols are coupled *via* a dirigent protein to form the furofuran lignan (+)-pinoresinol. This represents the entry point into the pathway. (+)-Pinoresinol is subsequently reduced, first to the tetrahydrofuran, (+)-lariciresinol, and then to the dibenzylbutane lignan, (-)-secoisolariciresinol. This subsequently undergoes a two-step dehydrogenation to form the dibenzylbutyrolactone lignan, (-)-matairesinol. These precursors can then be further transformed to give the lignan (-)-plicatic acid (Davin and Lewis, 1995; Davin *et al.*, 1997).

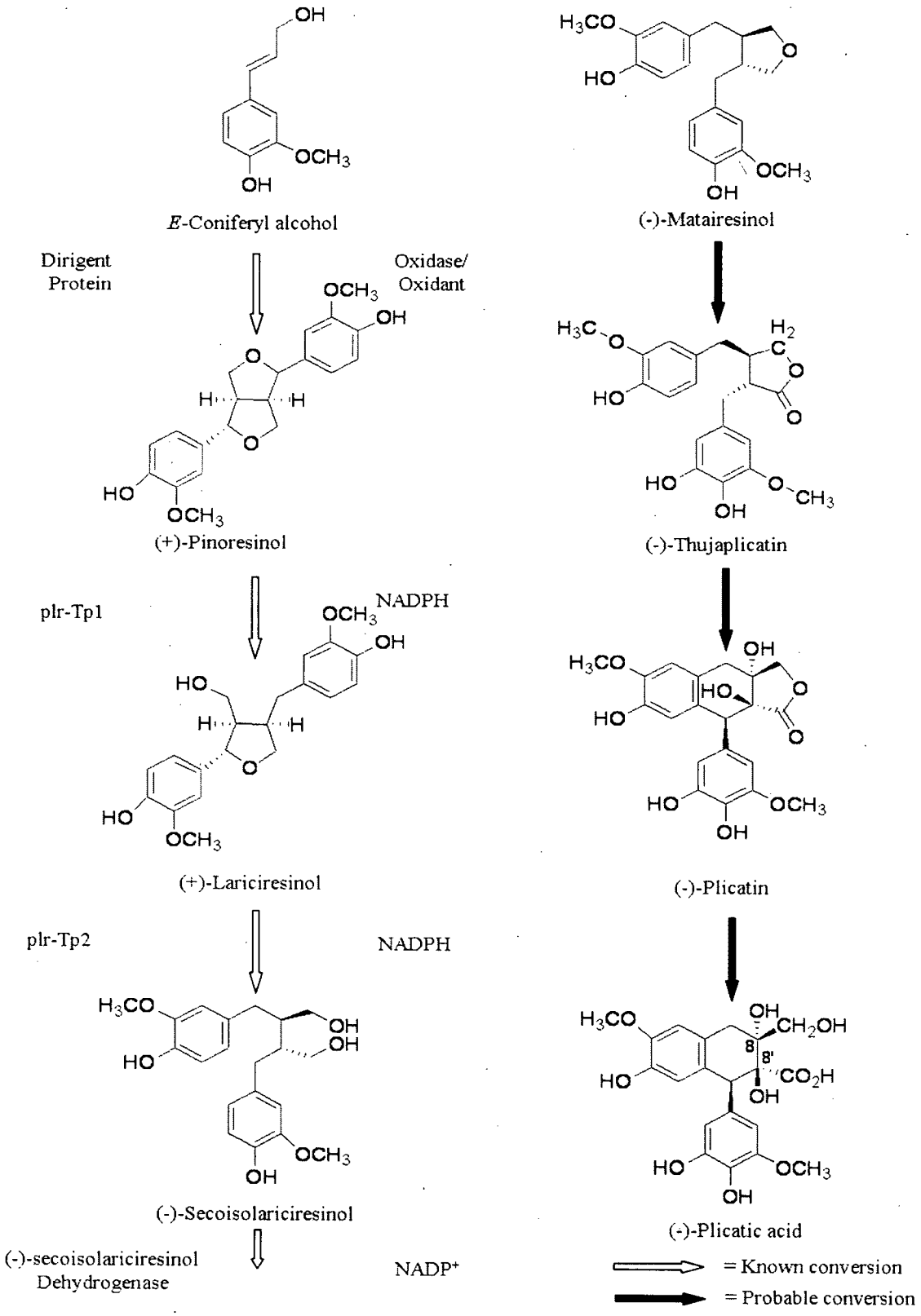


Figure 1.5 The proposed biosynthetic pathway of plicatic acid (Gang *et al.*, 1998)

Swan and Jiang (1970) compared the sapwood and heartwood phenolic extractive content. They estimated the concentration of individual extractive compounds for each growth ring along a transect spanning from the outer sapwood to the pith of two WRC trees. From this data they postulated a biosynthetic pathway. Their first observation was that the concentrations of most extractive compounds increased by a factor of around 100 times across the sapwood to heartwood boundary. In the sapwood region the thujaplicatin methyl ethers (T.M.E.s) had the highest concentration of all compounds at approximately 0.1 % DW. They noted that T.M.E. concentration only increased by an order of 7-10 times into the heartwood yielding an average of 0.73% DW. However, there is a much greater increase in (-)-thujaplicatin concentration from sapwood to heartwood reaching a level 1.44 % DW. They also noted that the thujaplicins were almost nonexistent in the sapwood, but become rapidly more abundant in the heartwood, reaching approximately 0.2 % DW. They concluded that in the heartwood, lignans are formed in the sequence of (-)-thujaplicatin to (-)-dihydroxythujaplicatin to (-)-plicatin to (-)-plicatic acid by a series of hydroxylation reactions. However, in the sapwood, the starting material thujaplicatin is preferably converted into T.M.E's by *O*-methylation. It seems that thujaplicins in the heartwood act as enzyme inhibitors that prevent *O*-methylation, thereby promoting hydroxylation of (-)-thujaplicatin leading to the formation of (-)-plicatic acid rather than the T.M.E.s. Swan and Jiang's proposed biosynthetic pathways are shown in Figure 1.6. The rapid decrease in (-)-plicatic acid content toward the pith after its maximum content in the outer heartwood is reached might be attributed either to the lesser ability of the tree to synthesize it when younger, or to its self-polymerization due to its strongly acidic nature, or to further oxidation of it to other lignans.

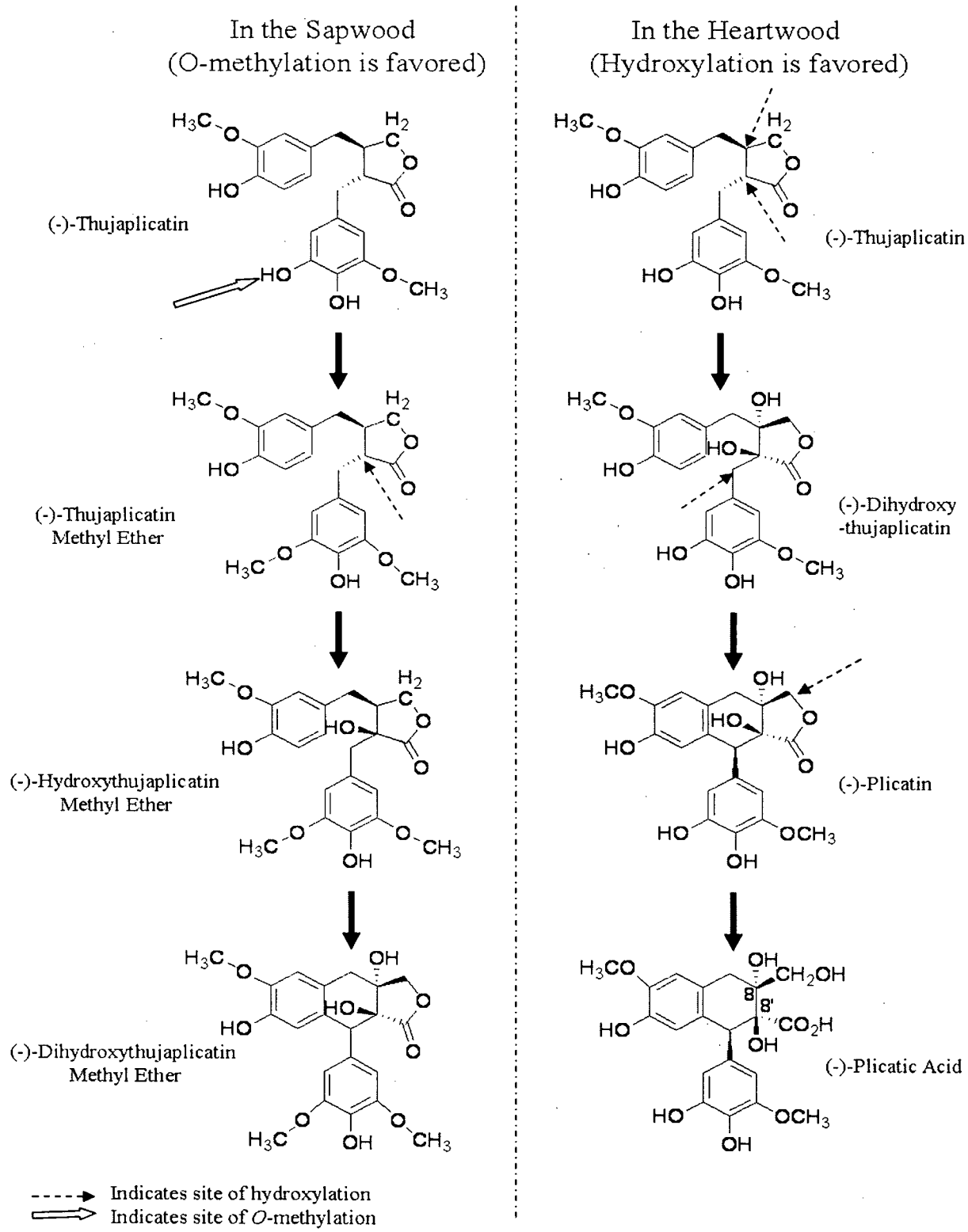


Figure 1.6 The proposed lignan biosynthesis pathways in the sapwood vs. the heartwood of WRC (Swan & Jiang, 1970)

Given the biological activity of several of the extractive compounds of WRC there is an increasing demand for them in an ever-growing natural products industry. Their antimicrobial activity may play an important physiological role in protecting standing WRC trees from pathogen invasion and subsequently confer durability to WRC wood products. In the forest products sector, use of naturally durable woods such as WRC offer a perceived environmentally friendly alternative to biocide treated products. Presently, the high production costs of compounds such as β-thujaplicin from callus cell cultures prevent their large scale application to non-durable woods such as pine but this may be a possibility if such tropolones can be artificially synthesized on an industrial scale. Such production may be driven by the fact that such compounds also have potential pharmaceutical applications that range from UV skin protection, to anti-cancer and anti-HIV treatments. Given the small molecular size of tropolone compounds such as β-thujaplicin in the field of cancer treatments may offer an attractive alternative to expensive, present day treatments such as Piclataxel, know commercially as 'Taxol', a large, complex molecule extracted from *Taxus cuspidate*, of the genus Taxus (Yews). In short, the extractives of WRC offer several intriguing prospects in the industries of wood protection, pharmaceuticals and nutraceuticals for the future.

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1.9 Objectives

The overall goal of this work was to provide information on the relationship between durability, the change in extractive content, and microorganisms on WRC products in service in order to help the industry to develop strategies to improve the service life of WRC products.

The main objective was to determine whether depletion or modification of natural extractives by microorganisms or weathering can explain the premature failure of WRC products

I will test the following hypotheses:

(a) Extractives are chemically modified / detoxified by extractive tolerant, 'pioneer' micro-organisms, paving the way for decay to occur.

(b) Extractives are depleted by weathering making products susceptible to decay.

(c) A combination of (a) and (b).

In reference to hypothesis (a), given the time limitations of this work, the primary focus was on examining extractive-tolerance of micro-organisms as being indicative that chemical modification of extractives may occur. Exploring the possible extractive detoxification mechanisms by micro-organisms will be examined in future work.

Chapter 2

Extracting and Quantifying Heartwood Extractives of Western Redcedar (*Thuja plicata* Donn) Using Ultrasonication and Reverse Phase HPLC

2.1 Abstract

Western redcedar (*Thuja plicata* Donn) (WRC) heartwood samples were extracted in methanol and reverse phase high performance liquid chromatography (HPLC) with an ultraviolet (UV) detection system was used for extractive separation and analysis. Six major extractives were quantified by comparing analyte response with the response factor of an internal standard by a single point calibration. The method's limits of detection were 0.6μg/ml for (-)-plicatic acid, 3.0μg/ml for γ-thujaplicin, 3.0μg/ml for β-thujaplicin, 3.0μg/ml for β-thujaplicinol, 0.6μg/ml for thujic acid and 1.2μg/ml for methyl thujate. Yields were 36% higher for powdered samples than for sliced samples. A temperature of 4°C during ultrasonication yielded 16% more (-)-plicatic acid than in non-cooled extractions but did not significantly increase yields for the remaining five compounds. We assessed the recovery and repeatability of the extraction method by adding the aromatic compounds methoxyhydroquinone and 2-acetonaphthone to heartwood samples. Recovery yield was ~90% with ~5% variability.

Key words: extractives; high performance liquid chromatograpghy; lignan; quantification; tropolone; western redcedar (*Thuja plicata* Donn).

2.2 Introduction

Western redcedar (*Thuja plicata* Donn) (WRC) is a naturally durable softwood species native to British Columbia in Canada as well as in Washington, Oregon and California in the USA. Its heartwood extractives are comprised of a mixture of tropolones (2-hydroxy-2,4,6-cycloheptatrien-1-one) (Dewar, 1945) and lignan (dimeric, phenolic metabolites) compounds (Gang *et al.*, 1998) with high commercial value (Barton and Macdonald, 1971; Jones and Falk, 2005). Several of these extractives, namely the 'thujaplicins' (α-, β-, and γ-thujaplicin) exhibit strong antimicrobial, insecticidal, and herbicidal activities with efficiency comparable to commercial pest control agents (Inamori *et al.*, 1999, 2000; Sakagami *et al.*, 2000; Arima *et al.*, 2003; Morita *et al.*, 2004). These and other structurally similar compounds also have cytotoxic activity against cancer cell lines *in vitro* (Baba *et al.*, 1998; Matsumura *et al.*, 2001; Inamori *et al.*, 2003, 2004) and can inhibit human immunodeficiency virus (HIV) replication (Budihas *et al.*, 2005).

To date, only β-thujaplicin is synthesized on a large scale using callus cultures of species such as Mexican cypress (*Cupressus lusitanica*). The remaining compounds are still obtained by extracting heartwood of WRC and other members of the Cupressaceae family. Formerly, wood extraction has been achieved using hot water or steam distillation (Barton and MacDonald, 1971; Mitsuhiko *et al.* 2002), soxhlet apparatus with organic solvents (Johansson *et al.*, 2000), or supercritical fluid extraction using carbon dioxide (Terauchi *et al.*, 1993; Ohira *et al.*, 1994, 1996; Eller and King, 2000). These methods are performed at temperatures ranging from 40-100°C. Other quantitative methods have been developed for analyzing WRC wood and foliage extractives using gas chromatograpghy (Nault, 1987; Kimball *et al.*, 2005) that inherently require temperatures in the range of 100-300°C for the volatilization of analytes. However,

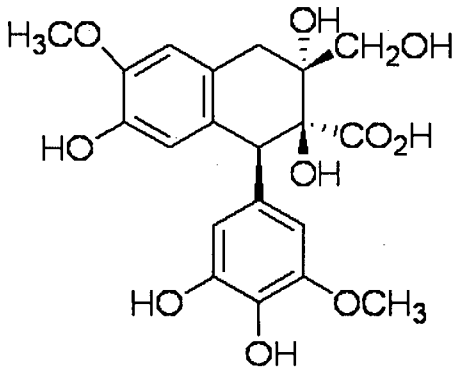
several of the compounds of interest such as (-)-plicatic acid, γ- and β-thujaplicin are thermo- or photo-sensitive (Coombs and Trust, 1973; Shibata *et al.*, 2003).

We describe a method to extract and quantify six major WRC extractives without exposing them to excess heat. We used reverse phase HPLC to separate extractives. Using extractive standards and an ultraviolet (UV) detection system we constructed calibration equations for quantifying six relevant extractives: (-)-plicatic acid, γ-thujaplicin, β-thujaplicin, β-thujaplicinol, thujic acid, and methyl thujate (Figure 2.1). We tested the effect of a) sample wood preparation and b) temperature on extractive yield using a methanol extraction by ultrasonication. Finally, we evaluated how method parameters affect extractive detection, recovery, and repeatability.

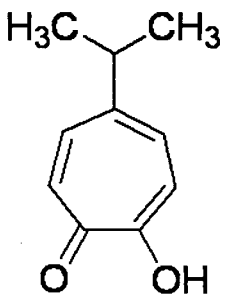
2.3 Materials and Methods

2.3.1 Chemicals and Solvents

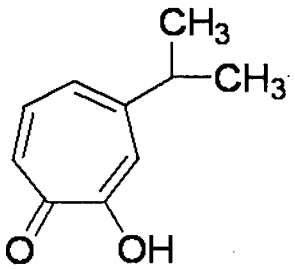
HPLC grade methanol and acetonitrile were purchased from the Fisher Scientific Company (Nepean, ON, Canada). Reagents/standards ethyl 4-hydroxy 3-methoxycinnamate (99% pure), methoxyhydroquinone (99% pure), 2-acetonaphthone (99% pure) and β-thujaplicin (99 % pure) were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). γ-Thujaplicin (96% pure), β-thujaplicinol (96% pure), and methyl thujate (96% pure) were provided by Forintek Canada Corp. (Vancouver, British Columbia, Canada). (-)-Plicatic acid (96% pure) and thujic acid (96% pure) were supplied by Xylon Biotechnologies Ltd., (Vancouver, British Columbia, Canada). All solvents and compounds were used as received.



2,3,6-Trihydroxy-7-methoxy2hydroxymethyl -4-(3',4'-dihydroxy-5'-methoxyphenyl)-tetralin-3-carboxylic acid
(-)-Plicatic Acid



2-Hydroxy-5-isopropyl-2,4,6-cycloheptatrien-1-one
***γ*-Thujaplicin**



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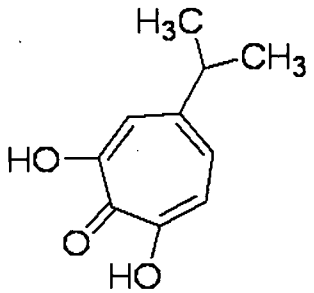
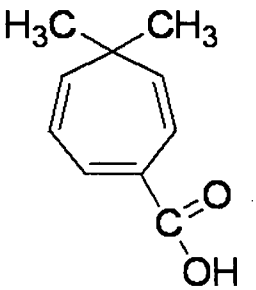
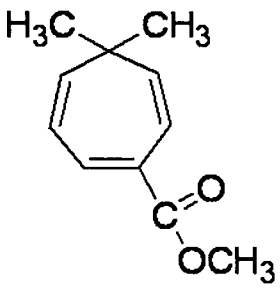
***β*-Thujaplicin**



2,7-Dihydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one
***β*-Thujaplicinol**



7,7-Dimethylcycloheptatriene-3-carboxylic acid
Thujic Acid



7,7-Dimethyl-3-carbomethoxy-1,3,5-cycloheptatriene
Methyl Thujate

Figure 2.1 Chemical structure and nomenclature used for WRC heartwood extractive compounds of interest.

2.3.2 Separation of Extractives by HPLC

Extractive compounds were separated by reverse phase HPLC as described in Daniels and Russell (2006) using a Waters 2695 HPLC from Waters Corp. (Mississauga, Ontario, Canada) equipped with a ODS3 C-18 reverse phase column (3μm, 4.6mm x 150mm) from Intersil Corp. (Milpitas, California, USA) and a Dionex AD20 ultraviolet (UV) detector from Dionex Corp. (Oakville, Ontario, Canada). MassLynx analytical software from Micromass Ltd (Manchester, UK) was utilized for chromatographic analysis. Mixed extracts were injected (15μl) on to the column and readily separated using the method described in Table 2.1.

2.3.3 Confirmation of Extractive Chemical Structures by NMR

The molecular structures of selected extractives were confirmed from pure, crystalline standards using nuclear magnetic resonance (NMR). ^{1}H, and ^{13}C NMR spectra were measured using a Bruker AVANCE-300 spectrometer from Bruker (Billerica, Massachusetts, USA) at 40 °C in either MeOD (deuterated methanol) or DMSO-d^6 (deuterated dimethyl sulfoxide). Chemical shifts were referenced to tetramethyl silane (TMS; 0.0 ppm), summary in Table 2.2. NMR data is consistent with structures reported in literature sources (Erdtman and Gripenberg, 1948; Barton and Gardner, 1954; Gripenberg, 1956; Gardner *et al.*, 1957, 1966). A mixed extract sample was then spiked with each of the standards and subject to HPLC analysis in order to confirm which peak corresponded to which extractive compound.

2.3.4 Chromatographic Sensitivity and Quantification of Extractives

Pure crystalline extractive standards were used to estimate the method limit of detection (MLOD) for the six extractives. MLOD was defined as the concentration of analyte (μg/ml) required to produce a chromatographic signal equal to three times the peak-to-peak noise. A

Table 2.1 HPLC program for the separation of WRC heartwood extractives (Daniels and Russell, 2006).

Time (min)	Solvent A (%)	Solvent B (%)	Flow Rate (ml/min)
0	10	90	1
50	60	40	1
60	10	90	1.5
62	10	90	1

Note: solvent A = 0.1% formic acid, 99.9% nano pure H_20. Solvent B = 99.9% acetonitrile with 0.1% formic acid. Column temperature was 50°C, and a UV wavelength of 230nm was used. A total elution was typically reached at 48 min.

Table 2.2 A Summary of NMR Data for the Extractives of Interest.

