What causes natural durability in *Eucalyptus bosistoana* timber?

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Abstract

What causes natural durability in *Eucalyptus bosistoana* timber?

This study investigated the natural durability of 8 and 60 year old *Eucalyptus bosistoana* (coast grey box). The sample's heartwood compounds were extracted with an optimised extraction process and then incorporated into agar. *Trametes versicolor* (white rot) and *Gloeophyllum trabeum* (brown rot) fungi were grown upon these agars and their growth rate was used to assess the fungicidal abilities of the extracts.

The extraction method of cell wall compounds was optimised. An Accelerated Solvent Extraction system (ASE) was used with the following settings:

- 2 cycles per sample
- 70°C extraction temperature
- 50% rinse
- 5 minute static time

Ethanol was found to extract the compounds of the highest fungicidal activity. Ethanol was found to extract similar amounts to water (~13% of dry weight for a 60 year old sample), however analysis of both water and ethanol extracts with a FTIR spectrometer, found that they were of different chemical composition.

A difference in fungicidal activity of extracts was found between the 8 year old and 60 year old samples. There was a large difference in the percentage of extracts present between the samples as well as the type of compounds present, shown by FTIR.

Key words:

Eucalyptus bosistoana, natural durability, ASE, extractives, *Trametes versicolor*, *Gloeophyllum trabeum*

Contents

Introduction

Wood has been a major building material for centuries. Timber is a biological product which is susceptible to biological decay; this paper focuses solely upon the decay of wood through fungal activity. Moist conditions (especially below ground) increase the rate at which timber decays by fungal attack. However decay resistance occurs naturally in some timbers where toxic extractives are located within the heartwood cell walls, this may also be achieved through chemical preservative treatment (Morrell & Zabel, 1992; Schultz & Nicholas, 2000). Timbers which possess natural durability are highly sought after as they are rare and do not contain preservative chemicals. A current global trend of restricting the use of treated timber has meant naturally durable timber is becoming a more valuable product. New Zealand is reliant upon a monoculture of *Pinus* r*adiata* (D. Don) in their forestry industry. However *P. radiata* does not produce naturally durable heartwood and therefore requires chemical treatment for outdoor use. CCA (copper, chromium & arsenic) treatment is often used for high durability products. However CCA treatments have been banned or restricted in many countries around the world due to health and environmental effects of the arsenic it contains. This highlights a significant market for durable timbers around the world.

The New Zealand Dry Land Forest Initiative (NZDFI) is a collaborative cross-sector research and development project that is dedicated to

"researching and promoting genetically-improved naturally durable eucalypt species for planting on drought prone and erodible pastoral land in New Zealand.

They do not wish to replace the current *P. radiata* market but to simply take advantage of the high value tropical hardwood market. They have selected six

eucalypts for trial establishments within New Zealand, of which; *Eucalyptus bosistoana* is one.

E. bosistoana (coast grey box) can be found naturally in the coastal areas of Victoria and New South Wales in areas of rain fall from 700-1200 mm per year. It is the largest of the box *eucalypts*, producing long straight stems. It is relatively resistant to waterlogged and drought conditions and prefers fertile lime soils (Nicholas & Millen, 2012). It produces class one durability timber, giving it a life time of 25+ years below ground and 40+ years above ground. This durability comes from its pinkish brown coloured heartwood which contains fungicidal extractives.

The extractives which give *E. bosistoana* its natural durability however have never been identified. There have been a number on studies on non-natural eucalypts and their heartwood extractives, this is due to their intense use in the pulp and paper industry. Little information is available on durable eucalypts and their heartwood extracts. Hillis and Rudman were two of the leading researchers of eucalypts and their heartwood extractives in the middle of the last century, little has been done since.

The exact nature of these compounds is unknown for most natural durable species (Da Costa & Rudman, 1958; Taylor, Gartner, & Morrell, 2002). Identifying the extractives which contribute to the fungicidal properties of *E. bosistoana* may help to develop methods to classify trees in terms of their natural durability. This would ensure *E. bosistoana* timber has a consistent durability and be accepted in the domestic market, Identification of essential fungicidal compounds may also lead to innovative methods of treating New Zealand's non-durable *P. radiata*.

Objectives

The overall aim of this study was to find what makes *E. bosistoana* timber resistant to biological decay?

The study examined the following:

- The most efficient method of extracting the most compounds?
- Which solvent extracts the most fungicidal compounds?
- Does the durability of *E. bosistoana* differ with age?