Compound	NMR Data
(-)-Plicatic acid	^{1}H NMR (DMSO-d^6, 300 MHz): δ = 2.75 (d, J = 6.9 Hz, 2H, CH_2), 3.44 (s, 2H, CH_2OH), 3.70 (s, 6H, OCH_3), 4.30 (s, 1H, CH), 6.06 (s, 1H, CH), 6.21 (s, 1H, CH), 6.30 (s, 1H, CH), 6.56 (s, 1H, CH). ^{13}C NMR (DMSO-d^6, 75.4 MHz): δ = 19.0, 34.62, 56.1, 56.5, 62.2, 93.2, 97.8, 102.4, 112.8, 116.4, 126.1, 132.1, 132.9, 144.1, 144.7, 145.9, 147.4, 177.5.
γ-Thujaplicin	^{1}H NMR (MeOD, 300 MHz): δ = 1.26 (d, J = 6.9 Hz, 6H, CH_3), 2.93 (q, J = 6.9 Hz, 1H, CH), 7.32 (d, J = 11.7 Hz, 2H, CH), 7.45 (d, J = 11.7 Hz, 2H, CH). ^{13}C NMR (MeOD, 75.4 MHz): δ = 22.5(2C), 37.5 (1C), 124.9 (2C), 136.5 (2C), 150.0 (1C), 171.0 (2C).
β-Thujaplicin	^{1}H NMR (MeOD, 300 MHz): δ = 1.27 (d, J = 6.9 Hz, 6H, CH_3), 2.93 (q, J = 6.9 Hz, 1H, CH), 7.08 (d, J = 10.2 Hz, 1H, CH), 7.22 (d, J = 10.8 Hz, 1H, CH), 7.33 (s, 1H, CH), 7.43 (t, J = 10.5 Hz, 1H, CH). ^{13}C NMR (MeOD, 75.4 MHz): δ = 22.3, 38.7, 122.7, 123.7, 127.7, 137.5, 160.4, 171.6.
β-Thujaplicinol	^{1}H NMR (MeOD, 300 MHz): δ = 1.26 (d, J = 6.9 Hz, 6H, CH_3), 2.92 (q, J = 6.9 Hz, 1H, CH), 7.12 (dd, J = 10.8, 1.5 Hz, 1H, CH), 7.39 (d, J = 11.1 Hz, 1H, CH), 7.43 (d, J = 1.5 Hz, 1H, CH). ^{13}C NMR (MeOD, 75.4 MHz): δ = 22.7, 38.1, 120.6, 121.5, 127.2, 151.2, 158.8, 160.6, 166.9.
Thujic Acid	^{1}H NMR (MeOD, 300 MHz): δ = 1.01 (s, 6H, CH_3), 5.25 (d, J = 10.5 Hz, 1H, CH), 5.49 (d, J = 9.9 Hz, 1H, CH), 6.28 (dd, J = 9.9, 6.9 Hz, 1H, CH), 6.67 (d, J = 10.2 Hz, 1H, CH), 7.56 (d, J = 6.9 Hz, 1H, CH). ^{13}C NMR (MeOD, 75.4 MHz): δ = 25.2, 34.7, 122.8, 123.2, 131.7, 132.1, 135.5, 138.3, 169.4.
Methyl thujate	^{1}H NMR (MeOD, 300 MHz): δ = 1.00 (s, 6H, CH_3), 3.81 (s, 3H, OCH_3), 5.24 (d, J = 10.2 Hz, 1H, CH), 5.49 (d, J = 10.2 Hz, 1H, CH), 6.27 (dd, J = 9.9, 6.9 Hz, 1H, CH), 6.66 (d, J = 10.2 Hz, 1H, CH), 7.54 (d, J = 6.6 Hz, 1H, CH). ^{13}C NMR (MeOD, 75.4 MHz): δ = 25.5, 34.7, 51.2, 122.5, 123.2, 131.3, 132.2, 135.4, 138.3, 168.0.

1mg/ml stock solution prepared for each standard in methanol was diluted to make solutions of 5, 4, 3, 2, 1μg/ml. Following chromatographic analysis an additional set of solutions was prepared with concentration points of smaller, more directed increments until the MLOD was estimated to within 0.1 μg/ml.

Calibration equations were constructed relative to the internal standard ethyl 4-hydroxy 3-methoxycinnamate whish possesses a chromatographic retention time (t_R) proximal to extractives of interest and its t_R does not overlap with extractive peaks. From stock standard solutions, a six-point concentration gradient was prepared that reflected the natural abundance of each compound in heartwood; the lowest point was the MLOD in each case. To the six solutions the IS was added at a concentration of 100μg/ml. Then six additional solutions with the compound of interest at 100μg/ml were spiked with the IS at the same concentrations. From these data a twelve-point scatter plot was constructed, where the y axis was defined as area ratio (extractive peak area divided by IS peak area); and the x axis as concentration (μg/ml) ratio ([extractive] divided by [IS]). This process was repeated three times and a scatter plot was constructed from the mean data obtained. The detector responses for each compound were subjected to linear regression analysis, and response factors were calculated. Statistical analysis was performed of the response factors to distinguish between significantly different values.

2.3.5 Sample Preparation and Optimization of Extraction

WRC cubes of 19mm on each dimension were manufactured from the outer heartwood of a sound 136yr old second growth WRC tree felled in the UBC Malcolm Knapp research forest, Maple Ridge, British Columbia, Canada. Samples originated from a single longitudinal axis parallel with growth rings. Blocks were sequentially numbered relative to their position. Even-

numbered blocks were utilized for chemical analysis. Odd-numbered blocks were oven dried at 105°C for 24 hours to estimate the dry weight (DW) of the neighboring even-numbered blocks. Each sample was typically 2.0 ± 0.2g DW.

Of twenty four samples, eight were sliced as thinly as possible; slices were typically 2mm in width. Sixteen samples were ground to a fine powder (<150 microns) using a freezer mill equipped with a coolant circulation chamber from Bel-art products (Pequannock, New Jersey, USA). A temperature of ~ 4°C was maintained during grinding to avoid generation of excess heat caused by friction. WRC Samples were steeped in 15ml methanol and exposed to ultrasonic frequency (40 kHz) for 120 minutes using a Branson 8510 ultrasonic bath (Danbury, Connecticut, USA). Samples were extracted once only in each case. Eight ground samples and eight sliced samples were extracted in a 4°C bath with a lid and cooled with ice. Another eight ground samples were extracted in a non-cooled bath. Extracts were filtered using a 0.2µm nylon syringe filter to remove any wood particles and stored at 4°C in the dark. Typically 12ml of filtered extract was recovered from each sample. This experiment followed a completely randomized design (CRD) with three treatments: grinding (cooled), slicing (cooled), and grinding (non-cooled) ($k = 3$). Eight replicate samples were used in each case ($n = 8$).

2.3.6 Method Evaluation

Method recovery and repeatability was assessed by spiking heartwood samples with two aromatic compounds, methoxyhydroquinone and 2-acetonapthone not typically present in WRC but which have t_R near to peaks of interest. 100µl of a solution containing 1.5mg/ml methoxyhydroquinone and 2-acetonapthone in methanol was added to ground samples prior to extraction. Eight spiked samples and eight control samples (not spiked) extracted and analyzed.

Response factors were calculated for methoxyhydroquinone and 2-acetonaphthone as stated previously. The mean recovery value and relative standard deviation (RSD) was calculated for both compounds as a measure of the repeatability.

2.3.7 Statistical Analysis

All experiments followed a completely randomized design (CRD) with treatments denoted as k and replicates as n. A one-way analysis of variance (ANOVA) ($\alpha = 0.05$) and Tukey's test for comparison of means (Tukey, 1949) were performed on data. Statistical analysis was performed using JMP IN software (version 4.0.3 (academic), SAS Institute Inc., North Carolina, USA).

2.4 Results and Discussion

A typical chromatogram of WRC heartwood extractives is shown in Figure 2.2. Chromatographic analysis using this method was highly sensitive, detecting extractive compounds at concentrations ranging from 0.6 to 3.0μg/ml depending on the compound. The method's limit of detection was estimated at 0.6μg/ml for (-)-plicatic acid, γ-thujaplicin (3.0μg/ml), β-thujaplicin (3.0μg/ml), β-thujaplicinol (3.0μg/ml), thujic acid (0.6μg/ml) and methyl thujate (1.2μg/ml). The best fits by regression analysis of the detector responses for each of the extractive compounds were linear ($R^2 > 0.96$) (Table 2.3) over the range of concentrations investigated, indicating that single point calibrations could be used. ANOVA (α=0.05) followed by Tukey's test of multiple comparisons (Tukey, 1949) suggested that mean response factor values for extractives could be separated into four groups. Response factor values for the isomeric isopropyl-tropolones γ- and β-thujaplicin were similar and it is common practice to assume equivalent response factors among compounds of identical carbon number and similar structure (Kimball *et al.*, 2005).

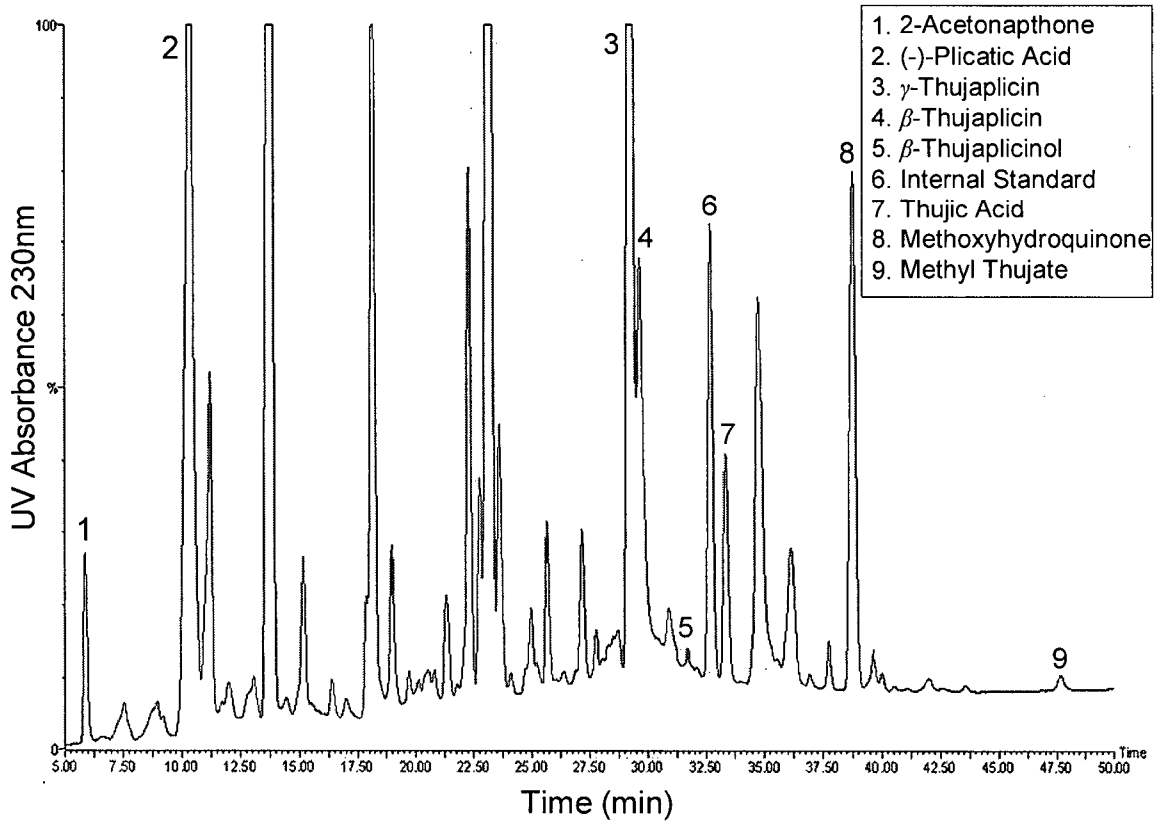


Figure 2.2 Chromatogram obtained from the analysis of WRC heartwood extractives.

Table 2.3 Linear regression analysis from mean detector response data for calibration standard solutions over specified concentration ranges.

Extractive compound	Range (μg ml^{-1})	Linear Formula	R^2
(-)-Plicatic acid †	0.6-1000	y = 0.4496x - 0.0074	0.97
γ-Thujaplicin ‡	3.0-200	y = 2.4223x - 0.5015	0.96
β-Thujaplicin ‡	3.0-200	y = 2.3401x - 0.5642	0.96
β-Thujaplicinol ¶	3.0-200	y = 1.171x - 0.3653	0.97
Thujic acid †	0.6-200	y = 0.1211x + 0.0083	0.98
Methyl thujate †	1.2-200	y = 0.185x - 0.0079	0.97
2-Acetonaphthone †	1.0-200	y = 0.443x - 0.0116	0.99
Methoxyhydroquinone §	1.0-200	y = 3.2781x + 0.0563	0.99
γ- & β-Thujaplicin*	3.0-200	y = 2.3812x - 0.5329	0.96

Note: compounds followed by the same symbol indicate that their mean response factor values were not significantly different according to Tukey's test of multiple comparisons. R^2 indicates correlation coefficient. Compounds 2-acetonapthone and methoxyhydroquinone are used for sample fortification and are not extractives of WRC heartwood. *Mean response factor and linear equation for isomers γ- and β-thujaplicin.

We compared two wood preparations, powders and slices. Grinding heartwood made extraction more efficient and uniform. During a cooled extraction, ground samples yielded ~ 36% more extractives than sliced (Table 2.4). The increased yield was statistically significant ($\alpha = 0.05$). Further, extractive concentration variability was higher for sliced than for ground samples (mean RSDs for μg/g DW were 9.2 and 4.0%, respectively). Grinding wood increased the ratio of surface area to volume, providing a greater contact area for the solvent and so improving the extraction efficiency. Grinding may also disrupt cells, promoting solvent penetration between cells and cell fragments, and into lignified xylem cells with aspirated pits.

Because some extractives are not thermostable, we examined the effect of cooling samples during ultrasonication. Without cooling, we recorded temperatures of up to 42°C during the ultrasonic extraction cycle. On average, ground samples extracted in a cooled ultrasonic bath yielded ~ 16% more (-)-plicatic acid than in ground samples extracted in an un-cooled bath. However, low temperatures (4°C) did not significantly increase the yield for the other five compounds.

Estimating the efficiency of extraction is difficult given that a majority of heartwood extractives are synthesized *in situ* at the sapwood/heartwood border and infuse into lignified cells (Swan and Jiang, 1970). However, recovery of spiked compounds gives a good indication of losses that may occur during the extraction process. To assess such losses we spiked eight samples with compounds methoxyhydroquinone and 2-acetonaphthone (Table 2.5). A 100% yield recovery equated to a final concentration of 100μg/ml when samples were extracted in 15ml of solvent. Recovery of methoxyhydroquinone from spiked samples was 95.5% with a RSD of 5.3%. Recovery of 2-acetonaphthone was marginally lower at 83.1% with a RSD of 5.9%.

Table 2.4 The effect of various extraction methods on extractive concentration (μg/g DW).

Extractive compound	Finely ground (cooled)	RSD*	Sliced (cooled)	RSD	Finely ground (non-cooled)	RSD
(-)-Plicatic Acid	11041.7 [1](393.1)[2a]	3.6	7033.3 (406.7)[c]	5.8	9182.3 (479.8)[b]	5.2
γ-Thujaplicin	1842.9 (45.8)[a]	2.5	1269.1 (83.6)[b]	6.6	1806.5 (54.1)[a]	3
β-Thujaplicin	1828.5 (45.1)[a]	2.5	1175.2 (64.5)[b]	5.5	1717.4 (73.8)[a]	4.3
β-Thujaplicinol	243.4 (2.7)[a]	1.1	154.3 (25.0)[b]	16.2	240.7 (23.5)[a]	9.8
Thujic Acid	5963.2 (248.5)[a]	4.2	4112.2 (171.4)[b]	4.2	5751.4 (246.3)[a]	4.3
Methyl Thujate	69.2 (7.1)[a]	10.3	35.8 (6.1)[b]	17	54.3 (13.4)[a]	24.7

Note: [1]numbers is parenthesis indicate standard deviation. ANOVA generated the following critical F values for extractive of interest: (-)-plicatic acid ($F_{(2,21)} = 279.6$), γ-thujaplicin ($F_{(2,21)} = 133.9$), β-thujaplicin ($F_{(2,21)} = 341.3$), β-thujaplicinol ($F_{(2,21)} = 71.4$), thujic acid ($F_{(2,21)} = 162.3$), and methyl thujate ($F_{(2,21)} = 31.9$). Values were considered significant if greater than the tabular value of $F_{(2,21)} = 2.57$ ($\alpha=0.05$). [2]Numbers followed by the same letter were not significantly different ($\alpha=0.05$) according to Tukey's test of multiple comparison of means. *RSD = relative standard deviation expressed as a percentage.

Table 2.5 Recovery and repeatability data for compounds 2-acetonapthone and methoxyhydroquinone.

	Concentration of Recovered Compounds (μg ml^{-1})	
	2-Acetonapthone	Methoxyhydroquinone
Sample 1	84.1	99.4
Sample 2	84.8	99.7
Sample 3	86.5	96.8
Sample 4	88.4	99.5
Sample 5	79.1	92
Sample 6	75.3	88.1
Sample 7	87.9	99.6
Sample 8	78.3	89
Mean Recovery	83.1 [1](4.9)	95.5 (5.0)
*RSD %	5.9	5.3

Note: [1]numbers is parenthesis indicate standard deviation. *RSD = relative standard deviation expressed as a percentage.

We estimated that the total extractive content of WRC heartwood was approximately 3.3% (w/w DW). This was achieved by conducting an exhaustive extraction of eight WRC heartwood samples in methanol, filtering the extract and then using a rotovaporator to bring extracts to dryness. Among the six extractives analyzed (-)-plicatic acid was the most abundant at 11708.7μg/g DW (RSD = 4.1%), accounting for 35.7% of the total extractives (w/w). Consistent with the literature (Barton and MacDonald, 1971) γ-thujaplicin (~1971.4μg/g DW; RSD = 2.9%) was slightly more abundant than β-thujaplicin (~1893.1μg/g DW; RSD = 2.9%), both chemicals accounting for 6% and 5.8% (w/w) of the total extractives respectively. β-Thujaplicinol at 244.6μg/g DW (RSD = 1.3%) (0.8% w/w) was equivalent to 12% of γ- and β-thujaplicin concentration. Thujic acid was the most abundant non-lignan extractive at 6094μg/g DW (RSD = 4.8%) (18.6% w/w), while methyl thujate was present in trace amounts 74.5μg/g DW (RSD = 11.1%) (0.2% w/w). Our data were consistent with those of Jones and Falk (2005) who compared extractive yields from WRC heartwood using various extraction techniques. Except for methyl thujate (RSD of 11.05%), very little variation was observed in the estimated concentration (μg/g DW) of the compounds of interest with RSDs generally 5% or lower. Variability in the data were minimized by a) processing the samples with the freezer mill, b) protecting experimental samples from excess heat and light and c) using heartwood experimental samples in close proximity to one another within the tree.

Only six extractive compounds have been examined in the work reported here, many more have been characterized with several more uncharacterized. However, we estimated that overall these six extractive compounds accounted for 67% of the total extractive content of WRC used in this work.

2.5 Conclusions

Extraction of finely ground wood samples using methanol under ultra-sonic frequency provided yield of extractives and compounds with a broad range of polarity. The method itself is capable of detecting individual extractives at very low concentrations (0.6-3.0 μg/ml). The extraction and analysis process showed good efficiency and repeatability with recovery two aromatic compounds which were added to samples prior to extraction of at ~ 90% and an RSD of ~ 5%. It is difficult to estimate the recovery yield of heartwood extractives given that they are synthesized *in situ* and secreted with specialized cells. Quantitative analysis of six analytes was achieved by employing single-point calibrations using a single internal standard which is commercially available. This method was subsequently used for screening extractive tolerant fungal species (part 2) (see Lim *et al.*, 2006). The method described is straightforward and could be applied to almost any situation where such extractive compounds are required to be quantitated, particularly given the interest in using such compounds for pharmaceutical applications.

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Chapter 3

Isolating and Testing Fungi Tolerant to Western Redcedar (*Thuja plicata* Donn) Extractives

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3.1 Abstract

Western redcedar (*Thuja plicata* Donn) (WRC) is a naturally durable softwood species native to British Columbia in Canada, as well as Washington, Oregon and California in the USA. Untreated WRC products are durable because they contain extractive compounds that have strong anti-microbial activity. However, products can still fail in service due to fungal colonization initiated by extractive tolerant species and to depletion of extractives caused by weathering. To screen for extractive tolerant species we developed a WRC-feeder strip assay. When WRC-feeder strips were placed on malt extract agar, extractives from the wood accumulated in the media and strongly inhibited growth of non-tolerant fungal strains. Extractives remaining in feeder strips following incubation on media were characterized. Of the many compounds leached out, γ- and β-thujaplicin, β-thujaplicinol, plicatic acid and thujic acid were quantified. The growth of selected fungal strains was not affected by plicatic acid; however, it was inhibited to different degrees by β- and γ-thujaplicin. *Pachnocybe ferruginea* was extractive tolerant and may play an important role in the initial stages of degradation of WRC products.

Key words: γ-thujaplicin, β-thujaplicin, Extractives, Pioneer fungi, Western redcedar, Decks

3.2 Introduction

Western redcedar (*Thuja plicata* Donn) (WRC) is a naturally durable softwood species native to British Columbia, Canada as well as Washington, Oregon and California in the USA. Its heartwood contains higher concentrations of aromatic and polyphenolic extractives than other softwood species (Barton and MacDonald, 1971). Several of these compounds exhibit strong antimicrobial activity (Inamori *et al.*, 1999, 2000; Arima *et al.*, 2003; Morita *et al.*, 2004). Because these compounds are highly effective against wood decay fungi, they may protect standing trees from being weakened and prone to wind-throw (Van der Kamp, 1986; DeBell *et al.*, 1997) and make WRC wood products naturally durable. Given its durability, cedar is extensively utilized for wood products for exterior residential applications and accounts for a significant proportion of Canada's forest products industry (Gonzalez, 2004).

WRC products can still fail in service due to a) depletion of extractives caused by weathering or b) colonization by extractive-tolerant fungal species. Weathering involves photo-degradation of extractives by UV radiation in sunlight and precipitation leaching out chemicals from the surfaces or ends of wood boards causing depletion of the overall extractive content (Coombs and Trust, 1973; Johnson and Cserjesi, 1980). This may permit colonization by decay fungi that are inhibited by the original extractive content. Pioneer fungi have been isolated from standing WRC trees and products in service, frequently from the inner regions of WRC products where extractives may be less affected by weathering (Van der Kamp, 1975; Lim *et al.*, 2005). Such species do not decay wood, but may detoxify fungicidal extractives, facilitating wood colonization by decay fungi, which are less tolerant to extractives (Jin *et al.*, 1988).

Preventing colonization by pioneer fungi may increase the service life of WRC products. To date only a few pioneer fungi have been isolated and characterized. In this work, we described a

method for screening WRC extractive tolerant fungi. Agar media with extractives or pure compounds at different concentrations have been used to screen microorganism tolerance to WRC extractives (Jin *et al.*, 1988, Lim *et al.*, 2005). Synthesis or isolation of total or specific extractive compounds is costly and using a single compound may not reflect the effects of total extractives. Given this, we used WRC- feeder strips (WRC-FS) to detect decay and pioneer fungi that are tolerant to a mixture of naturally occurring WRC extractives. Results for fungal growth with WRC-FS were compared to data obtained with single extractives.

3.3 Materials and Methods

3.3.1 Fungal Isolations and Identification

WRC boards were collected from house decks and experimental test sites located in Vancouver, British Columbia, Canada. The service life of decks ranged from 20-100 years. We sampled twelve boards from deck A (100 years), five boards from deck B (20 years) and seven boards from deck C (25 years). Two sections about 2 cm in thickness were cut from each board using a circular saw. Fungi were isolated within and near decay pockets as described by Lim *et al.* (2005). A 1% malt extract agar (MEA) was used for isolating general fungal flora and 1% MEA with benomyl (BMEA) was used for basidiomycetes fungi (Clubbe and Levy, 1977). Isolates were grouped by macro- and micro-morphological characteristics using taxonomic guides and standard procedures (Nobles, 1965; Stalpers, 1978; Wang and Zabel, 1990). This initial identification was complemented by molecular techniques.

DNA was extracted from mycelia and the internal transcribed spacer (ITS) region was amplified using the primers ITS5 and ITS4 (Lim *et al.*, 2005; White *et al.*, 1990). PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen Inc., Mississauga, Ontario, Canada).

Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc., Foster City, California, USA) at the DNA synthesis and Sequencing Facility, MACROGEN (Seoul, Korea). All the nucleotide sequences presented in this work have been deposited at Genbank and their accession numbers are shown in Table 3.1.

3.3.2 Inhibition of Fungal Growth by WRC Feeder Strips (WRC-FSs).

WRC-FSs, about 5 x 3 x 0.2cm in size, were obtained from the outer heartwood of a sound 80 year old WRC tree harvested at UBC Malcolm Knapp Research Forest, Maple Ridge, British Columbia. All wood samples originated from a single longitudinal axis parallel with growth rings. Blocks were sequentially numbered relative to their position. Samples were immediately placed into individual sealable bags, labeled and stored at -20°C until further use to minimize volatilization of extractives. The FSs used in this study were sterilized by irradiation using electron beam technology (Iontron Industries Canada, Port Coquitlam, British Columbia, Canada) which prevents alteration of extractives that may occur through sterilization by alternative methods such as autoclaving. To measure fungal growth rate (mm/day), an agar plug (5 mm in diameter) from a freshly grown fungal isolate on MEA, was transferred onto 1% MEA with a WRC-FS. The inoculum and the WRC-FS were placed on opposite sites of the MEA plate. Fungi were also grown on 1% MEA without FS (control). All plates (three replicates per isolate) were incubated at 20 °C in the dark to prevent extractive photo-degradation. Fungal growth from each culture was measured every 3 days by taking two perpendicular measurements from the inoculum to the maximum and minimum edge of the colony. The two measurements were averaged. The average growth rate (mm/day) was calculated after 21 days Growth inhibition tests were performed onto 1% MEA supplemented with individual extractives. The added concentration for each compound was calculated from the WRC-FS experiment (see below).

Table 3.1. Fungal isolates from WRC deck in service and fungal growth inhibition by WRC-FSs.

Fungal ID	Acc. No for ITS	Source				Growth (mm/day)[a]		
		A	B	C	Location	Con	FS	Inhibition (%)
Acanthophysium lividocaeruleum	AY618666	5	3	4	D / S	2.4	0.9	64.4
Coniophora puteana	DQ516523	2	1	-	D	2.6	1.1	56
Dacrymyces stillatus	DQ516524	1	2	2	D	0.6	0.1	83.5
Hyphoderma praetermissum	AY618668	3	-	2	D	1.7	0.1	94.5
Pachnocybe ferruginea	AY618669	9	14	7	D / S	0.8	0.8	8.1
Phellinus ferreus	DQ516525	2	-	5	D	2.2	0.1	95.6
Aureobasidium pullulans	DQ516526	2	-	2	D	0.3	0.1	69.4
Exophiala heteromorpha	DQ516527	5	-	5	D	1.4	0.4	71.4
Phialocephala dimorphospora	AY618688	12	8	9	D	2.1	0.3	86.6
Rhinocladiella atrovirens	AY618683	20	10	12	D	0.6	0.2	71.7
WRCF-A1	AY618686	22	5	12	D	1.3	0.2	82.1
Umbelopsis autotrophica	DQ516528	2	3	1	D	2.4	0.2	90.3

Note: Numbers below the source represent isolate frequency for A, 100 year old deck; B, 20 year old deck; C, 25 year old deck. From each source approximately 150 isolations were made. Site of isolation: D (decay areas), S (sound inner areas). [a]Average growth rate (mm day^{-1}) calculated after 21 days on 1% MEA without (control = con) or with WRC-FS (FS). Values were mean of three replicates.

3.3.3 Extractive Analysis of WRC-FSs

WRC-FSs were placed onto 1% MEA and then examined in order to estimate the amount of extractives that leached out of the WRC-FS and accumulated in the media. Five WRC extractive compounds of interest were extracted, separated and quantified (μg/g dry weight, DW) by reverse phase high performance chromatography (HPLC) equipped with an ultraviolet (UV) detection system as described in Chedgy et al. (2006) and Daniels and Russell (2006). Extractives of interest were (-)-plicatic acid, γ-thujaplicin, β-thujaplicin, β-thujaplicinol, and thujic acid. These compounds were selected on the basis that they are: (a) known to have antimicrobial activity; and (b) are major constituents of WRC heartwood. WRC-FSs were cut into two equal sections and weights were recorded for each section. One section was oven dried at 105°C for 24 hours to calculate the dry weight (DW). The second section was finely ground, and subjected to chemical analysis.

This experiment followed a completely randomized design (CRD) with three treatments: WRC-FSs (i) frozen at -20°C (control), (ii) placed in empty plates and stored at 20°C for 21 days; (iii) stored on 1% MEA at 20°C for 21 days ($k = 3$). Six replicates were made in each case ($n = 6$), and the mean concentration of extractives was then calculated.

3.3.4 Statistical analysis

All experiments followed a CRD with treatments denoted as k and replicates as n. A one-way analysis of variance (ANOVA) ($\alpha = 0.05$) and Tukey's test for multiple comparisons of means (Tukey, 1949) were performed on data. Treatment effects were considered significant if the resulting critical F value was greater than the appropriate tabular value ($F_{[(k-1)],\,[(k(n-1)]}$). Statistical analysis was performed using JMP IN software (version 4.0.3 (academic), SAS Institute Inc., North Carolina, USA).

3.4 Results and Discussion

3.4.1 Fungal Identification and Extractive Resistance Tests

A total of 242 fungal isolates were recovered from 24 boards taken from three WRC decks. Using macro- and microscopic characteristics, as well as molecular DNA data, 12 fungal taxa were isolated more than three times. Six species isolated on BMEA were identified as basidiomycetes. One zygomycete and five ascomycetes were identified because of their sporangia and asexual structures, respectively (Table 3.1). The initial fungal identification was complemented by ITS sequence analyses. ITS sequences often diverge at the species level, and are preferentially used for identification (Schmidt and Moreth, 2002, 2003; Högberg and Land, 2004). Except for one ascomycete species (WRCF-A1), sequence analyses confirmed morphological identifications, and allowed fungi that could not be identified by morphology to be grouped with known species (Table 3.1). WRCF-A1 had no distinct asexual morphology on MEA media and its ITS sequence showed 95% sequence similarity with unknown endophytic fungi and leaf litter ascomycetes. The basidiomycetes most frequently isolated were *Acanthophysium lividocaeruleum* and *Pachnocybe ferruginea*. They were present in both decay areas and sound inner areas which tend to have a higher extractive content as extractives in the inner regions are less prone to photo-degradation from UV and depletion by leaching (Coombs and Trust, 1973; Hon, 1991; Shibata *et al.*, 2003). The ascomycetes, *Phialocephala dimorphosphora*, *Rhinocladiella atrovirens,* and the species WRCF-A1 were commonly isolated but were present mainly in decay areas. It is likely that these species occupy decay areas because they have low to moderate extractive resistance and decay pockets contained lower extractive concentrations compared to sound areas. The four known fungal species reported here, have been also isolated from WRC products in service (Scheffer *et al.*, 1984; Wang and Zabel, 1990).

One or two representatives from each of the 12 taxa were used to determine tolerance to WRC extractives. When grown on 1% MEA with WRC-FS, most isolates showed less growth than the controls (Figure 3.1). Several of the extractives are water-soluble and diffuse from the WRC-FS into the media when placed in contact. In addition, WRC-FS were manufactured such that the transverse wood face was in contact with the media promoting infusion of moisture into the vesicles and tracheids of the wood, allowing more extractives to diffuse from the wood into the media. *P. ferruginea* exhibited the highest tolerance to WRC-FS extractives, displaying a growth rate comparable to controls. This species was the most common one in this work and has frequently been reported on WRC fences and creosote-treated WRC poles (Wang and Zabel, 1990; Lim *et al.*, 2005). It has also been reported to be commonly isolated from the heartwood of Douglas-fir and was characterized by Kropp and Corden (1986). Two basidiomycetes, *A. lividocaeruleum* and *Coniophora puteana*, and three ascomycetes, *Aureobasidium pullulans*, *Exophiala heteromorpha*, and *R. atrovirens,* had growth of 28 to 44% relative to controls, which represented moderate tolerance to WRC extractives (Table 3.1). Although *C. puteana* had higher WRC extractive tolerance than *A. lividocaeruleum*, it was found in or near decay pockets and was less frequently isolated. *A. lividocaeruleum*, one of the most frequently isolated species from WRC fences, was suggested as being a pioneer species with *P. ferruginea*, based on their tolerance to β-thujaplicin (Lim *et al.*, 2005). *A. pullulans, E. heteromorpha* and *R. atrovirens* were detected near or in decay pockets. *A. pullulans* is able to colonize weathered wood surfaces from a variety of tree species, including WRC, and to cause black stain (Schoeman and Dickinson, 1997; Chedgy *et al.*, 2005). It may play an important role in colonizing and modifying wood surfaces rather than inner areas of WRC products. The growth of other fungi was significantly reduced on plates with WRC-FS. The only zygomycete *Umbelopsis autotrophica* showed very low tolerance to extractives and thus is unlikely to have ability to

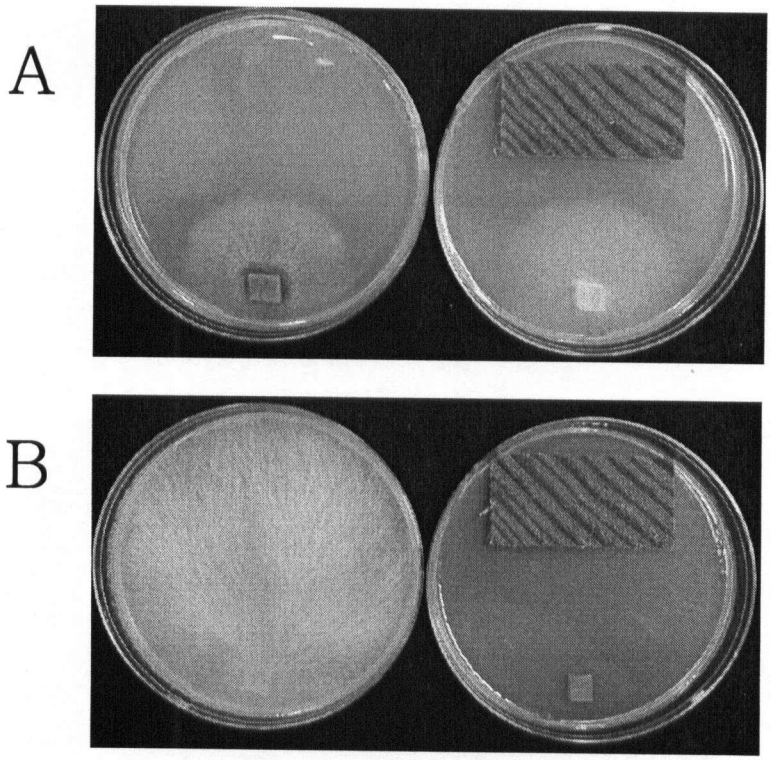

Figure 3.1. Fungal Growth on media with or without a WRC-FS.

A: *Pachnocybe ferruginea*, B: *Phellinus ferreus* growths on 1% MEA (control) and on 1% MEA with WRC feeder strips after 21 days incubation at room temperature.

modify extractives and is unlikely to cause decay of wood.

On WRC product surfaces, it has been reported that the pattern of fungal colonization consisted of staining fungi followed by soft-rot and decay fungi (Banerjee and Levy, 1971; Clubbe, 1980). The inner sound areas of WRC boards contained no staining or soft-rot fungi, only extractive tolerant basidiomycetes (e.g. *P. ferruginea*), suggesting that these species may play a pivotal role in the degradation of the inner parts of WRC products.

3.4.2 Extractives Analyses of WRC-FSs

Wood is a naturally variable material and its extractive concentrations vary within and between trees. We observed a similar trend in our control WRC-FSs. The total extractive content of WRC-FS was approximately 3.3% (w/w DW), and the five compounds quantified in this work represent about 67% (w/w DW) of this total. The overall concentration of the five extractives varied from 16.86-19.24 mg/g DW accounting for a variation of 14%. We determined that 57.5% of the extractives that we measured diffused into the media based of the amount of extractives recovered from the WRC-FSs placed on MEA. Among the five compounds measured, γ-thujaplicin and β-thujaplicin leached out more than plicatic and thujic acids or β-thujaplicinol from the WRC-FSs (Figure 3.2). Data analyses by ANOVA showed that the treatment effect was significant for each of the five extractives of interest. Incubation of WRC-FS on media caused a significant loss ($\alpha = 0.05$) of extractives from the wood to the media. We examined the effects of the above five pure compounds on fungal growth on MEA. Each compound was used at a concentration equivalent to its loss from the FS (Figure 3.2, Table 3.2).

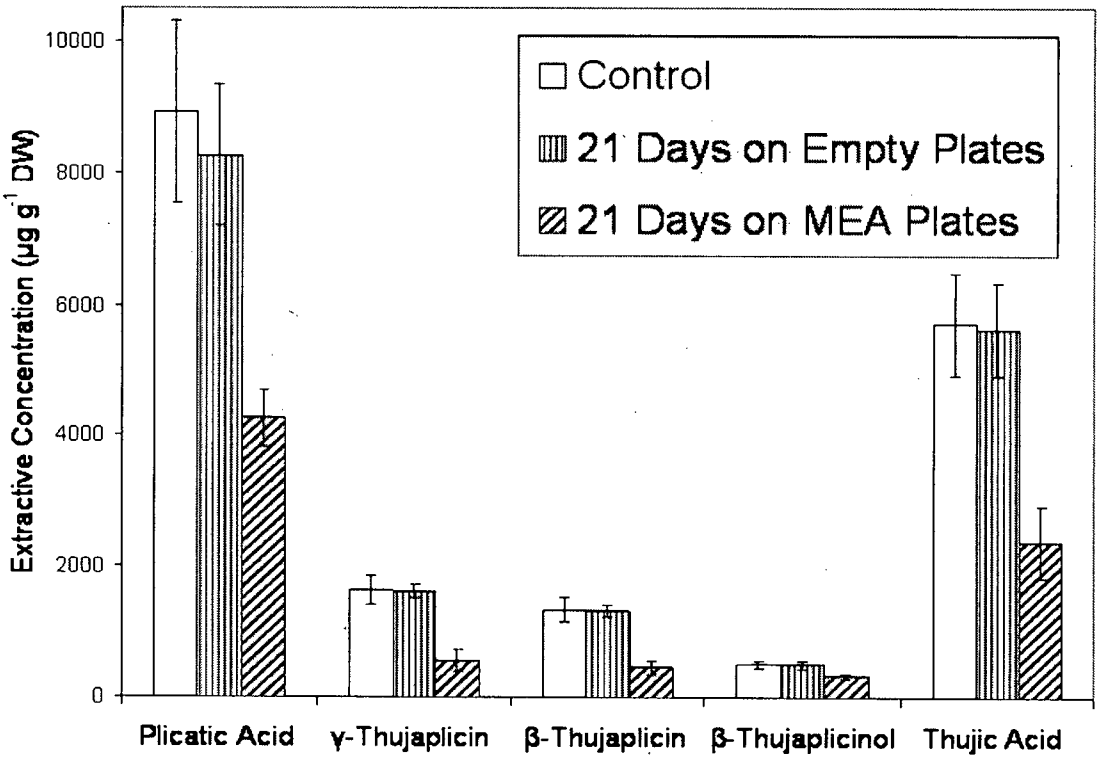

Figure 3.2 Extractive concentrations (µg/g DW) versus WRC feeder strip storage conditions.

Note: ANOVA was performed on the amount of individual extractive compounds lost on WRC-FSs on MEA compared to controls. Critical F values ($\alpha = 0.05$) were as follows: plicatic acid ($F_{(2,15)}$ 35.97); γ-thujaplicin ($F_{(2,15)}$ 79.34); β-thujaplicin ($F_{(2,15)}$ 87.90); β-thujaplicinol ($F_{(2,15)}$ 28.35); and thujic acid ($F_{(2,15)}$ 44.57). Values are considered significant if the critical F value is greater than the tabular value of $F_{(2,15)} = 3.68$.

Three fungi with high, moderate and low tolerance to WRC extractives were selected: *P. ferruginea, R. atrovirens* and *Phellinus ferreus*, respectively (Table 3.2). *P. ferruginea* showed high tolerance to the five compounds. *R. atrovirens'* growth was inhibited by γ-thujaplicin, reduced to level similar to FSs by β-thujaplicin and thujic acid. *P. ferreus* did not grow on MEA with γ-thujaplicin, β-thujaplicin and thujic acid, and its growth was slightly inhibited by plicatic acid and β-thujaplicinol. β-thujaplicinol, a very effective natural fungicide (Barton and MacDonald 1971), affected slightly the growth of the three fungi; however, its concentration in MEA resulting from FS leaching was much lower than the concentration previously tested (Rennerfelt, 1948; Roff and Whittaker, 1959; Lim *et al.*, 2005). Plicatic and thujic acid have been reported as having low antimicrobial activity (Rennerfelt, 1948; Barton and MacDonald, 1971). Consistent with this, relatively high concentrations (up to 150 ppm) of plicatic acid did not affect fungal growth significantly; however, high concentrations of thujic acid (107 ppm) inhibited growth of the less tolerant basidiomycetes. Many other chromatographic peaks were present in HPLC chromatograms from FS, and some of these compounds diffused into MEA (data not shown). Although more compounds than the five tested have been identified (Barton and MacDonald, 1971) and several remain uncharacterized, the purification of such compounds is difficult, and they are not available commercially. Given this, the microbial toxicity of these compounds and their synergistic effects could not be assessed.

Extractives such as the thujaplicins also occur in plant species throughout the Cupressaceae family (Zavarin *et al.*, 1967). Similar antimicrobial activity has been reported for tropolone compounds in species such as aomori hiba cedar (*Thujopsis dolabrata*) (Inamori and Morita, 2001), Mexican cypress (*Cupressus lusitanica*) (Zhao *et al.*, 2006) and Taiwan incense-cedar (*Calocedrus formosana*) (Ono *et al.*, 1998).

Table 3.2 Concentration of extractives that leached into media and their effect of fungal growth.

Treatment	Chemical Concentration in MEA (ppm)	Growth rate (mm/day)[a]		
		P. ferruginea	*R. atrovirens*	*P. ferreus*
Control	-	0.9 (0.1)	0.6 (0.0)	2.2 (0.3)
Plicatic Acid	149.4	0.9 (0.0)	0.6 (0.0)	1.9 (0.1)*
γ-Thujaplicin	34.0	0.8 (0.1)	0.0 (0.0)*	0.0 (0.0)*
β-Thujaplicin	27.6	0.7 (0.0)	0.2 (0.0)*	0.0 (0.0)*
β-Thujaplicinol	5.6	0.8 (0.0)	0.5 (0.0)*	1.8 (0.1)*
Thujic Acid	107.0	0.8 (0.0)	0.2 (0.1)*	0.0 (0.0)*
WRC-FS		0.8 (0.0)	0.2 (0.0)*	0.0 (0.0)*

Note: 1% MEA plates were infused with extractives at the concentration that they accumulated in media having leached from WRC-FSs. [a]Media were then inoculated with three representative fungal species and the growth rate (mm/day) was calculated. Asterisk indicates that growth rate was significantly different from the control ($\alpha = 0.05$) following ANOVA.

While ascomycetes may appear from the literature to be more tolerant than basidiomycetes to β-thujaplicin, basidiomycetes tolerance has been characterized for relatively few species (Morita *et al.*, 2004). The basidiomycete *P. ferruginea* often has been misidentified because its morphology is graphium-like. In this work we showed that this species was highly tolerant to antimicrobial WRC extractives. Currently, there is no information on the mechanism by which it detoxifies WRC extractives. The only work reporting fungal detoxification of thujaplicins is Jin *et al.* (1988). They suggested that two species that they isolated from red cedar heartwood, a *Sporothrix* species, *Kirschsteiniella thujina*, and a *Phialophora* species, could convert thujaplicins into a non-toxic lactone termed 'thujin'. However, many groups have failed to isolate similar species from WRC. In future work we intend to characterize the ability of 'pioneer' species like *P. ferruginea* to detoxify WRC extractives.