Review of Literature

Fungal Attack

Some fungi have the potential to cause major physical damage to wood (Morrell & Zabel, 1992). These fungi require favourable conditions of nutrients, water, air, temperature and pH to inhabit wood and damage it (Schmidt, 2006). Given suitable conditions fungi may decay woody cell walls at an alarming rate. As cellulose, hemicellulose and lignin are insoluble and too large for fungal hyphae to absorb, extra cellular enzymes are released to degrade the polymers to monosaccharide's. These are absorbed and metabolized by intracellular enzymes, producing energy and fungal biomass (Morrell & Zabel, 1992; Schmidt, 2006).

Brown-rot is the most common and destructive type of fungal decay. Only the cellulose and hemicelluloses are utilised, leaving the lignin behind in a demethylated and oxidised state. As a result the wood darkens, shrinks, breaks and crumbles into blocks.

White-rots however attack all cell wall polymers. Dependent on the type of white rot fungi different components of the walls are attacked at different rates. Preferential white rots decay the hemicellulose and lignin initially which causes the dissolution of the middle lamella. Simultaneous white rots however remove both lignin and carbohydrates at a similar rate resulting in a uniform degradation of the cell walls (Morrell & Zabel, 1992; Pandey & Pitman, 2003)

Durability

When constructing a building it is essential to employ a timber of known strength and durability, especially when subject to high moisture conditions (Harju & Martti, 2006). This will ensure the structure will last as long as its intended service life. Durability of timber is best ranked by benchmarking it against other available timbers, usually a timber highly susceptible to wood decaying fungi and other organisms (Harju & Martti, 2006). The European standard classifies the natural durability of solid wood into five classes, these values are found through standardized field and lab tests (Harju & Martti, 2006). Some timbers have a naturally occurring heartwood of high durability which may be used as is, while others need preservative treatment(Schultz & Nicholas, 2000).

Chemical Treatment

Timbers of low natural durability often require treatments which deposit biocides and preservatives into the cell walls (Schultz & Nicholas, 2000). Chrome copper arsenate (CCA) has been used for decades as a chemical treatment for timber of low natural durability to produce products of a high durability (Schultz & Nicholas, 2000).

The use of CCA has been heavily restricted around the globe due to rising concerns over the use of chromium and arsenic salts due to the environmental and health hazards associated with them. It has been banned for many uses in America as well as the EU and Australia. The future of CCA treated timber is therefore becoming more uncertain on a global scale and New Zealand is one of the few first world countries continuing the practice (Schultz & Nicholas, 2000). The toxicity of arsenic is of highest concern as when leached from timber it may enter water ways or soils,

posing a danger to the health of humans as well as the environment (García-Valcárcel & Tadeo, 2006). This has caused unrest in New Zealand wineries that use CCA posts, and driven the use of naturally durable posts. CCA treated timber currently does not have an acceptable disposable facility other than secure landfills which is of high cost (e.g. in Marlborough, New Zealand, $21 per cubic metre in a secure landfill (Nicholas & Millen, 2012)). Such problems do not exist for naturally durable wood.

Natural Durability

Natural durability of heartwood is caused by extractives located within the cell walls (Rudman, 1962).These extractives can be toxic to fungi, inhibiting fungal decay of the cell walls (Harju & Martti, 2006).

It has been speculated by Hart and Hillis (1974) that tannins possess fungicidal properties and Rudman and DaCosta (1961) state that phenolics present are responsible (these are essentially the same). Harju and Martti (2006) have found that the number of total phenolics present in *Pinus sylvestris* is an appropriate measure for the natural durability and are potentially able to replace decay test by specific brown rot fungi.

The natural durability of wood may vary within the stem and between individuals (Amusant, Beauchene, Fournier, Janin, & Thevenon, 2004). Klumpers et al. (1994) has suggested that the amount of phenolic extractives decreases towards the pith, in part explaining the differences in timber properties such as durability, colour, dimensional stability, adhesive bonding etc.

Intra-tree natural durability follows a radial and vertical pattern, with higher durability in the juvenile outer wood and lower durability higher up and closer to the

pith (Gartner, Morrell, Freitag, & Spicer, 1999; Klumpers, Scalbert, & Janin, 1994; Wilkes, 1985). Figure 1 illustrates this for *Eucalyptus maculate* & *Eucalyptus sieberiana* species (Rudman, 1965). The reason behind this has only been speculated. Rudman et al. (1965) believes that after the formation of toxic extractives they begin to chemically age and become less toxic. However as some species such as *E. wandoo* do not experience a decrease in fungicidal properties towards the pith, it is believed that some species have such high amounts of fungitoxic extractives that after aging the remaining extracts will have sufficient toxicity to deter fungal decay (Rudman, 1965).

Rudman and Da Costa (1961) were unable to find any relationship between basic density and decay resistance in ten durable eucalypts and concluded that durability was largely due to the extractives (Rudman & DaCosta, 1961).