3.5 Conclusions

Using FSs from sound WRC heartwood permits screening fungi for tolerance to the mixture of extractive compounds that they would encounter in trees or wood products, and overcomes the limited availability and cost of pure extractive compounds. Results indicated that *P. ferruginea* is an extractives-tolerant pioneer fungus that may play an important role in the initial modification or detoxification of WRC extractives in wood products. These issues are being addressed in ongoing work.

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Chapter 4

Black Stain of Western Redcedar (*Thuja plicata* Donn) by *Aureobasidium pullulans*: the Role of Weathering

4.1 Abstract

Western redcedar (*Thuja plicata* Donn) (WRC) is valued for its natural durability conferred by fungicidal extractive chemicals. However, weathered surfaces of WRC products are susceptible to black stain caused by fungi such as *Aureobasidium pullulans*. The effect of weathering on extractive concentrations at the wood surface was characterized and correlated with the ability of this fungal species to colonize weathered surfaces. UV plus water spray treatments substantially reduced extractives but did not promote fungal colonization. In contrast, UV-only treatments reduced extractive contents less than the other treatments but stimulated fungal colonization. *A. pullulans* exhibited high tolerance to the tropolone β-thujaplicin *in vitro*; thus loss in tropolone content may not be required for colonization. Water spray most likely washed away products of lignin photo-degradation, which resulted in decreased fungal colonization.

Key words: *Aureobasidium pullulans*, black stain, extractive resistance, weathering, western redcedar (*Thuja plicata* Donn).

4.2 Introduction

WRC is utilized in the manufacture of wood products for exterior residential applications. It is valued for its natural durability conferred by fungicidal extractive compounds (Barton and MacDonald, 1971). However, WRC products are susceptible to black stain caused by staining

fungi which can significantly reduce wood aesthetic qualities and lead to premature replacement. *Aureobasidium pullulans* (de Bary) G. Arnaud causes black stain and is a major colonizer of weathered wood surfaces (Schoeman and Dickinson, 1997) and painted wood (Bardage and Bjurman, 1998; Jakubowsky et al.,1983; O'Niel, 1986; Shirikawa et al., 2002). It can also penetrate many protective coatings (Sharpe and Dickinson, 1992b). The black coloration is attributed to the presence of melanin in the fungal hyphae (Yurlova et al., 1999), which protects fungal cells from the damaging effects of UV radiation (Kawamura, 1999).

Weathering of the wood surface promotes *A. pullulans*' growth since the fungus metabolizes the complex aromatic organic molecules that are formed as a result of lignin photo-degradation (Bourbonnais and Paice, 1987; Dickinson, 1972; Schoeman and Dickinson, 1997; Sharpe and Dickinson, 1992a, 1993). This may provide a significant competitive advantage to this fungal species and may explain why *A. pullulans* is predominantly isolated from weathered surfaces (Dickinson, 1972). Many of the fungicidal extractive compounds present in WRC are aromatic and polyphenolic in nature (Barton and MacDonald, 1971). Of the array of WRC extractive compounds characterized to date are a series of compounds known as the tropolones. Several of these compounds, namely the thujaplicins (α-, β-, and γ-thujaplicin) are reported to exhibit strong antimicrobial activity against a range of wood-inhabiting and plant pathogenic fungi (Inamori et al., 2000; Morita et al., 2004a, 2004b).

Extractive contents at the surface of WRC products may form the first line of defense in preventing fungal spore germination. At this early developmental stage, microorganisms may be less resistant to the toxic effect of extractives. However, extractives near the wood surface may be prone to leaching from precipitation (Chedgy et al., 2005) and are degraded by ultra-violet

(UV) radiation from the sun (Coombs and Trust, 1973; Shibata et al., 2003) that penetrates the upper 0.75mm of the wood surface (Hon, 1991). It is not known whether *A. pullulans* is tolerant to WRC extractives or if they are simply depleted by weathering at exposed surfaces, paving the way for colonization. To address WRC black stain, it is necessary to understand the interactions between WRC extractives, weathering and fungal colonization. Therefore, the aims of this research were to 1) establish whether or not *A. pullulans* has resistance to fungicidal tropolone compounds present in WRC *in vitro*, and 2) assess the ability of this fungal species to colonize weathered wood surfaces.

4.3 Materials and Methods

4.3.1 Isolation and Identification of Black Staining Fungi

Black staining fungi were isolated from in service WRC siding located in Vancouver, British Columbia, Canada. Wood flecks that exhibited visible signs of black stain were removed from the surface of siding and placed onto 1% malt extract agar (MEA) plates then incubated at 20°C for several weeks. Fungi growing on MEA were routinely sub-cultured from mycelial margins to new MEA plates to obtain pure cultures. Fungal identification was achieved by macro- and micro-morphological analyses using taxonomic guides (de Hoog and Yurlova, 1994). This was complemented by molecular technique for species identification as described by Lim et al. (2005). The internal transcribed spacer (ITS) region was used for molecular identification and amplified using PCR with the primers ITS5 and ITS4 (Schmidt and Moreth, 2002; White et al., 1990). Sequencing from three representative strains of each isolated taxon was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc., Wellesley, Massachusetts) at the DNA synthesis and Sequencing Facility, MACROGEN (Seoul, Korea).

4.3.2 β-Thujaplicin Resistance

Two isolated black stain fungal species, *A. pullulans* and *Hormonema dematioides* Lagerb & Melin, were tested. Mycelial growth was measured on 25ml MEA plates containing various concentrations of β-thujaplicin (99% pure, Sigma-Aldrich Ltd, Oakville, Ontario). A 10 mg ml^{-1} stock solution prepared in 50% ethanol was filter-sterilized and kept in the dark at 4°C. Concentrations of 0, 2, 8, 16, 32 and 64 ppm (parts per million) were added to MEA. Control MEA plates with and without ethanol showed that ethanol had no effect on fungal growth at the low concentrations used to prepare the β-thujaplicin plates. Media were inoculated with a 5 mm plug of agar taken from the edge of actively growing isolate colonies. The cultures were maintained in the dark at 20°C and the growth (mm) was evaluated by measuring two perpendicular diameters of the colony after 21 days. This experiment followed a completely randomized design (CRD) with treatments defined as the various β-thujaplicin concentrations (k = 6). Three replicate plates were used per strain at each concentration, and three strains of each species were used (n = 9). Statistical analysis was performed on data obtained with this experimental design.

4.3.3 Weathering and Fungal Effects on Wood Chemistry

Wood samples - WRC outer heartwood was obtained from a 136 year old standing tree harvested at the UBC Malcolm Knapp research forest, Maple Ridge, British Columbia. All wood samples originated from a single longitudinal axis parallel with growth rings. Siding pieces were manufactured of dimensions 160mm x 65mm x 10mm with the radial face on the largest face. Ponderosa pine (*Pinus ponderosa* P. & C. Lawson) sapwood was also used as a control. The sapwood of this species contains small amounts of extractives that are not fungicidal like those of WRC.

Weathering of siding material - WRC and pine siding pieces were exposed to simulated weather conditions using a Weather-Ometer® (Ci65A, Atlas Material Testing Technology LLC, Chicago, Illinois) located at the Forintek Canada Corp. laboratory, British Columbia. Four treatments were used: i) water spray (WS), ii) UV, iii) WS and UV, and iv) no weathering (Table 4.1). Each treatment was run continuously for a period of 200 hrs, at a temperature of 50°C to prevent mould growth and a relative humidity ranging from 40-95% depending on treatment to provide some moisture to facilitate chemical reactions. Eighteen WRC and pine siding pieces were subjected to each of the weathering treatments. Each treatment was repeated three times with a new set of samples. Following the weathering process twelve WRC and pine pieces from each treatment were used for chemical analyses.

For chemical analysis the upper 1mm of the weathered surface was removed using a computer controlled Precix 3600 router (Precix, Surrey, British Columbia). Four pieces were processed each time and the shavings from these samples were combined. This reduced the number of replicates from twelve to three for each weathering treatment. Samples were placed into clean glass vials in methanol and extracted for 120 minutes at an ultrasonic frequency of 40 kHz using a Branson 8510 ultrasonic bath (Branson Ultrasonics Corp., Connecticut). A temperature of 4°C was maintained throughout the ultrasonication process by the addition of ice to the water bath, and a lid was used to shield samples from incandescent light to avoid thermal and photo-degradation of extractive compounds of interest. Extract solutions were filtered using a 25mm 0.2μm nylon syringe filter to remove any wood particles and stored at 4°C in the dark.

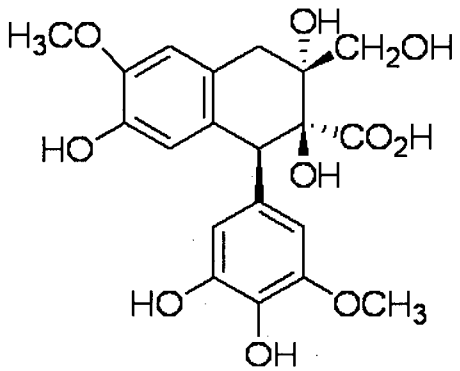
Table 4.1 Weathering treatments.

	Program cycle			
	1	2	3	4
Control cycle	-	-	-	-
WS only	30 mins WS* (dark)	30 mins (dark)	30 mins WS (dark)	30 mins (dark)
UV only	30 mins UV*	30 mins (dark)	30 mins UV	30 mins (dark)
UV and WS	30 mins UV	30 mins WS (dark)	30 mins UV and WS	30 mins (dark)

*WS = water spray; UV = ultraviolet. The duration of each weathering regime was 200hrs. WS rate was approximately 9.08 liters per hour. The UV wavelength was set at 340nm, and we measured 120Kj/m^2 total for each of the 200 hours weathering cycles with UV.

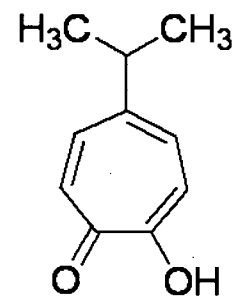
Reverse phase HPLC analysis - Separation and quantification of extractive compounds was carried out by reverse phase high performance liquid chromatography (HPLC) coupled with an ultraviolet detection system as described in Chedgy et al. (2006a). Five extractives of interest were quantified (μg/g DW) by comparing analyte response with the response factor of an internal standard by a single point calibration. Extractives of interest were (-)-plicatic acid, *γ*-thujaplicin, *β*-thujaplicin, *β*-thujaplicinol, and thujic acid (Figure 4.1). A Waters 2695 HPLC separation module (Waters Corp., Milford, Massachusetts) equipped with an Intersil ODS3 C-18 (3μm, 4.6mm x 150mm) reverse phase separation column (Intersil Corp., Milpitas, California) was used for extractive separation. Mixed extracts were injected (15μl) on to the column and separated based on their hydrophobic character using a mobile phase of 0.1% formic acid, 10% acetonitrile, and 89.9% nano-pure H_20 which run against an increasing linear gradient of 99.9% acetonitrile with 0.1% formic acid. The column chamber was heated to temperature of 50°C, and a Dionex AD20 UV absorbance detector (Dionex Corp., Sunnyvale, California) at wavelength of 230nm was used for the extractive detection. A total elution of extractives was typically reached after approximately 48 minutes and MassLynx analytical software (version 4.0, Micromass Ltd, Manchester, England) was utilized for chromatographic analysis.

A CRD was used to assess the effect of weathering on extractive concentration. Experimental treatments were defined as the different weathering treatments ($k = 4$). The surface wood was analyzed from twelve replicate siding pieces from each weathering treatment. Surface wood was removed from four pieces simultaneously using the automated router and the dust was pooled together. This reduced the replicate number from twelve to three. Therefore, $n = 9$ (3 x analytical surface wood samples, 3 x weathering treatments).



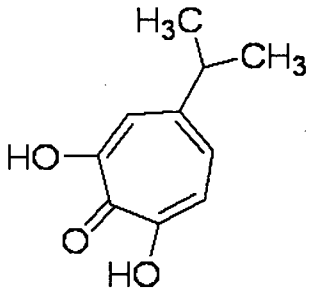
2,3,6-Trihydroxy-7-methoxy2hydroxymethyl -4-(3',4'-dihydroxy-5'-methoxyphenyl)-tetralin-3-carboxylic acid

(-)-Plicatic Acid



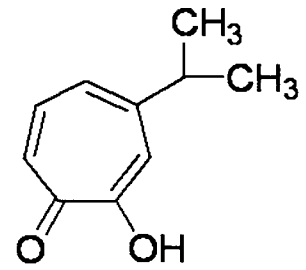
2-Hydroxy-5-isopropyl-2,4,6-cycloheptatrien-1-one

***γ*-Thujaplicin**



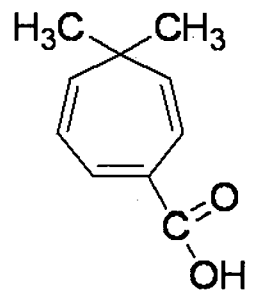
2,7-Dihydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one

***β*-Thujaplicinol**



2-Hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one

***β*-Thujaplicin**



7,7-Dimethylcycloheptatriene-3-carboxylic acid

Thujic Acid

Figure 4.1 Chemical structure and nomenclature used for WRC heartwood extractive compounds of interest (Barton and MacDonald, 1971).

4.3.4 Inoculation of *A. pullulans* on Weathered Siding

Weathered and un-weathered (control) WRC and pine siding pieces were sprayed with a liquid culture of *A. pullulans* and incubated in growth chambers. Three *A. pullulans* strains were grown in 250ml flasks containing 100ml of 1% malt extract media. The inoculum was approximately $1x10^6$ spores. Shake cultures were incubated at 22°C, 200rpm, for fourteen days. Cells were collected by centrifugation and re-suspended in nano-pure H_20. The culture suspension from three *A. pullulans* strains were mixed together in equal parts. The resulting solution was sprayed homogeneously over all of the weathered siding pieces. Six WRC and pine siding pieces from each weathering treatment were completely randomized and divided among four growth chambers. Samples were suspended over a layer of water. The incubation was carried out for 6 months at 20°C and close to 100% relative humidity. To provide a surface film of water, the samples were sprayed with a distilled water mist every 3-4 days. The degree of colonization was assessed in two ways: i) qualitatively, by scoring siding pieces to reflect the degree of black staining; ii) quantitatively, using a Minolta CM-2600d portable integrated sphere spectrophotometer (Konica-Minolta Ltd, Ontario) which yields an index of white-black coloration ranging from 0-100 (0 = Black, 100 = White). Discoloration of wood after exposure to *A. pullulans* could be calculated by taking readings before and after colonization at six regions of each siding piece. As before, a CRD was used to assess the effect of weathering on the colonization ability of *A. pullulans* on weathered surfaces. Experimental treatments were defined as the different weathering treatments ($k = 4$). Six siding pieces were analyzed for the degree of discoloration from each weathering run, and each weathering treatment was repeated three times ($n = 18$) (6 replicate siding pieces x 3 weathering treatments).

4.3.5 Statistical Analysis

All experiments followed a completely randomized design (CRD) with treatments denoted as k and replicates as n. Analysis of variance (ANOVA) ($\alpha = 0.05$) and Tukey's test for comparison of means (Tukey, 1949) were performed with this experimental design. Treatment effects were considered significant at the 95% significance level if the resulting critical F value was greater than the appropriate tabular value ($F_{[(k-1)], [(k(n-1)]}$). All statistical analyses were performed using JMP IN software (version 4.0.3 (academic), SAS Institute Inc., Cary, North Carolina).

4.4 Results and Discussion

Macro- and microscopic characterization of the fungal isolates, along with the ITS sequences allowed us to recognize two fungal taxa, *Aureobasidium pullulans* and *Hormonema dematioides*. *Aureobasidium* and *Hormonema* species are related to the bitunicate Ascomycete-family Dothideaceae and form black, yeast-like cells (Yurlova et al., 1999). Because both species have similar morphological characteristics and cannot be easily differentiated (Takeo and de Hoog, 1991), we sequenced ITS regions as suggested by Ray et al. (2004) to confirm their identification. See Appendix 2 for light microscope photographic images of *A. pullulans* and *H. dematioides*. Sequence dissimilarity was not observed within the species, but was found between the two species at a level of 12.38%. *A. pullulans* was the most commonly isolated species and our results agreed with previous research (Dickinson, 1972, Bardage and Bjurman, 1998), but only a few strains (three) of *H. dematioides* were isolated from WRC siding. The ITS sequences of our isolates have been deposited in GenBank with the accession numbers DQ787427 (*A. pullulans*) and DQ787428 (*H. dematioides*).

Resistance to β-thujaplicin *in vitro* was assessed for both *A. pullulans* and *H. dematioides*. Statistical analysis suggested that β-thujaplicin concentration had a significant effect on the growth of both *A. pullulans* and *H. dematioides* (Table 4.2). *A. pullulans* isolates exhibited high tolerance to β-thujaplicin. They were able to grow at 32 ppm (5.5 ± 1.1 mm), although growing more slowly than the controls (33.3 ± 1.5 mm). Similar tolerance was observed with some pioneer and decay fungi identified in WRC standing trees and other wood products (Lim et al., 2005). *H. dematioides* showed little β-thujaplicin resistance, with growth completely inhibited at 8 ppm and severely impaired at 2 ppm (14.7 ± 2.1 mm) compared to the control (31.9 ± 1.8 mm).

The five extractives of interest were quantified following analysis of the upper 1mm of WRC wood surfaces following different weathering treatments (figure 4.2). Weathering was found to have a significant effect on extractive concentration (see figure 3 for critical F values). Overall losses for the five extractives measured were 29.8%, 79.9% and 89.4% for UV, WS and UV + WS treatments, respectively. UV alone had the least effect on extractive content.

Our data were consistent with the results of Johnson & Cserjesi (1980) for the depletion of β- and γ-thujaplicin in WRC shakes exposed in natural conditions in Vancouver, British Columbia, Canada. They established that β- and γ-thujaplicin depletion was 25% after a year of exposure and 90% after three years, although this may have been due in part to biodegradation. Extractive loss in our UV + WS treatment using a weather-Ometer was approximately equivalent to one year's weathering loss in natural conditions. Johnson & Cserjesi (1980) also noted that the β- to γ-thujaplicin concentration ratio (average 1:1.5) remained constant during weathering. Compounds such as β-thujaplicin are prone to photo-degradation. Shibata et al. (2003)

Table 4.2 Total fungal growth (mm) of black staining isolates after 21 days on MEA containing various concentrations of β-thujaplicin.

	β-Thujaplicin concentration (ppm)					
	0	2	8	16	32	64
A. pullulans 1	33.33 [1](1.53)[2a]	31.00 (0.93)[a]	26.67 (2.31)[b]	9.33 (2.08)[c]	5.00 (1.00)[d]	-
A. pullulans 2	32.33 (2.52)[a]	32.33 (2.67)[a]	22.67 (5.03)[b]	11.50 (0.5)[c]	5.17 (1.04)[d]	-
A. pullulans 3	34.33 (0.58)[a]	30.33 (2.47)[a]	25.33 (2.52)[b]	10.67 (0.58)[c]	6.33 (1.15)[d]	-
Mean	33.33 (1.54)[a]	31.22 (2.03)[a]	24.89 (3.29)[b]	10.50 (1.05)[c]	5.50 (1.07)[d]	-
H. dematioides 1	30.00 (2.00)[a]	15.00 (3.25)[b]	-	-	-	-
H. dematioides 2	32.33 (0.58)[a]	14.67 (2.09)[b]	-	-	-	-
H. dematioides 3	33.33 (2.87)[a]	14.33 (0.9)[b]	-	-	-	-
Mean	31.89 (1.82)[a]	14.66 (2.08)[b]	-	-	-	-

Note: [1]numbers is parenthesis indicate standard deviation (mm). ANOVA indicated that treatment effect was significant for both *A. pullulans* ($F_{(5,48)} = 324.6$) and for *H. dematioides* ($F_{(5,48)} = 559.6$). Values were considered significant if greater than the tabular value of $F_{(5,48)} = 2.41$ ($\alpha=0.05$). [2]Numbers followed by the same letter were not significantly different ($\alpha=0.05$) according to Tukey's test of multiple comparison of means.

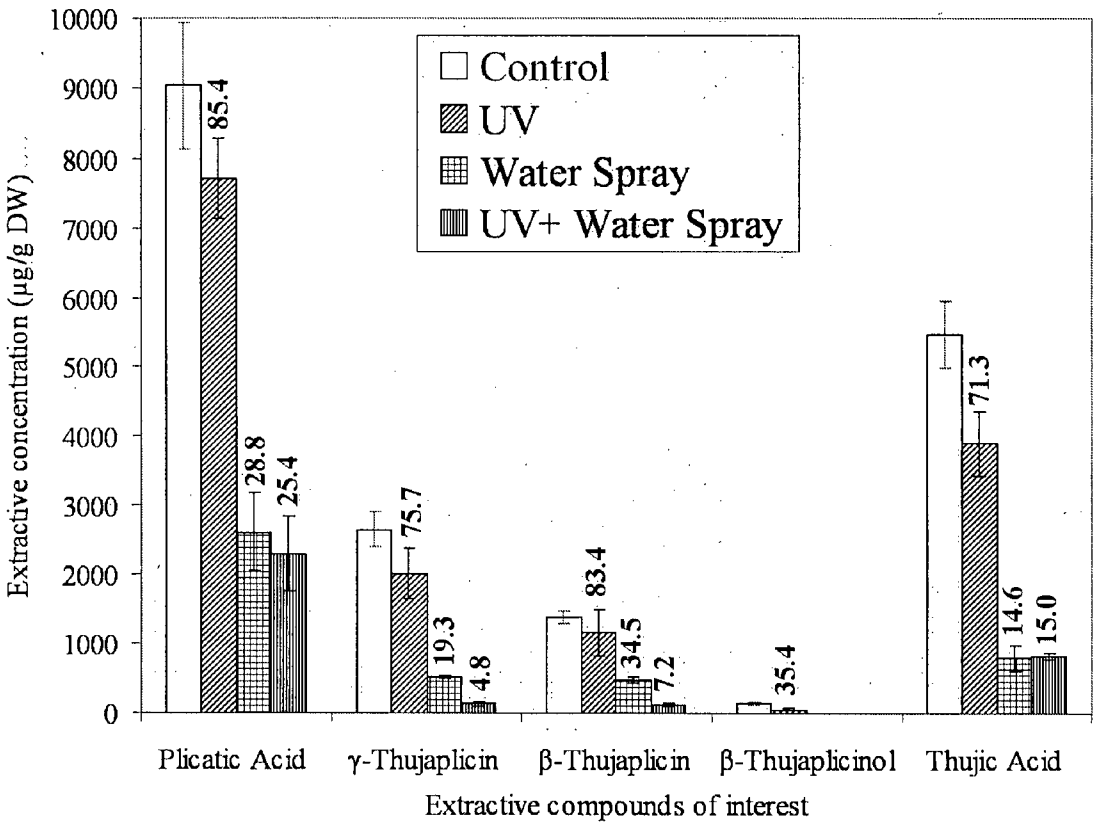


Figure 4.2 Weathering treatment effects on the concentrations (µg/g DW) of WRC extractive compounds of interest.

Note: Y error bars indicate standard deviation. Numbers above error bars represent concentration expressed as a percentage of the control. Critical F values are as follows for each extractive of interest: plicatic acid ($F_{(3,32)} = 68.2$); γ-thujaplicin ($F_{(3,32)} = 253.5$); β-thujaplicin ($F_{(3,32)} = 615.8$); β-thujaplicinol ($F_{(3,32)} = 292.3$); and thujic acid ($F_{(3,32)} = 397.8$). Values were considered significant if greater than the tabular value of $F_{(3,32)} = 2.90$ (α=0.05).

demonstrated that irradiation (210-380 nm) caused photochemical decomposition of *β*-thujaplicin and a measurable loss of antibacterial activity.

We assessed whether *A. pullulans* was likely to colonize WRC when extractives were depleted by weathering compared to un-weathered wood. Growth of *A. pullulans* isolates on weathered siding pieces after six months incubation was estimated qualitatively and quantitatively. Table 4.3.a shows the mean qualitative growth scores that were assigned by visual inspection following different weathering treatments. In addition we recorded photograph images of *A. pullulans* growing on weathered surfaces using a stereo microscope (Figure 4.3). Growth of *A. pullulans* was greatest on surfaces weathered by UV.

Quantitative assessment of growth using spectrophotometer readings to calculate the mean degree of discoloration of weathered wood before and after colonization by *A. pullulans* agreed with these observations (Table 4.3.b). Statistical analysis suggested that treatment effects had a significant impact on the discoloration of weathered surfaces by *A. pullulans* on pine and on cedar. In every case, *A. pullulans* was successfully re-isolated and identified from all siding pieces used in the trial. As expected, *A. pullulans* grew more vigorously on pine siding than on cedar. Weathering by UV-only gave the greatest degree of fungal colonization and discoloration on both cedar and pine, while treatment by UV + WS gave the least. UV consistently promoted fungal colonization. Lignin is highly susceptible to photo-degradation (Chang, 2002; Crestini and Auria, 1996). *A. pullulans* can metabolize products of lignin photo-degradation (Bourbonnais and Paice, 1987; Schoeman and Dickinson, 1997; Sharpe and Dickinson, 1992a, 1993). Our data suggested that UV-only caused less decrease in extractive concentrations in contrast to other weathering treatments. *A. pullulans* showed a high tolerance to the fungicidal

Table 4.3.a *A. pullulans* growth on weathered wood surfaces estimated qualitatively.

	Weathering Treatment			
	No weathering	UV	UV+ water spray	Water spray
Ponderosa Pine	3.58	4.15	2.21	2.01
Cedar	2.25	3.71	1.51	1.47

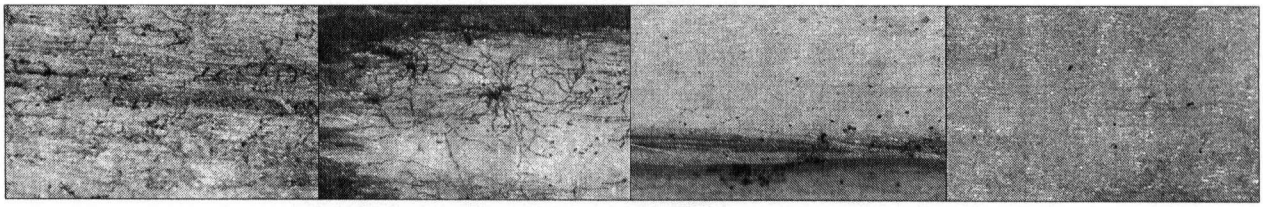
0 = no visible growth; 1 = trace growth, some mild grey/black coloration; 2 = obvious coloration; 3 = significant black stain; 4 = some regions completely covered; 5 = total coverage, no unstained wood.

Table 4.3.b Spectrophotometer measurements of weathered wood discoloration before and after colonization by *A. pullulans*.

	Weathering Treatments			
	Control	UV	UV + WS	WS
Ponderosa Pine	35.66 [1](4.47)[2a]	31.94 (5.92)[a]	5.15 (2.69)[c]	21.17 (0.88)[b]
WRC	8.49 (3.37)[b]	18.98 (7.92)[a]	8.68 (3.37)[b]	8.77 (3.49)[b]

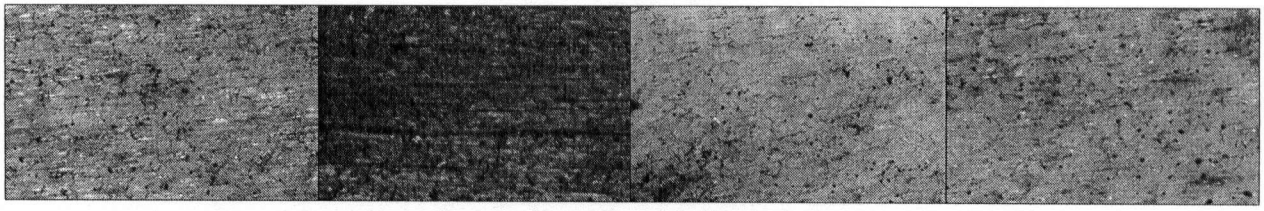
Note: [1]numbers in parenthesis indicate standard deviation from the three replications of the experiment. Critical F values were as follows: on pine ($F_{(3,68)} = 194.4$) and on cedar ($F_{(3,68)} = 25.7$). These values were much greater than the tabular value of $F_{(3,68)} = 2.7$ ($\alpha = 0.05$). [2]Numbers followed by the same letter in each row are not significantly different ($\alpha=0.05$) according to Tukey's test for multiple comparison of means.

WRC



Un-weathered **UV** **UV+WS** **WS**

Ponderosa pine



Un-weathered **UV** **UV+WS** **WS**

Figure 4.3 ***A. pullulans*** **colonization of weathered wood surfaces.**

effects of β-thujaplicin; this suggests that extractive depletion is not necessary for fungal colonization. *A. pullulans* could also colonize un-weathered wood surfaces. Furthermore, the depletion of extractives was greater when water spray was introduced into the treatments and resulted in less colonization than on un-weathered wood surfaces.

4.5 Conclusions

Weathering treatments of WRC siding caused significant changes in the extractive content of exposed surface. UV plus water spray severely reduced extractives but did not lead to increased fungal colonization compared to un-weathered wood. Water spray most likely washed away products of lignin photo-degradation, leaving the wood surface void of accessible carbon sources resulting in decreased fungal growth. In contrast, UV-only treatments reduced extractive contents less than the other treatments but stimulated fungal colonization. *A. pullulans* exhibited high tolerance to the tropolone β-thujaplicin *in vitro*, suggesting that tropolone reductions by weathering may not be required for colonization. It is likely that *A. pullulans* may have competitive advantages in colonizing exposed WRC surfaces because it can use lignin breakdown products as a carbon source, it is resistant to UV due to its melanized cells and it tolerates tropolones.

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Chapter 5

Effects of Leaching on Fungal Growth and Decay of Western Redcedar (*Thuja plicata* Donn)

5.1 Abstract

We tested the effect of leaching on the concentration of second-growth Western redcedar (*Thuja plicata* Donn) (WRC) heartwood extractives and compared the ability of six commonly isolated fungal species to decay leached versus non-leached WRC in standard soil block decay tests. We leached WRC blocks and used reverse phase high performance liquid chromatograpghy (HPLC) and ultra-violet (UV) detection to separate and quantify five key extractives compounds: (-)-plicatic acid, γ-thujaplicin, β-thujaplicin, β-thujaplicinol, and thujic acid. Leaching reduced the concentration of extractives by ~80%. We assessed the extractive-tolerance *in vitro* of fungal species: *Acanthophysium lividocaeruleum, Coniophora puteana, Heterobasidion annosum, Pachnocybe ferruginea, Phellinus sulphurascens,* and *Phellinus weirii* by measuring growth rate (mm/day) on media infused with WRC leachate compared to controls. These data were correlated with the ability of species to decay pine, leached WRC and non-leached WRC.

P. sulphurascens exhibited the lowest extractive-tolerance and caused minimal decay in non-leached WRC but could decay pine and to a lesser degree, leached WRC. *C. puteana, H. annosum,* and *P. weirii* displayed moderate to high tolerance to leachate and caused decay in non-leached as well as in leached WRC, but decay was always greatest on leached WRC and pine suggesting that depletion of extractives promotes decay in these fungi. *A. lividocaeruleum* and *P. ferruginea* exhibited high tolerance to leachate and did not require depletion of

extractives for decay to occur. While *A. lividocaeruleum* clearly caused decay on all types of wood, only small but consistent amounts of decay were observed with *P. ferruginea,* perhaps due to its slow growth rate or its inability to decay wood.

Key words: extractives, extractive-tolerance, decay, fungi, leaching, western redcedar (*Thuja plicata* Donn).

5.2 Introduction

WRC wood products are valued for their natural durability conferred by fungicidal extractive compounds (Barton & MacDonald, 1971) and are used heavily in the manufacture of wood products with exterior residential applications which account for a significant portion of Canada's forest products industry (Gonzalez, 2004). However, such products are still prone to decay and this may be attributable in part to extractive depletion caused by weathering (Chedgy et al., 2005, 2006b). Ultraviolet (UV) radiation from sun light is known to cause photo-degradation of extractive compounds (Coombs and Trust, 1973; Shibata et al., 2003) but can only penetrate the upper 1mm of exposed surfaces (Hon, 1991) and may have a limited effect on the extractive content. Leaching of extractives by precipitation that can penetrate deep within WRC products is more likely affecting extractive concentration. This could result in an increased susceptibility to wood-destroying micro-organisms and a potentially reduced service life.
The extractives of WRC are comprised of a mixture of lignans and tropolone compounds. Several of the tropolones, namely the 'thujaplicins' are reported to exhibit strong antimicrobial activity against a range of wood-inhabiting and plant pathogenic fungi *in vitro* (Inamori et al., 2000; Morita et al., 2004a, 2004b). Tropolones are 2-hydroxy-2,4,6-cycloheptatrien-1-one molecules and their derivatives which possess special characteristic properties due to the 1,2

arrangement of the carbonyl and hydroxyl groups on an unsaturated seven-membered carbon ring (Dewar, 1945). These compounds act as a natural chemical defense shield against pathogen invasion in standing trees (DeBell et al., 1997; Van der Kamp, 1986) and confer durability in cedar wood products (Barton & MacDonald, 1971; Rennerfelt, 1948; Rudman, 1962). Johnson and Cserjesi (1980) reported that in WRC shakes, the two most abundant compounds with anti-microbial activity, γ- and β-thujaplicin were depleted by 25% after one year exposure to natural weather conditions, and by 90% after three years. Biodegradation of extractives by microorganisms may have also contributed to this depletion. Premature replacement of WRC wood products resulting from extractive depletion may be further compounded by the fact that WRC product manufacture increasingly relies on second growth lumber which contains approximately half the extractive content of the best old growth lumber (Barton & MacDonald, 1971; Nault, 1988). This may lead to potentially less durable WRC wood products. To address the decay of WRC, it is necessary to understand the interactions between WRC extractives, leaching and fungal decay. Therefore, the aims of this research were to 1) characterize the effect of leaching on the concentration of extractives in second-growth WRC, and 2) compare the ability of several decay fungal species to decay leached versus non-leached WRC.

5.3 Materials and Methods

5.3.1 Wood Materials

WRC blocks of 19mm on each dimension were manufactured from the outer heartwood of an 80yr old second growth WRC tree which was felled in the UBC Malcolm Knapp research forest, Maple Ridge, British Columbia. Ponderosa pine (*Pinus ponderosa* P. & C. Lawson) sapwood blocks were used as a control species which does not contain any known fungicidal extractives similar to those present in WRC.

5.3.2 Leaching of WRC Blocks and Chemical Analysis

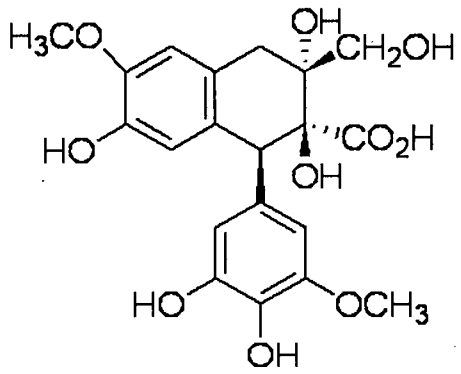
In total, 84 WRC blocks were leached and an additional 84 were not leached, and frozen at -20°C until further use. We followed the American Wood-Preservers' Association (AWPA) standard method of testing wood preservatives by laboratory soil-block cultures (E10-01) (AWPA, 2004) which includes guidelines for leaching of wood samples. WRC blocks were submerged in a volume 50ml of distilled H_20 per block which was replaced daily with fresh water for a period of 14 days. Wood samples were extracted and extractive compounds were quantified as described in Chedgy et al. (2006a). Samples were sliced into 2mm thick sections then finely ground under liquid nitrogen and extracted in methanol with ultrasonication. Separation and analysis of extractives were carried out by reverse phase high performance liquid chromatography coupled with an ultraviolet (UV) detection system. Extractives were quantified (µg/g dry weight, DW) by comparing analyte response with the response factor of an internal standard by a single point calibration. The five compounds of interest were (-)-plicatic acid, γ-thujaplicin, β-thujaplicin, β-thujaplicinol, and thujic acid (Figure 5.1). This experiment followed a completely randomized design (CRD) with two treatments: no leaching and leaching (k=2). Six replicates WRC blocks were used in each case (n=6).

5.3.3 Fungal Growth with WRC Leachate

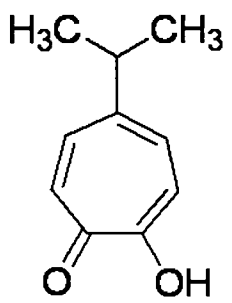
The growth and tolerance of six fungal species to WRC leachate was tested. The fungal species examined were *Acanthophysium lividocaeruleum, Coniophora puteana, Heterobasidion annosum, Pachnocybe ferruginea, Phellinus sulphurascens,* and *Phellinus weirii*. Two strains of each species were used, except for *P. sulphurascens* and *P. weirii* where only one strain was available. The strains used were isolated from in-service WRC products (Lim et al., 2005), and were the most frequently isolated species.

Following the leaching of WRC blocks, the resulting leachate was retained and stored at 4°C in the dark. Approximately 25% of this volume was freeze-dried using an Edwards Modulyo freeze dryer equipped (BOC Edwards Pharmaceutical Systems, Wilmington, Massachusetts) with a ThermoSavant VLP 200 vacuum pump (Thermo Electron Corp., Waltham, Massachusetts). This was accomplished by filling multiple 50ml falcon tubes with 35ml of leachate and frozen at -80°C in a horizontal position. Once frozen, a small hole was pieced in the cap of the falcon tubes to allow air flow. Tubes were then placed into a pre-cooled freeze dryer (-45°C) under a vacuum of 1mbar to freeze-dry for a period of 48 hours. The resulting leachate powder was re-suspended to a concentration of 50mg ml^{-1} in 50% ethanol and filter sterilized. The agar dilution method was used for the antifungal activity tests. Mycelial growth was measured on 25ml 1% malt extract agar (MEA) plates containing various concentrations of WRC leachate.

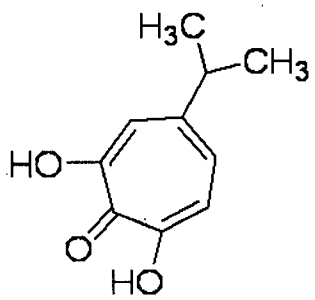
Concentrations of 0, 16, 32, 64, 128 and 256ppm (parts per million) were used and prepared by homogenously spreading an appropriate volume of the stock solution onto the surface of each media plate. Control plates were simultaneously prepared containing 50% ethanol only at the same volumes used to prepare the various leachate containing plates. The medium was then inoculated with a 5 mm plug of agar taken from the edge of actively growing isolate colonies. The cultures were maintained in the dark at 20°C and the growth was evaluated by measuring two perpendicular diameters of the colony every three days. The growth rate (mm/day) was then



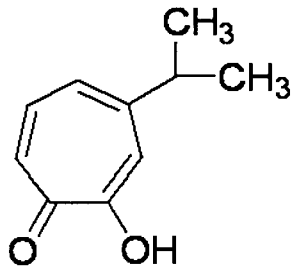
2,3,6-Trihydroxy-7-methoxy2hydroxymethyl
-4-(3',4'-dihydroxy-5'-methoxyphenyl)-
tetralin-3-carboxylic acid
(-)-Plicatic Acid



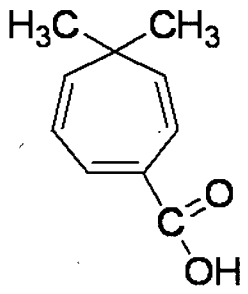
2-Hydroxy-5-isopropyl-2,4,6-
cycloheptatrien-1-one
***γ*-Thujaplicin**



2,7-Dihydroxy-4-isopropyl-2,4,6-
cycloheptatrien-1-one
***β*-Thujaplicinol**



2-Hydroxy-4-isopropyl-2,4,6-
cycloheptatrien-1-one
***β*-Thujaplicin**



7,7-Dimethylcycloheptatriene-
3-carboxylic acid
Thujic Acid

Figure 5.1 Chemical structure and nomenclature used for WRC heartwood extractive compounds examined (Barton and MacDonald, 1971).

estimated for each isolate during its exponential growth phase. Three replicate plates were prepared for each isolate at each leachate concentration.

This trial followed a CRD with six treatments defined as the varying concentrations of WRC leachate (k=6). Three replicates cultures were established for each species strain, at each leachate concentrations, and two strains were used per species. Statistical analysis was performed the mean growth rates (mm/day) calculated from both strains together (n=6), apart for species *P. sulphurascens,* and *P. weirii* were only one strain was used in which case separate statistical analysis was performed (k=6, n=3).

5.3.4 Soil Block Decay Tests

The decay ability of the six fungal species was compared on leached and non-leached WRC blocks, as well as on blocks of ponderosa pine sapwood. The extent of decay was measured by estimating the percentage mass loss (dry weight, DW) of blocks compared to controls after a period of incubation with the various fungal isolates. We followed the standard procedure for laboratory soil block cultures (E10-01) as outlined by the AWPA (2004). All wood blocks used were free of knots, with 2 to 4 rings per cm. Blocks showed no visible evidence of infection by mold, stain or wood-destroying fungi. In the case of pine, no visible concentrations of resin were observed. Before use, pine and WRC blocks were placed in a conditioning room at 20°C and a relative humidity of 65% for 48 hours then sorted by weight into narrow weight range groups. Pine blocks used in the experiment were 3.3g ± 0.1g, and all WRC blocks used in the experiment were approximately 2.1g ± 0.1g. Pine blocks were numbered and the dry weight (DW) of each was calculated by oven drying blocks at 105°C for 24 h. For WRC blocks it was only possible to estimate the DW as several of the extractive compounds of interest are prone to thermal as well

as photo-degradation (Johnson & Cserjesi, 1980; Shibata et al. 2003). In this instance, all WRC blocks originated from heartwood on a single longitudinal axis parallel with growth rings. Blocks were sequentially numbered relative to their position. Prior to use, all blocks (pine, leached and non-leached WRC) were ion beam sterilized (Iotron Industries Canada Ltd., Port Coquitlam, British Columbia).

Glass jars containing soil were prepared as described in the AWPA standard protocol. Feeder strips of dimensions 3 x 28 x 34mm were manufactured from lodgepole pine (*Pinus contorta*) sapwood and placed on the soil surface (one per jar). Jars were autoclaved (with lids) at 103.4 KPa for 30 minutes on two consecutive days. Jars were then inoculated with fungal colonies by placing 10mm x 30mm agar blocks removed from near the leading edge of the mycelium of actively growing colonies. Agar blocks were placed in contact with one edge of the feeder strip and in contact with the soil. Jar lids were loosened by ¼ turn from the fully tightened position to allow limited oxygen flow and placed in an incubation room (25°C with a relative humidity of 70%) until feeder strips were completely covered with mycelium. Test blocks were bought to a moisture content (M_c) of 40% by placing sterilized blocks at 100%+ M_c into a sterile fume hood and allowing them to air dry until the appropriate M_c had been reached. Blocks were then placed on the surface of feeder strips with the tracheids in the vertical orientation to encourage mycelial penetration. All samples were incubated for a period of sixteen weeks. Six replicates were used for each fungal isolate and for each wood type: WRC leached, WRC non-leached and pine sapwood. In addition, for each wood type six controls were used, which were not inoculated with fungal cultures. To reduce the number of jars used in the experiment, two blocks were used per jar.

5.3.5 Statistical Analysis

All experiments followed a completely randomized design (CRD) with treatments denoted as k and replicates as n. A one-way analysis of variance (ANOVA) ($\alpha = 0.05$) and Tukey's test for comparison of means (Tukey, 1949) were performed on data. Statistical analysis was performed using JMP IN software (version 4.0.3 (academic), SAS Institute Inc., North Carolina).

5.4 Results and Discussion

Leaching of WRC blocks resulted in significant losses of the five key extractives. Leached WRC contained ~80% less extractives than non-leached WRC (Figure 5.2). The ability of fungal species *A. lividocaeruleum, C. puteana, H. annosum, P. ferruginea, P. sulphurascens,* and *P. weirii* to grow on media supplemented with varying concentrations of WRC leachate was examined. Table 5.1 shows the mean growth rate (mm/day) of fungal species with different WRC leachate concentrations (ppm) *in vitro*. Statistical analysis suggested that the presence of WRC leachate had a significant effect on the mycelial growth of *A. lividocaeruleum, C. puteana, H. annosum, P. sulphurascens,* and *P. weirii*. However, the presence of WRC leachate did not significantly affect the growth of *P. ferruginea*, even at the highest concentration of 256ppm. The growth rate of *P. ferruginea* seemed to marginally increase with extractive concentration suggesting that it may have the ability to utilize extractives as a carbon source. This will be further explored in future work. *A. lividocaeruleum* and *H. annosum* were also able to grow on plates with 256ppm leachate but at a slower growth rate than on the control plates. Soil block test experiments showed that, in most cases, the six fungal species were able to decay pine sapwood to a greater degree than second growth WRC heartwood. Overall, leached WRC wood was decayed more readily than non-leached WRC (Figure 5.3).