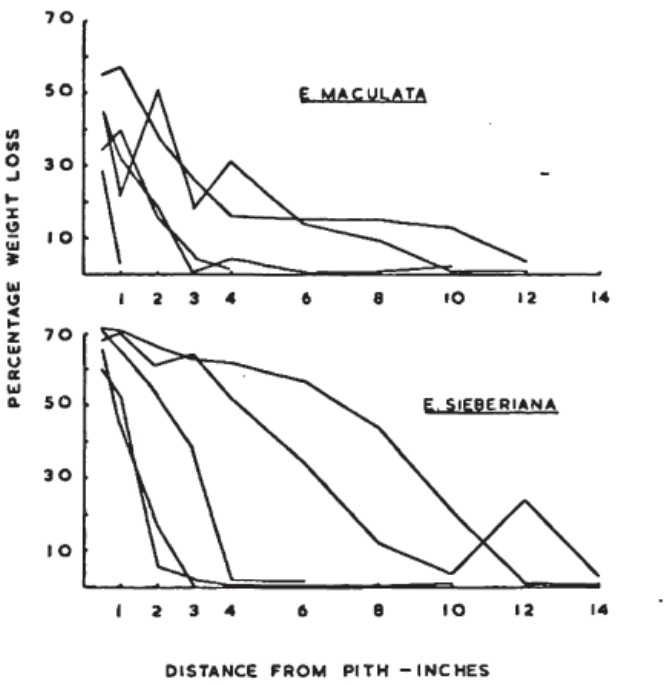

Figure 1 - The pattern of radial variation in decay resistance in trees of E. Maculta & E. Sieberiana (Rudman, 1965)

Heartwood

Formation

The International Association of Wood Anatomists (IAWA) has defined heartwood as being "*the inner layers of the wood, which, in the growing tree, have ceased to contain living cells, and in which the reserve materials (*e.g. *starch) have been removed or converted into heartwood substance*" (Taylor et al., 2002). The transformation of sap wood to heartwood is still not completely understood but many of the processes have been studied (Rudman, 1966; Taylor et al., 2002). The death of the parenchyma cells is by definition the point that sapwood transforms into heartwood (Taylor et al., 2002). It is known that heartwood extractives are deposited at the transition zone between heartwood/sapwood (Taylor et al., 2002). These extractives often are responsible for the distinctive change in colour, odour and lustre of heartwood. Streit and Fengel (1994) (as cited in Taylor, Labbé, and Noehmer (2001)) noted that the extractives formed within the transition zone impregnate the cell walls, starting within the middle lamella and then moving into the secondary walls. This can be likened to the process of lignification and is often referred to as *pseudo lignification.* This micro deposition of extractives clearly plays a role in the durability of heartwood of some species, and has been shown through the impregnation of non-durable timbers with these extractives resulting in an increase in durability (Taylor et al., 2001).

Wood Extracts

Wood is made up of three large insoluble polymers; lignin, cellulose and hemicelluloses but also contains compounds of a much lower molecular weights such as volatile and non-volatile acids, sugars, steroids, terpenes, phenols, lactones etc. (González-Rodríguez, Pérez-Juan, & Luque de Castro, 2003). There has been numerous studies upon the total amount of lignin, cellulose and pentosan contents within E*ucalyptus* wood but information on the phenolic extracts is scarce (Conde, Cadahia, Garciavallejo, & Desimon, 1995; Hillis, Hart, & Yazaki, 1974). Many tree species have large quantities of these polyphenols accumulated within their heartwood, which provide the fungicidal properties of heartwood (Hart & Hillis, 1974; Klumpers et al., 1994). Most of these extractives can be removed using a series of organic solvents (Smith, Campbell, Walker, & Hanover, 1989). Some of the extracts are strongly bonded to the cell wall polymers, as a result when extracted numerous times, some timbers may still possess a degree of natural durability. The extractives may be used to increase the natural durability of non-durable timbers through impregnation. Rudman and DaCosta (1961) found that decay resistance within various eucalyptus species was mainly related to the toxicity of their methanol soluble extractives

Methods of Assessing Natural Durability

Previous studies have used multiple methods for the extraction of compounds and the determination of their fungicidal activity, all of which were lengthy processes.

Anderson (1961) performed 24 hour soxhlet extractions on blocks of 1 x 1 x ¼ inch dimensions to remove and measure the extractives located within *Sequoia sempervirens*. Extracted and non-extracted blocks were weighed, subjected to six weeks of exposure to brown rot fungus and then re-weighed to measure the fungicidal capacity of the extracted compounds. This method is very lengthy.