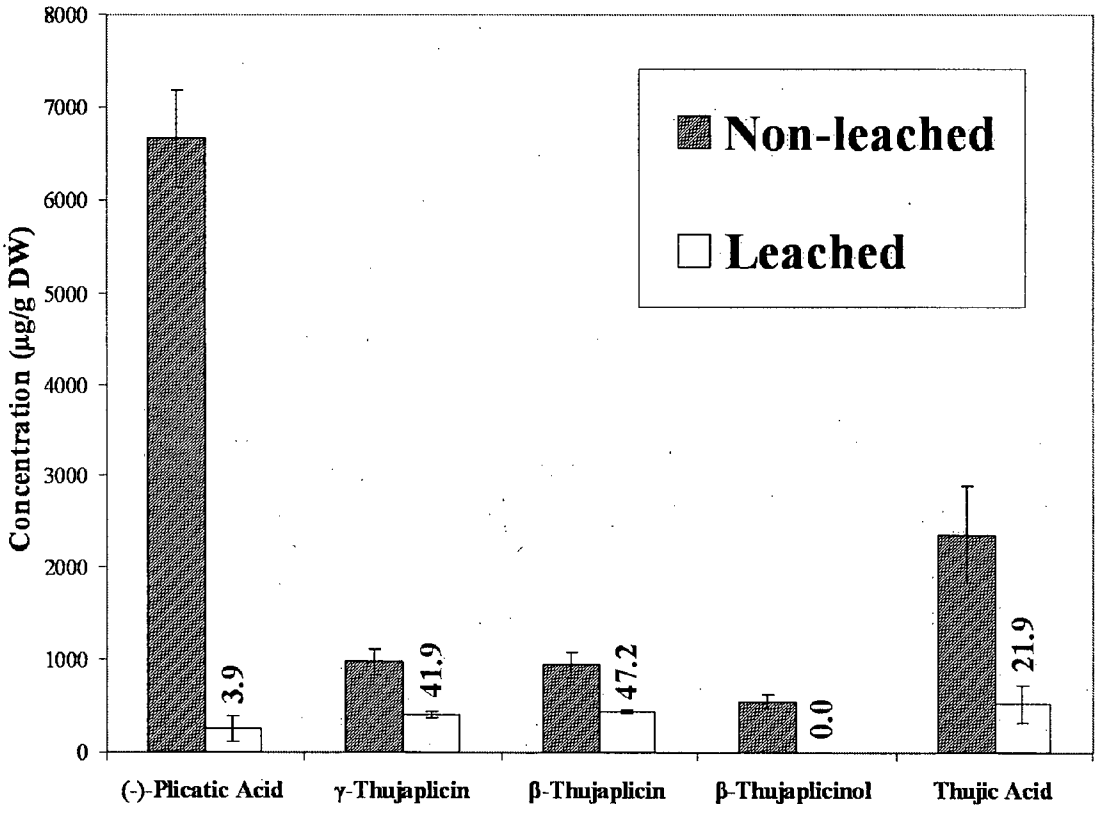


Figure 5.2 Concentration of extractives (µg/g DW) in non-leached and leached WRC heartwood blocks.

Note: Y error bars indicate standard deviation. Numbers above error bars represent concentration expressed as a percentage of the non-leached WRC blocks. ANOVA generated the following critical F values for extractives of interest: (-)-plicatic acid ($F_{(1,10)}$850.3); γ-thujaplicin ($F_{(1,10)}$85.1); β-thujaplicin ($F_{(1,10)}$76.4); β-thujaplicinol ($F_{(1,10)}$36613.7); and thujic acid ($F_{(1,10)}$63.9). Treatment effect is significant if F value is greater than tabular value of $F_{(1,10)}$4.96.

Table 5.1. Mean fungal growth rate (mm/day) on media containing various concentrations (ppm) of leachate.

Species	Leachate Concentration (ppm)					
	0	16	32	64	128	256
A. lividocaeruleum 1	4.17 [1](0.30)	4.31 (0.27)	4.22 (0.19)	3.67 (0.33)	2.64 (0.13)	1.63 (0.15)
2	3.94 (0.24)	4.72 (0.19)	4.89 (0.10)	4.64 (0.27)	1.96 (0.13)	0.99 (0.28)
Mean	4.06 (0.27)[a]	4.51 (0.23)[a]	4.56 (0.14)[a]	4.15 (0.30)[a]	2.30 (0.13)[b]	1.31 (0.22)[c]
C. puteana 1	4.06 (2.34)	3.00 (1.67)	2.06 (1.75)	1.69 (0.34)	0.0 (0.0)	0.0 (0.0)
2	3.86 (1.71)	3.22 (1.39)	2.83 (0.17)	2.19 (0.76)	0.0 (0.0)	0.0 (0.0)
Mean	3.96 (2.02)[a]	3.11 (1.53)[a]	2.44 (0.96)[b]	1.94 (0.55)[c]	0.0 (0.0)[d]	0.0 (0.0)[d]
H. annosum 1	9.58 (0.30)	8.94 (0.63)	9.39 (0.54)	9.28 (0.54)	3.53 (0.42)	0.69 (0.6)
2	5.94 (0.24)	5.81 (0.13)	5.86 (0.13)	4.86 (0.19)	3.81 (0.46)	2.61 (0.53)
Mean	7.76 (0.27)[a]	7.38 (0.38)[a]	7.63 (0.33)[a]	7.07 (0.36)[a]	3.67 (0.44)[b]	1.65 (0.57)[c]
P. ferruginea 1	0.51 (0.30)	0.69 (0.13)	0.61 (0.10)	0.64 (0.02)	0.66 (0.15)	0.67 (0.11)
2	0.47 (0.38)	0.69 (0.05)	0.68 (0.31)	0.52 (0.17)	0.67 (0.12)	0.67 (0.1)
Mean	0.49 (0.34)[a]	0.69 (0.09)[a]	0.65 (0.21)[a]	0.58 (0.10)[a]	0.67 (0.14)[b]	0.67 (0.11)[b]
P. sulphurascens	6.00 (0.17)[a]	1.36 (0.42)[b]	0.0 (0.0)[c]	0.0 (0.0)[c]	0.0 (0.0)[c]	0.0 (0.0)[c]
P. weirii	6.22 (0.35)[a]	4.78 (0.75)[b]	4.47 (0.05)[b]	3.33 (0.17)[c]	0.0 (0.0)[d]	0.0 (0.0)[d]

Note: [1]numbers in parenthesis indicate standard deviation (mm). ANOVA generated the following critical F values for the six species examined: *A. lividocaeruleum* ($F_{(5,30)}$56.87), *C. puteana* ($F_{(5,30)}$13.26), *H. annosum* ($F_{(5,30)}$21.51), *P. ferruginea* ($F_{(5,30)}$2.12), *P. sulphurascens* ($F_{(5,12)}$509.35), and *P. weirii* ($F_{(5,12)}$169.68). Treatments are considered to have a significant effect if the critical F values are greater than the appropriate tabular values, these were $F_{(5,30)}$2.53 and $F_{(5,12)}$3.11 (α=0.05) for this experiment. [2]Numbers followed by the same letter were not significantly different (α=0.05) according to Tukey's test of multiple comparison of means.

For *C. puteana, H. annosum, P. sulphurascens, and P. weirii* statistical analysis suggested that leaching treatments had a significant effect on the amount of decay observed, in this case, loss of extractives resulted in greater decay of WRC. For these species the greatest amount of decay occurred on pine, followed by leached WRC and the least amount of decay was recorded in non-leached WRC. For example, *C. puteana* produces a mean mass loss of ~57% for pine, ~37% for leached WRC, and ~13% for on non-leached WRC. Tukey's test for multiple comparison of means also showed that for *C. puteana* the degree of decay was significantly different ($\alpha = 0.05$) on the three wood types tested. A similar, but less defined pattern was observed for *H. annosum* and *P. weirii* with no significant difference between pine and leached WRC wood with mean % mass loss in the ranges of 30-40% for pine and leached WRC. Non-leached WRC blocks were decayed to a lesser extent at approximately 18-26% mass loss with both fungal species. *P. sulphurascens* only caused decay on pine (~32% loss) and leached WRC (~12%) but could barely grow on non-leached WRC (~2%).

However, treatment effects were not significant for *A. lividocaeruleum and P. ferruginea* suggesting that loss of extractives is not necessary for decay to occur. *A. lividocaeruleum* provided conclusive results from the decay test as it grew well on the three wood types causing mass loss between 18-24%. *P. ferruginea* caused small amounts of decay (~5%) on all three wood types suggesting that extractive content does not affect the growth and decaying ability of this species. However, this result is inconclusive given the low amounts of decay observed, perhaps a function of its slow growth rate. Also noteworthy was the fact that aggressive wood decayers: *A. lividocaeruleum* and *H. annosum* caused mass losses in the range of 25-30% were observed in pine blocks, lower that would have been expected. This may be due to the fact that each fungal species requires different M_c for optimal growth. In the work reported here a M_c of

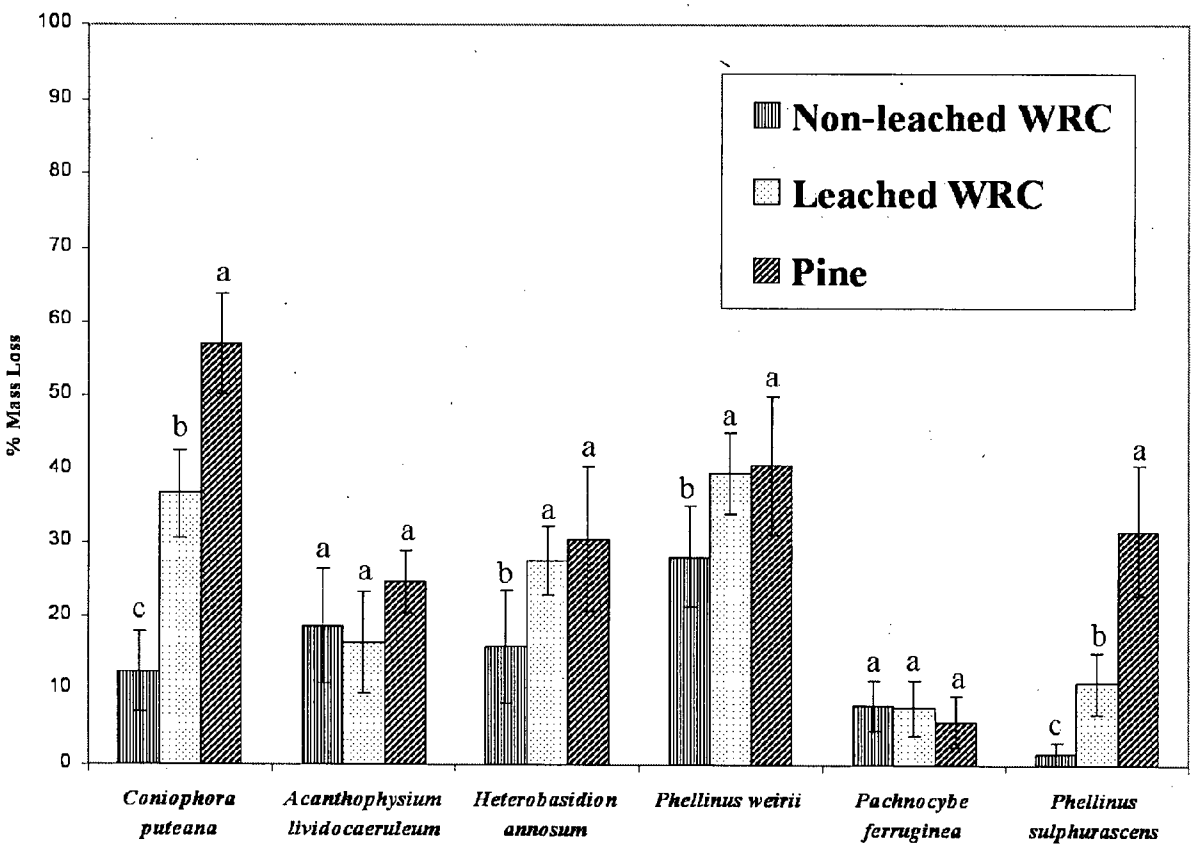


Figure 5.3 Estimation of the degree of decay (% mass loss) of wood blocks by six fungal species of interest.

Note: Y error bars indicate standard deviation. Critical F values are as follows for each of the fungal species: *C. puteana* ($F_{(2,15)} = 94.14$); *A. lividocaeruleum* ($F_{(2,15)} = 1.39$); *H. annosum* ($F_{(2,15)} = 2.25$); *P. weirii* ($F_{(2,15)} = 7.39$); *P. ferruginea* ($F_{(2,15)} = 0.83$).; and *P. sulphurascens* ($F_{(2,15)} = 49.43$). Values were considered significant if greater than the tabular value of $F_{(2,15)} = 3.68$ (α=0.05). Bars with the same letter were not significantly different according to Tukey's test for multiple comparison of means ($\alpha = 0.05$).

40% was created which may be lower than the M_c encountered in natural conditions. *H. annosum* for example is typically causes rot in standing trees, Neilson et al. (1985) reported a typical M_c of 58% in freshly cut WRC. To this end, we intend to repeat the decay test at a slightly higher M_c in order to further explore this aspect.

5.5 Conclusions

We observed that in laboratory conditions, leaching of second-growth WRC heartwood blocks resulted in an 80% loss of extractives. Extractive loss generally resulted in a greater degree of decay of WRC in a standard decay block tests using six of the most commonly isolated fungal species from in-service WRC wood products. This was typified by species such as *P. sulphurascens,* which only caused decay on pine and leached WRC but could barely grow on non-leached WRC. This pattern was apparent to a lesser extent with species *C. puteana*, *H. annosum*, and *P. weirii* which were able to decay non-leached WRC blocks but to a lesser degree than leached WRC and pine blocks. This suggests that for these species leaching of extractives promotes fungal decay of WRC. However, this was not the case for species *A. lividocaeruleum* and *P. ferruginea,* which were able to decay pine, leached WRC and non-leached WRC to a statistically similar degree. Wood moisture content may have influenced the amount of decay observed by the various fungal species in this work, with each species requiring different moisture levels for optimal growth. Several aggressive wood decaying species such as *H. annosum* and *A. lividocaeruleum* caused less decay than expected. We intent to re-explore these aspects of the experiment to ensure that we have a clear representation of the nature of decay of WRC wood products. However, we did establish a clear correlation between tolerance to WRC leachate *in vitro* and the ability to decay leached versus non-leached second growth WRC in soil block decay tests.

P. ferruginea is of particular interest as it exhibited high tolerance to WRC leachate *in vitro* and could decay non-leached WRC blocks. This species is a candidate 'pioneer' species that may detoxify extractives in wood products, paving the way for colonization by less extractive-tolerant decay fungi. We observed low amounts of decay by this species, perhaps due to its slow growth rate. To our knowledge there is no evidence in the literature to suggest that this species can cause wood decay by degrading cellulose, hemicellulose or lignin. We also observed that the growth rate of this species marginally increased when grown on media supplemented with WRC leachate compared with controls. To this end, it is a possibility that it may utilize such extractives as a carbon source. The small mass losses observed our decay test may be attributable to loss of simple sugars or extractives. Further work is required to better understand its role in the decay of WRC products.

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Chapter 6

General Conclusions and Future Work

The aim of this work was to explore the relationship between extractive content of WRC wood products and colonization by micro-organisms that may reduce service life either through decay or discoloration. We investigated the role of weathering in causing depletion of anti-microbial extractives and how this affected colonization by fungi. In addition, we identified extractive-tolerant fungi that did not require depletion of extractives for colonization. These 'pioneer' species may play an important role in the initial modification or detoxification of WRC extractives in wood products, paving the way for colonization by less specialized decay fungi.

We successfully modified a method to extract, separate and accurately quantify extractive compounds using ultra-sonication and reverse phase HPLC. Six compounds that accounted for approximately 67% (w/w) of the total extractive content of WRC heartwood were quantified by comparing analyte response with the response factor of an internal standard by a single point calibration. This method was highly sensitive with limits of detection at 0.6µg/ml for (-)-plicatic acid, 15.6µg/ml for γ-thujaplicin, 15.6µg/ml for β-thujaplicin, 15.6µg/ml for β-thujaplicinol, 1.0µg/ml for thujic acid and 1.8µg/ml for methyl thujate. We showed that grinding heartwood samples under liquid nitrogen produced a 36% increase in extractive recovery yield compared to sliced samples. Finally, extraction using methanol with ultra-sonic frequency provided good recovery of extractives (~90%) with high repeatability (RSD ~5%). This approach enabled multiple samples to be analyzed simultaneously and provided a controlled environment in which a low temperature could be maintained and samples shielded from incandescent light. This

approach was straightforward, highly sensitive and detected extractives present at very low concentrations with a high level of precision. This method could be applied to almost any situation where WRC extractives are required to be quantitated, particularly given the interest in using WRC extractives for pharmaceutical applications ranging from anti-cancer and HIV treatments.

We then developed a novel technique to screen fungi for extractive-tolerance *in vitro*. This was achieved by monitoring growth rates of isolated fungal strains on media inoculated with WRC-FSs. This enabled fungal strains to be exposed to a mixture of extractives that they would encounter in WRC wood products. Furthermore, researchers can overcome the limited availability and cost of pure extractive compounds. From the twelve species screened, results indicated that the basidiomycetes, *Acanthophysium lividocaeruleum* and *Coniophora puteana*, and three ascomycetes, *Aureobasidium pullulans*, *Exophiala heteromorpha*, and *Rhinocladiella atrovirens* had moderate tolerance to WRC extractives. The basidiomycetous species *Pachnocybe ferruginea* exhibited a high level of tolerance to WRC extractives. The five fungal species were commonly isolated from WRC wood products.

In order to determine which of the extractive constituents were affecting fungal growth we estimated the concentrations of individual extractives that leached from WRC-FSs and accumulated into the media using our chemical analytical method. We prepared media supplemented with individual extractive compounds at concentrations corresponding to amount leached from WRC-FSs and inoculated this media with three representative species, *P. ferruginea* (high extractive-tolerance), *R. atrovirens* (moderate extractive-tolerance), and *Phellinus ferreus* (low extractive-tolerance). We concluded that (-)-plicatic acid even at high

concentrations had little fungicidal activity, and β-thujaplicinol also had a minimal effect on growth at the low concentrations leached from WRC-FSs. γ-Thujaplicin and β-thujaplicin exhibited the highest fungicidal activity, while thujic acid also decreased fungal growth at high concentrations. Again, *P. ferruginea* consistently showed a high tolerance to all extractive compounds.

Weathered surfaces of WRC products are also susceptible to black stain caused by fungi such as *Aureobasidium pullulans* which is reported to utilize products of lignin photo-degradation as a carbon source. Extractive contents at the surface of WRC products may form the first line of defense in preventing fungal spore germination. The effect of weathering on extractive concentrations at the surface was characterized and correlated with the ability of this fungal species to colonize weathered surfaces. Simulated weathering treatments using a Weather-Ometer® caused significant changes in the extractive content of exposed surface. UV-only treatments reduced extractive contents less than the other treatments but stimulated fungal colonization. However, UV plus water spray severely reduced extractives but did not lead to increased fungal colonization compared to un-weathered wood. Water spray most likely washed away products of lignin photo-degradation, leaving the wood surface void of accessible carbon sources resulting in decreased fungal growth. *A. pullulans* exhibited moderate to high tolerance to the tropolone β-thujaplicin *in vitro*, suggesting that tropolone reductions by weathering may not be required for colonization. It is likely that *A. pullulans* may have competitive advantages in colonizing exposed WRC surfaces because it can use lignin breakdown products as a carbon source, it is resistant to UV due to its melanized cells and it tolerates tropolones.

Finally, in standard laboratory tests we determined that the effect of leaching on the extractive content of second growth WRC was an ~80% reduction of the five key compounds (-)-plicatic acid, γ-thujaplicin, β-thujaplicin, β-thujaplicinol, and thujic acid. For six fungal species that had been commonly isolated from in service WRC wood products, we observed a correlation between tolerance to WRC leachate *in vitro* and the ability to decay leached versus non-leached second growth WRC in soil block decay tests. *Phellinus sulphurascens* exhibited the lowest tolerance to WRC leachate and caused minimal decay on non-leached WRC but was able to decay leached WRC and Ponderosa pine blocks. *C. puteana*, *H. annosum*, and *Phellinus weirii* exhibited moderate to high tolerance to extractives were able to decay non-leached WRC blocks but to a lesser degree than leached WRC and pine blocks. This suggests that for these species leaching of extractives promotes fungal decay of WRC. *A. lividocaeruleum* and *P. ferruginea* were the most tolerant to WRC extractives and could grow on all types of wood to an equal degree suggesting that these species do not require depletion of extractives by leaching for decay to occur. However, we observed little wood decay by *P. ferruginea* in this work, perhaps due to the slow growth rate of this species.

Extractive tolerant micro-organisms such as *P. ferruginea* are of particular interest for future work as they may be a 'pioneer' species playing an important role in the initial modification or detoxification of WRC extractives in wood products, paving the way for colonization by less specialized decay fungi. In this work we have only demonstrated their tolerance to extractives, yet to date there is no evidence to suggest that they can detoxify fungicidal extractives. In addition, to our knowledge there is no evidence in the literature that this species is capable of decaying wood. The mass loss recorded could be attributable to loss of extractives or simple sugars present in wood. The next step will be to develop techniques in which candidate pioneer

fungal species can be exposed to individual extractive compounds *in vitro* which enable us to monitor the any chemical modifications that may occur to extractives over time. This will provide the industry with a better understand the role of this species and others in causing decay of WRC wood products.

Appendicies Table of Contents

List of Tables

Table of Figures

Appendix I

Fungal Diversity from Western Redcedar Fences and Their Resistance to β-Thujaplicin.

AI.1 Abstract

The work reported here investigated the fungal community inhabiting western redcedar fence material with a focus on species colonizing wood below the surface of which little is known. From seven pieces of fence material, twenty-three different fungal species were isolated and characterized using both traditional morphology and molecular identification methods. The species identified included thirteen ascomycetous and ten basidiomycetous fungi. Isolates were tested for their resistance to β-thujaplicin – one of the principle fungicidal agents of western redcedar heartwood extractives. Generally, ascomycetous fungi exhibited greater resistance to β-thujaplicin than basidiomycetous fungi. Interestingly, three ascomycetous and two basidiomycetous species frequently isolated had high tolerance to this compound. These species could be candidate 'pioneer' species that invade and detoxify WRC extractives, paving the way for colonization by decay fungi.

Key words: ascomycota, basidiomycota, β-thujaplicin, pioneer fungi, western redcedar (*Thuja plicata* Donn.), wood products.

AI.2 Introduction

Canada's and BC's value-added forest product industries depend in part on the unique qualities of some of this country's native wood species. Western redcedar (WRC) (*Thuja plicata* Donn) is a well-known and commercially important coniferous tree species common in the Pacific northwest. Its heartwood is valued for the natural durability conferred by fungicidal agents in its extractives (Wethern, 1959); in particular, by a group of tropolone compounds known as 'thujaplicins' (Rennerfelt, 1948). Of the several classes of thujaplicins characterized, β-thujaplicin (2-hydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one) appears to be the most prevalent and effective against decay fungi (Arima et al., 2003; Erdtman and Gripenberg, 1948; Inamori et al., 1999; 2000; Trust and Coombs, 1973). Despite such extractives, decay fungi are still a major factor in product failure in service. Furthermore, products manufactured from second growth WRC may have lower extractive contents than the best of the old growth (Nault, 1986). Optimizing the service life and value of products that rely on such natural protection requires an understanding of how extractives and fungal communities interact and evolve in service.

In WRC trees 'pioneer' fungal species can detoxify fungicidal extractives, clearing the way for less specialized fungi to colonize and decay wood freely (Jin, 1987; Van der Kamp, 1975). However, to our knowledge, the fungal succession in biodeterioration has not been documented for WRC products, and little is known about the microbial communities that these products harbor. While research groups have reported a limited number of decay fungi from WRC utility poles (Eslyn and Highlery, 1976; Morrell et al., 2001; Scheffer et al., 1984) and shingles/shakes (Smith and Swan, 1975), these studies relied on species identification by morphology only. This approach has two major limitations. Firstly, fungi in artificial cultures often exhibit fewer

morphological features than in their natural environments. This impedes identification, especially for fungi that lack asexual spores; e.g. Homobasidiomycetes. Secondly, a fungal species' characteristics can vary when it is grown on different media or under different culture conditions. The wealth of sequence information that has been compiled in databases means that it is now possible to identify fungi at a far higher resolution using molecular techniques than can be achieved using morphological methods. However, databases contain sequences from only a fraction of all known species to date, in this case morphological methods are still being relied on heavily (Allen et al., 2003; Wirsel et al., 2001).

AI.3 Materials and Methods

AI.3.1 Collection Sites and Fungal Isolations

WRC fence material was collected in Vancouver, B.C, Canada. Five pieces (125 X 19 X 4.5 cm) were from a fence in service between 1960 to 2001 and two (166.5 X 23 X 8 cm) from a fence in service between (1970 to 2001). Each piece was sliced horizontally into 2 cm blocks. Each block was further divided into five lateral sections. Wood flecks taken from within and near decay pockets were aseptically detached, briefly flamed to remove contaminating surface microflora and plated (Figure A1.1). Eight decay areas from seven fence pieces were labeled fence A - G. A 1% malt extract agar (MEA) was used for isolating the general microflora and 1% MEA with benomyl (BMEA) was used for the basidiomycetous fungi (Clubbe and Levy, 1977). The plates were incubated at room temperature for several weeks with fungi routinely sub-cultured from mycelial margins to new plates in order to obtain pure cultures. Species identification via classical methodology was achieved by macro- and micro-morphological analyses using taxonomic guides and standard procedures (Arx, 1981; Barnett and Hunter, 1987; Carmichael et

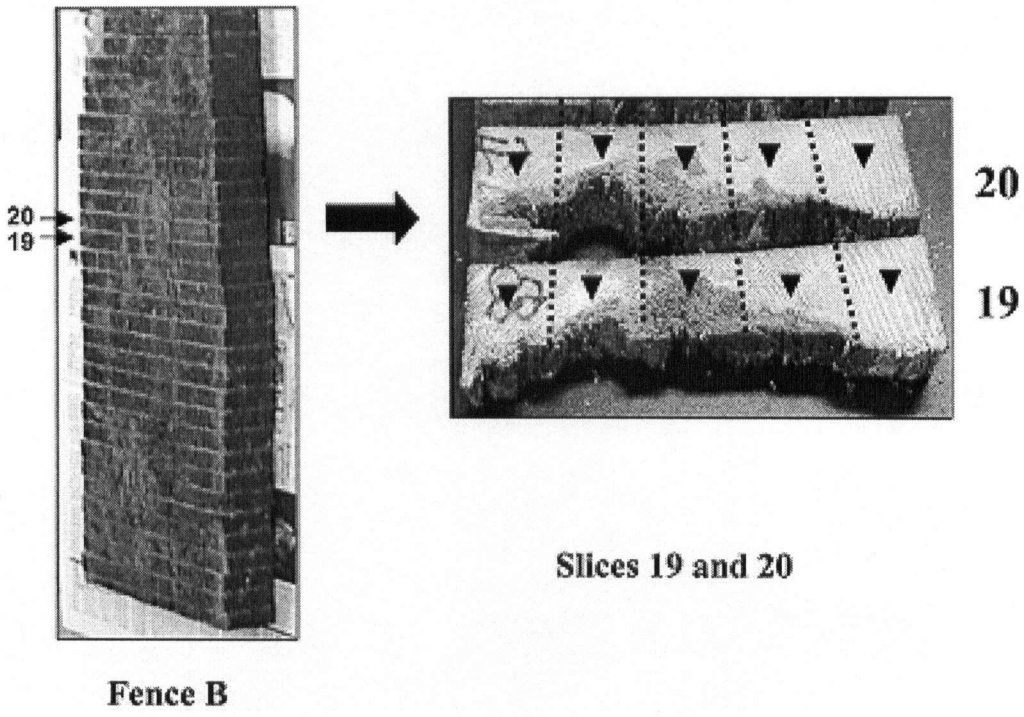

Figure AI.1 Schematic diagram for fungal isolation from WRC fences. In the example shown, fence material (Fence B) was cut horizontally into 2 cm slices and then divided further into five lateral sections. Wood flecks (shown by bold arrows) from the central regions were aseptically detached and inoculated onto plates.

al., 1980; Cole and Kendrick, 1973; Ellis, 1971; Nobles, 1965; Schol-Schwarz, 1970; Stalpers, 1978; Wang and Zabel, 1990). This was complemented by molecular techniques for species identification.

AI.3.2 DNA Extraction, PCR and Sequencing

DNA was extracted from mycelium scraped from the fungal colonies and placed into micro-centrifuge tubes with 300 µl of extraction buffer [100 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS]. The mixture was vortexed for 10 s, incubated at 75°C for 30 min. 4/5 volumes of glass beads were then added into the tube and vortexed for a further 10 min. DNA was purified via a two step phenol-chloroform extraction and precipitated with one volume of iso-propanol then centrifuged immediately at 12,000 rpm at room temperature for 10 min. After removing the supernatant, the pellet was washed with 70% ethanol, allowed to air dry and resuspended in 40 µl of distilled water. The extracted DNA was stored at -20°C until further use. To achieve PCR amplification of the internal transcribed spacer (ITS) regions, fungal universal primers (ITS5 and ITS4) and the basidiomycetous specific reverse primer (ITS4B) with ITS5 primer (Gardes and Bruns, 1993; White et al., 1990) were used. Amplification was performed as described by Lee et al. (2000). Usually 3 µl of each PCR product was used for the electrophoresis on 0.5% agarose gel containing EtBr in Tris-acetate EDTA (TAE) buffer. The PCR product sizes were determined by comparison to a 1 kb DNA marker (GIBCO BRL, U.S.A.). The PCR products were purified using a Qiaquick PCR Purification Kit (Qiagen Inc.). Sequencing was performed on an ABI 3700 automated sequencer (Perkin-Elmer Inc. USA) at the DNA synthesis and Sequencing Facility, MACROGEN (Seoul, Korea). All of the nucleotide sequences determined in this work have been deposited in the GenBank, their accession numbers

are shown in Table A1.1. The ITS region sequences were analyzed using BLAST in order to find the most similar available database sequences. The closest matched sequence for each species was shown in Table A1.1.

AI.3.3 Inhibition of Fungal Growth by β-Thujaplicin

The agar dilution method was used for the antifungal activity tests. Mycelial growth was measured on MEA plates with various concentrations of β-thujaplicin. A 10 mg ml^{-1} stock solution was prepared in 50% ethanol and kept in the dark at 4°C. Concentrations of 0, 2, 8, and 32 ppm were prepared. Vigorous mixing of the medium prevented the precipitation of the chemical. Ethanol had no effect on the hyphal growth at the low concentrations present in the growth medium. The medium was inoculated with a 5 mm plug of agar taken from the edge of actively growing isolate colonies. The cultures (three replicates used) were maintained in the dark at 20°C and growth was evaluated after 12 days by measuring two perpendicular diameters of the colony.

AI.4 Results and Discussion

AI.4.1 Isolation and Morphological Grouping of Fungi

Regions of wood decay generally occurred in or around the ends of fence panels. A total of 144 fungal isolates were recovered from 303 sampling sites in 8 regions of decay present on 7 WRC fence sources. The highest count of fungal isolates was recorded in the decay area of fence G (39 isolates: 27.1%), while the lowest was recorded in fence A and B (7 isolates each : 4.7%). The fungal diversity was highest in decay area F1 (10 different types) and lowest in decay area A (3 types). The macro- and microscopic characterization of the isolates allowed us to recognize 23

different fungal taxa, including thirteen ascomycetous fungi and ten basidiomycetous fungi. The basidiomycetes were identified using three characteristics: growth on BMEA, presence of clamp connection, and PCR amplification using forward primer ITS5 and basidio-specific reverse primer ITS4B, (Gardes and Bruns, 1993). However, PCR using ITS5 and ITS4B failed to amplify the ITS regions of two basidiomycetous isolates, *Cerinosterus luteoalba* and WRCF-B2. BMEA permits isolation of basidiomycetous fungi and prevents the growth of most microfungi (Clubbe and Levy, 1977). Two ascomycetous fungi, WRCF-A2 and *Phialophora* sp. 1, grew on BMEA while a basidiomycetous fungus, WRCF-B2, did not.

Among the ascomycetous species we recognized *Oidiodendron griseum*, *Phialophora* spp., *Rhinocladiella atrovirens*, *Sporothrix* spp., and three unidentified Ascomycota (WRCF-A1, WRCF-A2, and WRCF-A3). *O. griseum, Phialophora* spp., *Sporothrix* spp., and WRCF-A2 were isolated from a limited number of sites, while three species, *R. atrovirens,* WRCF-A1, and WRCF-A3 were isolated from a broad range of decay areas. These ascomycetous fungi were easily identified by their asexual structures. The most commonly isolated species was from the genus *Phialophora* (Table A1.1). In this genus six species, including *P. lignicola*, *P. versicola* and four unidentified, were recognized. Two *Sporothrix* species were also isolated but their specific identification was not pursued.

Most ascomycetous fungi found in this study were ubiquitous; this is concurrent with other research groups' findings. For example, *O. griseum* has been isolated from soil of cedar and spruce bogs, wood pulp (Barron, 1962), and pulp and paper samples (Wang. 1965), it causes a superficial discoloration of wood (Käärik, 1980). *Phialophora* species are recognized as staining agents of wood products in service and also as important soft rot fungi (Eaton and Hale, 1993).

Table AI.1 List of fungi isolated in this work; source, their characteristics and GenBank accession numbers.

Fungal Species	GenBank Acc No.	Source (no. of isolates)[1]	Isolation site[2]	BMEA Growth	CC	ITS5 / ITS4B	ITS5 / ITS4	Closest match in BLAST	Identity [%][3]
Basidiomycota									
Acanthophysium lividocaeruleum	AY618666	C(2), D(2), G(9)	B	G	P	A	A	Acanthophysium lividocaeruleum [AF506400]	282/289 (97%)
Cerinosterus luteoalba	AY618667	D(1), F1(1)	D	G	NP	NA	A	Sporobolomyces symmetricus [AY364836]	119/123 (96%)
Hyphoderma praetermissum	AY618668	A(2), E(3), F1(4)	D	G	P	A	A	Hyphodontia flavipora [AF455399]	168/173 (97%)
Pachnocybe ferruginea	AY618669	A(2), B(1), D(1), F1(10), F2(3), G(2)	B	G	NP	A	A	Septobasidium sp. [AB043972]	211/229 (92%)
Stereum sanguinolentum	AY618670	G(2)	D	G	P	A	A	Stereum sanguinolentum [AY089730]	443/466 (95%)
WRCF-B2	AY618671	F1(6), G(2)	D	NG	P	NA	A	Rhodotorula nothofagi [AF444641]	151/155 (97%)
WRCF-B4	AY618672	B(2), F1(1)	D	G	NP	A	A	Hyphodontia radula [AF145580]	555/557 (99%)
WRCF-B5	AY618673	C(2), G(5)	D	G	P	A	A	Butlerelfia eustacei [U85800]	386/413 (93%)
WRCF-B7	AY618674	F2(2)	D	G	NP	A	A	Phanerochaete sordida [AY219381]	580/594 (97%)
WRCF-B9	AY618675	F2(2)	D	G	NP	A	A	Phlebia livida [AB084618]	363/391 (92%)
Ascomycota									
Oidiodendron griseum	AY618676	E(1), F1(4), F2(1)	B	NG	NP	NA	A	Oidiodendron griseum [AF062794]	345/345 (100%)
Phialophora lignicola	AY618677	E(3)	O	NG	NP	NA	A	Salal root associated fungus [AF149081]	482/485 (99%)
Phialophora versicola	AY618678	B(1), F1(1)	B	NG	NP	NA	A	Ectomycorrhizal isolate [AJ430410]	464/465 (99%)
Phialophora sp. 1	AY618679	E(4)	D	G	NP	NA	A	Ascomycete sp. [AY354276]	335/350 (95%)
Phialophora sp. 2	AY618680	E(3), F1(2)	B	NG	NP	NA	A	Cadophora fastigiata [AY249073]	497/497 (100%)
Phialophora sp. 3	AY618681	F2(3), G(3)	B	NG	NP	NA	A	Phialophora sp. [AY465463]	469/471 (99%)
Phialophora sp. 4	AY618682	D(2)	D	NG	NP	NA	A	Phialophora sp. [AY465462]	467/500 (93%)
Rhinocladiella atrovirens	AY618683	E(2), F1(6), F2(1), G(9)	B	NG	NP	NA	A	Rhinocladiella atrovirens [AB091215]	563/567 (99%)
Sporothrix sp. 1	AY618684	F1(2), F2(1)	D	NG	NP	NA	A	Sporothrix schenckii [AF484468]	424/440 (96%)
Sporothrix sp. 2	AY618685	C(3)	D	NG	NP	NA	A	Ophiostoma grandicarpum [AJ293884]	171/174 (98%)
WRCF-A1	AY618686	A(3), B(1), C(1), D(1), E(2)	D	NG	NP	NA	A	Leaf litter ascomycete [AF502745]	422/441 (95%)
WRCF-A2	AY618687	C(3), G(4)	O	G	NP	NA	A	Oidiodendron myxotrichoides [AJ635314]	267/288 (92%)
WRCF-A3	AY618688	B(2), F1(2), F2(3), G(3)	B	NG	NP	NA	A	Phialocephala dimorphospora [AF486121]	486/495 (98%)

Note: G, growth; NG, no growth; CC, Clamp connection; P, present; NP, not present; A, amplification; and NA, no amplification. [1] Number of sampling point (isolates) collected from each fence: A, 16(7); B, 35(7); C, 33(11); D, 26(7); E, 30(18); F1, 56(39); F2, 25(16); and G, 82(39). [2]D, decay pocket; O, outside of decay pocket; and B, both regions.

[3]Identity [% similarity] was derived from matched nucleotide/compared nucleotide in GenBank.

R. atrovirens originally isolated from material on decayed wood was also found in wood products (Barnett and Hunter, 1987). Many *Sporothrix* species are the anamorphs of *Ophiostoma* and *Ceratocystis* (Domsch et al., 1980), some of them are commonly found in creosote-treated wood products (Wang and Zabel, 1990).

Among the Basidiomycetes five isolates were identified to the genus or species level by morphological features. The most frequently isolated species was *Pachnocybe ferruginea*; it was present in most of the decay areas except in fence C and E. This species has also been reported on creosote-treated western redcedar poles by Warren and Marshall (1986). Two other species, *Acanthophysium lividocaeruleum,* easily recognized by its scattered clamp connections, and gloeocystidia (Nakasone, 1990), and *Hyphoderma praetermissum,* with its white mat with subtomentous to short-woolly, nodous septate, and spathulate cystidia, were also frequently isolated. One isolate of *Stereum sanguinolentum* was identified by its simple septate, scattered single, double, or multiple clamps, and cystidium-like structures. This species is the only *Stereum* species that occurs primarily on gymnosperms. All of the above basidiomycetous fungi have been associated with white rot of various softwoods and wood products in North America (Eslyn, 1970; Gilbertson, 1974; Ginns, 1986; Lemke, 1964; Scheffer et al., 1984; Zabel et al., 1985).

An orange *Sporothrix* colony found in D and F1 fences that we initially identified as *Sporothrix luteoalba* based on its morphology, but using DNA sequence analysis it was renamed *Cerinosterus luteoalba.* Some *Sporothrix* species are reported as the anamorphs of the basidiomycetous genus *Cerinomyces* (Dacrymycetaceae), and Moore (1987) erected the new genus *Cerinosterus* to accommodate species of *Sporothrix* having dolipores and imperfect

parenthesome septa. Finally, some unknown Basidiomycetes WRCF-B2, and WRCF-B5 were isolated with high frequencies but rarely from the decay sites, while WRCF-B7 and WRCF-B9 were isolated only from a decay site (Table A1.1).

AI.4.2 Molecular Analysis of Fungal Isolates

In order to discriminate the isolates to the species level, the ITS regions were amplified. The amplified products ranged from 640 to 770 bp for the Basidiomycota and from 570 to 660 bp for the Ascomycota. However, for two isolates, *Phialophora versicola* and WRCF-A3, the amplified products were larger than the other species reported in this work, at about 1100 bp and 927 bp, respectively. The two fungi have introns of 520 bp for *P. versicola* and 340 bp for WRCF-A3 located near the 3' end of the 18S rDNA (data not shown). These introns contained four conserved regions, a characteristic of group I introns (Cech, 1988; Dujon, 1989). Blast searches revealed high similarity between the intron sequences of WRCF-A3 and a *Phialographium*-like fungus (AB038422), while the intron sequence of *P. versicola* matched closely those of *Lachnum sclerotii* (AF505520), *Rhabdocline parkeri* (AF462428), *Cadophora gregata* f. sp. *adzukicola* (AF056487), and *Hymenoscyphus ericae* (AY394907). Although phylogenetically distant, these species are coniferous pathogens or ectomycorrhizal fungi.

Sequences of 18S and LSU rDNA regions have been used for fungal identification in many ecological studies (Hunt et al., 2004; Kernaghan et al., 2003; Tedersoo et al., 2003). In addition to these regions, ITS sequence comparison is regarded as an excellent tool for identifying unknown fungi to broad species groups or genera (Horton and Bruns, 2001). The ITS sequence data enabled the linkage of most morphologically unidentifiable fungi to established genera. For example, WRCF-B5 belongs to the genus *Butlerelfia*, WRCF-B7 to *Phanerochaete*, WRCF-B9

to *Phlebia*, WRCF-A2 to *Oidiodendron*, and WRCF-A3 to *Phialocephala* (Table A1.1). At this stage of analysis, WRCF-B4 was identified as *Hyphodontia radula* since both sequences were 99% similar. *Phialophora* sp. 2 was identified as *Phialophora fastigiata* (telemorph – *Cadophora fastigiata*) with 100% sequence similarity.

Five *Phialophora* species were positioned in five distantly related clades in the ITS phylogenetic tree (data not shown). This result is consistent with previous work that suggested that the genus *Phialophora* is clearly polyphyletic (Gams, 2000). Interestingly *P. versicola* and *P. lignicola* were closely related to salal root associated fungi, which were isolated from Vancouver Island (Allen et al., 2003). Some *Phialophora* species are known to form ectendomycorrhizal relationships with *Pinus* and *Larix*, and have also been observed forming ericoid mycorrhizal with *Gaultheria shallon* (Monreal et al., 1999; Yu et al., 2001), though their ecological functions are not well understood. It is important to note that *Phialophora* species have different phylogenetic histories and ecological roles.

Pachnocybe ferruginea, Cerinosterus luteoalba and WRCF-B2 were matched to members of a primitive order of Basidiomycota. These results might explain why *C. luteoalba* and WRCF-B2 were not amplified by basidio-specific primers. *P. ferruginea* was closely related to *Septobasidium* sp. which was classified as Urediniomycetes. This is consistent with previous results on 5S rDNA and large subunit rDNA sequence analysis (McLaughlin, et al., 1995; Walker, 1984). *P. ferruginea* described first as a Hyphomycetes with reddish brown synnemata by Ellis (1971) was later transferred to Heterobasidiomycetes because of its simple septal pore structure (Kropp and Corden, 1986; Oberwinkler and Bandoni, 1982). The ITS sequence of *C.*

luteoalba confirmed its position within the Basidiomycota instead of Ascomycota, which coincided with Moore's (1987) suggestion.

Overall there was a good agreement between morphological and ITS-sequence based approaches. However, due to the limited ITS sequence data within the database the closest matches of some fungal isolates could not be established. Specifically, ITS sequences of *C. luteoalba, H. praetermissum* and WRCF-B2 had matches to only 5.8S rDNA region sequence of *Sporobolomyces symmetricus* (AY364836), uncultured fungus (AY241671) and *Rhodotorula nothofagi* (AF444641), respectively.

AI.4.3 β-Thujaplicin Resistance and Fungal Colonization

Although β-thujaplicin concentration varies within WRC trees (MacLean and Gardner, 1956), it inhibits many decay fungi at concentrations between 10 to 20 ppm (Inamori et al., 2000; Rennerfelt, 1948). In our work, β-thujaplicin showed some antifungal activity against most of the fungi examined (Table A1.2). Most basidiomycetous fungi tested were inhibited by concentrations between 2 and 8 ppm, while most of the ascomycetous fungi tested were affected at concentrations between 8 and 32 ppm. Our results were consistent with those of Rennerfelt (1948), who also showed that some ascomycetous fungi had higher resistance to thujaplicin than decay fungi. However, two basidiomycetous species, *P. ferruginea* and *A. lividocaeruleum*, and three ascomycetous species, *Oidiodendron* sp. (WRCF-A2), *Phialophora fastigiata* (*Phialophora* sp. 2) and *Phialophora* sp. 3, had high tolerance to this compound (Table A1.2). That these fungi were isolated both outside and in the center of decay areas suggests that they may be pioneer fungi in WRC fences. Such fungi may tolerate high concentrations of inhibitors,

Table AI.2 Effect of the various concentrations of β-thujaplicin on fungi isolated.

Fungi (identification by ITS sequence)	Fungal growth[1] (mm)			
	0 (Control)	2 ppm (mg/L)	8 ppm (mg/L)	32 ppm (mg/L)
Basidiomycota				
Acanthophysium lividocaeruleum	31.5 (1.3) a[2]	27.0 (1.3) b	23.8 (0.3) c	15.5 (0.9) d
Cerinosterus luteoalba	5.7 (0.6) a	0.0 (0.0) b	0.0 (0.0) b	0.0 (0.0) b
Hyphoderma praetermissum	13.8 (0.8) a	3.8 (0.8) b	0.0 (0.0) c	0.0 (0.0) c
Pachnocybe ferruginea	7.1 (0.5) a	6.9 (0.6) a	7.0 (0.3) a	5.1 (0.3) a
Stereum sanguinolentum	39.0 (1.0) a	14.3 (1.2) b	0.8 (0.3) c	0.0 (0.0) c
WRCF-B2	9.8 (0.3) a	8.1 (0.4) a	1.8 (0.3) b	0.0 (0.0) b
WRCF-B4 (*Hyphodontia radula*)	12.3 (0.6) a	0.0 (0.0) b	0.0 (0.0) b	0.0 (0.0) b
WRCF-B5 (*Butlerelfia* sp.)	15.3 (0.6) a	0.8 (0.3) b	0.0 (0.0) b	0.0 (0.0) b
WRCF-B7 (*Phanerochaete* sp.)	85.3 (0.6) a	23.0 (1.0) b	4.8 (0.3) c	0.0 (0.0) d
WRCF-B9 (*Phlebia* sp.)	35.7 (0.6) a	32.7 (1.2) b	2.8 (0.3) c	0.0 (0.0) d
Ascomycota				
Oidiodendron griseum	3.2 (0.3) a	2.3 (0.3) a	2.0 (0.0) ab	0.0 (0.0) b
Phialophora lignicola	10.7 (1.2) a	9.8 (1.0) ab	7.7 (1.3) b	0.0 (0.0) c
Phialophora versicola	6.7 (0.3) a	5.2 (0.8) a	3.8 (0.3) b	0.0 (0.0) c
Phialophora sp. 1	16.0 (1.0) a	15.7 (1.2) a	7.3 (1.5) b	0.0 (0.0) c
Phialophora sp. 2 (*P. fastigiata*)	17.8 (0.3) a	17.2 (0.3) a	16.5 (0.5) ab	14.3 (0.6) b
Phialophora sp. 3	7.3 (0.3) a	7.0 (0.0) ab	7.0 (0.5) ab	4.8 (0.3) b
Phialophora sp. 4	9.7 (0.6) a	7.2 (0.3) b	0.0 (0.0) c	0.0 (0.0) c
Rhinocladiella atrovirens	3.7 (0.6) a	2.8 (0.3) a	1.4 (0.4) b	0.0 (0.0) b
Sporothrix sp. 1	11.7 (0.6) a	10.8 (0.8) ab	9.7 (0.6) b	0.0 (0.0) c
Sporothrix sp. 2	13.0 (0.5) a	11.8 (0.3) a	9.8 (0.3) b	0.0 (0.0) c
WRCF-A1	8.7 (0.6) a	7.2 (0.3) ab	5.0 (0.5) b	0.0 (0.0) c
WRCF-A2 (*Oidiodendron* sp.)	31.7 (0.6) a	31.2 (0.3) a	26.8 (0.3) b	8.8 (0.3) c
WRCF-A3 (*Phialocephala* sp.)	10.8 (0.8) a	6.8 (0.3) b	1.8 (0.3) c	0.0 (0.0) c

[1] Fungal growth was measured 12 days after exposure on MEA. Values are mean of three replicates and standard error in parenthesis; [2] Numbers followed by the same letter in each row are not significantly different ($\alpha = 0.05$) according to the Duncan's method.

and, by detoxifying them, permit other ascomycetous and basidiomycetous fungi to become established.

These suggestions agree with the conclusions of Findlay (1966), Chesters (1950), Meredith (1960) and Shigo (1967) on WRC logs and fallen trees, and of Van der Kamp (1975) on standing trees. They showed that heartwood is invaded by a succession of fungi that allow decay to occur. Later, Jin et al. (1988) demonstrated that a pioneer *Sporothrix* fungus that was consistently isolated from the outer heartwood in WRC tree transformed thujaplicins into thujin, which was nontoxic to decay fungi.

AI.5 Conclusions

Complementing molecular techniques with traditional morphology based methods greatly increased the accuracy and speed of fungal species identification. Compared to earlier studies on WRC wood products, a variety of different types of wood-rotting basidiomycetous fungi were isolated from WRC fence materials. Identified Homobasidiomycetes in the present study were corticioid fungi, which cause white rot, and most of them were not commonly associated with WRC products. The isolates' tolerance to β-thujaplicin, as well as location and frequency of isolation may provide evidence of pioneer species involved in a succession of fungi that ends in decay of WRC products. Two basidiomycetous species: *Pachnocybe ferruginea* and *Acanthophysium lividocaeruleum*; three ascomycetous, soft-rot fungal species: *Oidiodendron* sp. (WRCF-A2), *Phialophora fastigiata* (*Phialophora* sp. 2), and *Phialophora* sp. 3 might be pioneer fungi in WRC fence decay. These findings contrast with previous research that suggested

basidiomycetes generally followed ascomycetous, soft-rot fungi (Butcher, 1968; Corbett and Levy, 1963; Duncan, 1960). They might facilitate the entry of other ascomycetous and basidiomycetous (decay fungi) into WRC fence materials. Therefore, these pioneer fungi may play an important role in deterioration of WRC fences. Further research is needed to establish chemical mechanisms of extractive detoxification by these pioneer species.

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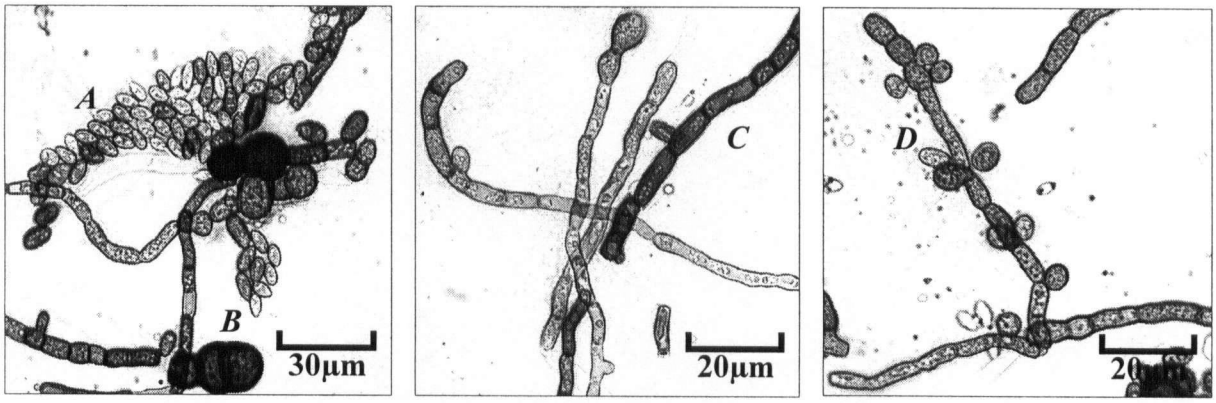
Appendix II

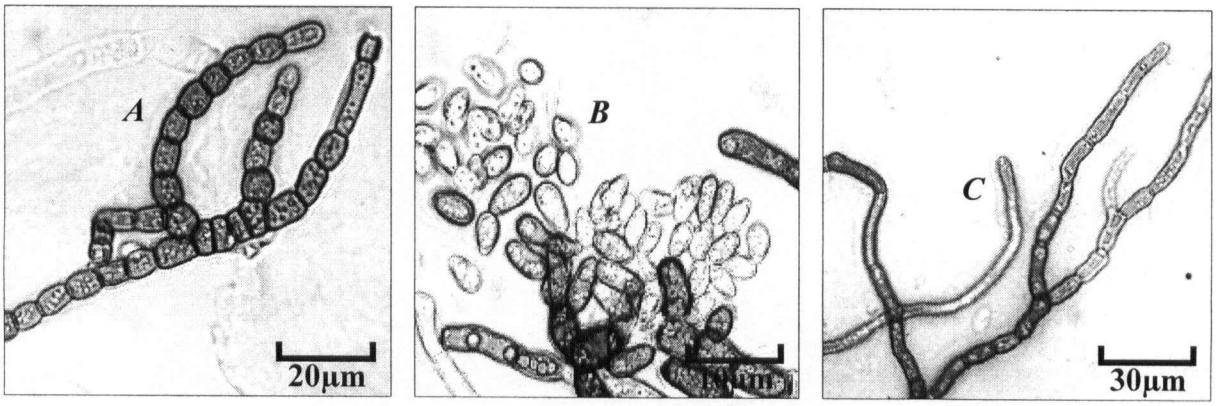
Morphology of Black Staining Fungi: *Aureobasidium pullulans* and *Hormonema dematioides.*

Aureobasidium pullulans



A. conidia; ***B***. dark arthroconidia; ***C***. dark hyphae; ***D***. conidial apparatus.

Hormonema dematioides



Hormonema dematioides; ***A***. dark hyphae; ***B***. conidia; ***C***. fertile hyphae.

Appendix III

ANOVA Source Tables

Displayed below are the analysis of variance (ANOVA) tables following statistical analysis of data reported in each chapter of this thesis. Each table is accompanied by a brief description of contents.

All experiments followed a completely randomized design (CRD) with treatments denoted as k and replicates as n. A one-way analysis of variance (ANOVA) ($\alpha = 0.05$) and Tukey's test for comparison of means (Tukey, 1949) were performed with this experimental design. Treatment effects were considered significant at the 95% significance level if the resulting critical F value was greater than the appropriate tabular value ($F_{[(k-1)],\,[(k(n-1)]}$). All statistical analyses were performed using JMP IN software (version 4.0.3 (academic), SAS Institute Inc., Cary, North Carolina).

Note: DF = degrees of freedom; WRC-FS = western redcedar feeder strips.

AIII.1 ANOVA Tables Relating to Chapter 2: Extracting and Quantifying Western Redcedar (*Thuja plicata* Donn) Heartwood Extractives Using Ultrasonication and Reverse Phase HPLC

AIII.1.1 Comparing Response Factors Among Extractive Compounds

Table A3.1: Comparing response factors among extractive compounds

Source	DF	Sum of Squares	Mean Square	F Ratio
Extractive compound	3	31.005	4.429	205.6
Error	16	0.344	0.022	
Total	23	31.349		

AIII.1.2 The Effect of Various Extraction Methods on Extractive Concentration (µg/g DW)

Table A3.2: (-)-Plicatic acid

Source	DF	Sum of Squares	Mean Square	F Ratio
Extraction Method	2	102553112	51276556	279.6
Error	21	3850608	183362.3	
Total	23	106403721		

Table AIII.3: γ-Thujaplicin

Source	DF	Sum of Squares	Mean Square	F Ratio
Extraction Method	2	1072062	536031	133.9
Error	21	84057.5	4003	
Total	23	1156119.5		

Table AIII.4: β-Thujaplicin

Source	DF	Sum of Squares	Mean Square	F Ratio
Extraction Method	2	2646601.5	1323301	341.3
Error	21	81432.1	3878	
Total	23	2728033.6		

Table AIII.5: β-Thujaplicinol

Source	DF	Sum of Squares	Mean Square	F Ratio
Extraction Method	2	56513.2	28256.6	71.4
Error	21	8313.1	395.9	
Total	23	64826.2		

Table AIII.6: Thujic acid

Source	DF	Sum of Squares	Mean Square	F Ratio
Extraction Method	2	16421174	8210587	162.3
Error	21	1062525	50596	
Total	23	17483699		

Table AIII.7: Methyl thujate

Source	DF	Sum of Squares	Mean Square	F Ratio
Extraction Method	2	5686.2	2843.1	31.9
Error	21	1867.8	88.9	
Total	23	7554		

AIII.2 ANOVA Tables Relating to Chapter 3: Isolating and Testing Fungi Tolerant to Western Redcedar (*Thuja plicata* Donn) Extractives

AIII.2.1 Extractive Concentrations (µg/g) Versus WRC Feeder Strip Storage Conditions

Table AIII.8: (-)-Plicatic acid

Source	DF	Sum of Squares	Mean Square	F Ratio
WRC-FS Storage Condition	2	76732233	38366117	35.9
Error	15	15999464	1066630.9	
Total	17	92731697		

Table AIII.9: γ-Thujaplicin

Source	DF	Sum of Squares	Mean Square	F Ratio
WRC-FS Storage Condition	2	4458827.4	22294.1	79.3
Error	15	421494.2	28100	
Total	17	4880321.6		

Table AIII.10: β-Thujaplicin

Source	DF	Sum of Squares	Mean Square	F Ratio
WRC-FS Storage Condition	2	3136438.7	1568219	87.9
Error	15	267602.8	17840	
Total	17	3404041.5		

Table AIII.11: β-Thujaplicinol

Source	DF	Sum of Squares	Mean Square	F Ratio
WRC-FS Storage Condition	2	138023.51	69011.8	28.4
Error	15	36507.1	2433.8	
Total	17	174530.6		

Table AIII.12: Thujic acid

Source	DF	Sum of Squares	Mean Square	F Ratio
WRC-FS Storage Condition	2	43566181	21783090	44.6
Error	15	7331562	488770.8	
Total	17	50897743		

AIII.3 ANOVA Tables Relating to Chapter 4: Black Stain of Western Redcedar (*Thuja plicata* Donn) by *Aureobasidium pullulans*: the Role of Weathering

AIII.3.1 The Effect of β-Thujaplicin on the Growth of Black Staining Fungi:

Table AIII.13: *Aureobasidium pullulans*

Source	DF	Sum of Squares	Mean Square	F Ratio
β-Thujaplicin concentration	5	10402.926	2080.59	324.6
Replicates	2	26.037	13.02	2.0
Error	46	294.852	6.41	
Total	53	10723.815		

Table AIII.14: *Hormonema dematioides*

Source	DF	Sum of Squares	Mean Square	F Ratio
β-Thujaplicin concentration	5	10999.648	2199.93	559.6
Replicates	2	51.37	25.69	6.5
Error	46	180.852	3.93	
Total	53	11231.87		

AIII.3.2 The Effect of Weathering on the Concentration of Individual Extractives

AIII.15: (-)-Plicatic acid

Source	DF	Sum of Squares	Mean Square	F Ratio
Weathering effect	3	324347588	108115863	68.2
Replicates	2	6347507	3173753.6	2.0
Error	30	47528915	1584297.2	
Total	35	378224011		

AIII.16: γ-Thujaplicin

Source	DF	Sum of Squares	Mean Square	F Ratio
Weathering effect	3	38801060	12933687	253.5
Replicates	2	73496	36747.927	0.7
Error	30	1530787	51026.249	
Total	35	40405343		

AIII.17: β-Thujaplicin

Source	DF	Sum of Squares	Mean Square	F Ratio
Weathering effect	3	10203725	3401242	615.8
Replicates	2	1076	538	0.1
Error	30	165698	5523	
Total	35	10370499		

AIII.18: β-Thujaplicinol

Source	DF	Sum of Squares	Mean Square	F Ratio
Weathering effect	3	110846.4	36948.8	292.3
Replicates	2	195.58	97.8	0.8
Error	30	3792.78	126.4	
Total	35	114834.75		

AIII.19: Thujic acid

Source	DF	Sum of Squares	Mean Square	F Ratio
Weathering effect	3	146329522	48776507	397.8
Replicates	2	189457	94728.251	0.8
Error	30	3678423	122614.09	
Total	35	150197401		

AIII.3.2 The Degree of Colonization of Weathered Wood Surfaces by *A. pullulans* (Quantitative Assessment)

AIII.20: Ponderosa pine sapwood

Source	DF	Sum of Squares	Mean Square	F Ratio
Weathered surfaces	3	10102.02	3367.34	194.4
Replicates	2	20.236	10.12	0.6
Error	66	1143	17.32	
Total	71	11265.256		

AIII.21: WRC heartwood

Source	DF	Sum of Squares	Mean Square	F Ratio
Weathered surfaces	3	1444.2095	481.403	25.7
Replicates	2	43.6553	21.828	1.2
Error	66	1235.157	18.714	
Total	71	2723.0218		

AIII.4 ANOVA Tables Relating to Chapter 5: Effects of Leaching on Fungal Growth and Decay of Western Redcedar (*Thuja plicata* Donn)

AIII.4.1 The effect of leaching on extractive concentration:

Table AIII.22: (-)-Plicatic acid

Source	DF	Sum of Squares	Mean Square	F Ratio
Leaching effect	1	122669190	122669190	850.3
Error	10	1442675	144267.53	
Total	11	124111865		

Table AIII.23: γ-Thujaplicin

Source	DF	Sum of Squares	Mean Square	F Ratio
Leaching effect	1	967317.2	967317	85.1
Error	10	113638.8	11364	
Total	11	1080956.1		

Table AIII.24: *β*-Thujaplicin

Source	DF	Sum of Squares	Mean Square	F Ratio
Leaching effect	1	754914	754914	76.4
Error	10	98812.69	9881	
Total	11	853726.69		

Table AIII.25: *β*-Thujaplicinol

Source	DF	Sum of Squares	Mean Square	F Ratio
Leaching effect	1	911153.61	911154	36612.7
Error	10	248.86	25	
Total	11	911402.48		

Table AIII.26: Thujic acid

Source	DF	Sum of Squares	Mean Square	F Ratio
Leaching effect	1	10204156	10204156	63.9
Error	10	1597652	159765.24	
Total	11	11801809		

AIII.4.2 The Effect of Leachate on Fungal Growth *In Vitro*

Table AIII.27: *Acanthophysium lividocaeruleum*

Source	DF	Sum of Squares	Mean Square	F Ratio
Leachate concentration	5	52.007215	10.4014	56.9
Block (strains)	1	0.066736	0.0667	0.4
Error	29	5.30375	0.1829	
Total	35	57.377701		

Table AIII.28: *Coniophora puteana*

Source	DF	Sum of Squares	Mean Square	F Ratio
Leachate concentration	5	79.32813	15.8656	13.3
Block (strains)	1	0.42612	0.4261	0.4
Error	29	34.69541	1.1964	
Total	35	114.44965		

Table AIII.29: *Heterobasidion annosum*

Source	DF	Sum of Squares	Mean Square	F Ratio
Leachate concentration	5	198.05652	39.6113	21.5
Block (strains)	1	39.2363	39.2363	
Error	29	53.39911	1.8413	
Total	35	290.69194		

Table AIII.30: *Pachnocybe ferruginea*

Source	DF	Sum of Squares	Mean Square	F Ratio
Leachate concentration	5	1.997772	0.399554	12.1
Block (strains)	1	0.0054186	0.005419	0.2
Error	29	0.9557504	0.032957	
Total	35	2.958941		

Table AIII.31: *Phellinus sulphurascens*

Source	DF	Sum of Squares	Mean Square	F Ratio
Leachate concentration	5	86.464892	17.293	509.4
Error	12	0.407407	0.034	
Total	17	86.872299		

Table AIII.32: *Phellinus weirii*

Source	DF	Sum of Squares	Mean Square	F Ratio
Leachate concentration	5	101.14082	20.2282	169.7
Error	12	1.43056	0.1192	
Total	17	102.57137		

AIII.4.3 Estimation of the Degree of Decay (% mass loss) of Wood Blocks by Six Fungal Species of Interest

Table AIII.33 *Acanthophysium lividocaeruleum*

Source	DF	Sum of Squares	Mean Square	F Ratio
% Decay	2	117.9	58.9	1.39
Error	15	632.7	42.2	
Total	17	750.7		

Table AIII.34 *Coniophora puteana*

Source	DF	Sum of Squares	Mean Square	F Ratio
% Decay	2	5942.4	2971.2	94.14
Error	15	473.4	31.6	
Total	17	6415.8		

Table AIII.35 ***Heterobasidion annosum***

Source	DF	Sum of Squares	Mean Square	F Ratio
% Decay	2	361.3	180.7	2.25
Error	15	1200.3	80	
Total	17	1561.7		

Table AIII.36 ***Pachnocybe ferruginea***

Source	DF	Sum of Squares	Mean Square	F Ratio
% Decay	2	21.1	10.5	0.83
Error	15	190.8	12.7	
Total	17	211.8		

Table AIII.37 ***Phellinus sulphurascens***

Source	DF	Sum of Squares	Mean Square	F Ratio
% Decay	2	2900.2	1450.1	49.43
Error	15	440.1	29.3	
Total	17	3340.3		

Table AIII.38 ***Phellinus weirii***

Source	DF	Sum of Squares	Mean Square	F Ratio
% Decay	2	767.4	383.7	7.39
Error	15	778	51.9	
Total	17	1545.4		