Da Costa and Rudman (1958) performed extractions on 2mm chipped samples and impregnated non-durable blocks with the extracts in an attempt to identify the durability in *Eucalyptus microcorys* (tallow wood). The method of extraction was however not stated but likely a soxhlet extraction. These impregnated as well as non-impregnated wood blocks were subjected to a lengthy exposure to brown rot fungi and their mass loss was determined (10 week process).

The duration of extraction and fungicidal properties tests are all very lengthy and are not suitable for breeding purposes which aim to assess >10,000 trees for natural durability. The NZDFI is planning on carrying out such extensive trials, therefore require a quicker process of assessment.

Methods

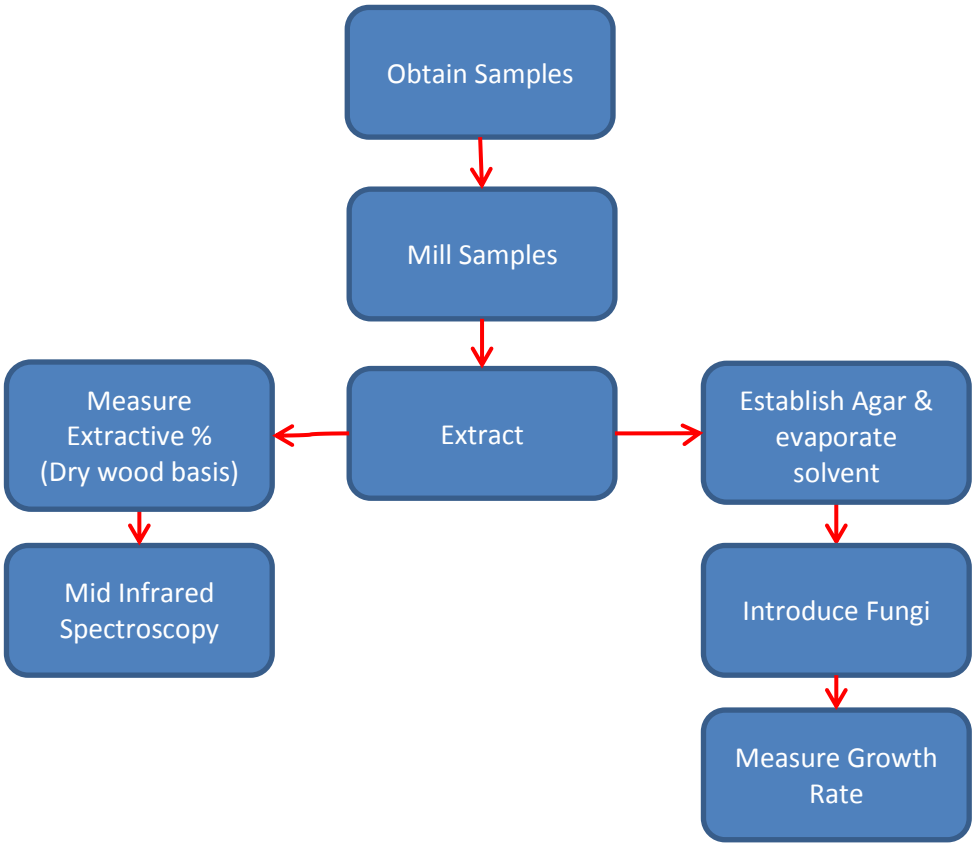

Figure 2 - Flow chart of the experimental procedure

Samples

Three dry samples of *Eucalyptus bosistoana* (coast grey box) were obtained from The New Zealand Dry Land Forest Initiative (NZDFI), one 8 year old and two 60 years old samples. The history of the samples was however unknown. The 60 year old samples were grown on another site as the 8 year old sample. Where the 60 year old samples were taken from within the stem was unclear, in relation to the pith or height up the tree. The younger sample was found to have some rot already developed in the sapwood as well as borer attack (Figure 3). The heartwood was isolated and sapwood discarded.

Figure 3 - 8 year old *E. bosistoana* sample displaying rot and insect attack in the sap wood but not heartwood

Milling

The heartwood of the samples was chiselled into 5x5mm splinters, which were subsequently chipped with a Thomas Wiley Mill to pass a 2mm mesh. The chipped samples were placed in a desiccator over silica gel to remove excess moisture.

Extraction

An Accelerated Solvent Extractor (ASE) was used to extract the compounds present within the wood. An ASE can replace traditional soxhlet extractions taking a fraction of the time (from 8 to 12 hours down to 15 minutes per cycle). ASEs also promise to reduce variation of extractions and give more consistent results.

Extractions of the 60 year old (B) sample were carried out at various temperatures with the ASE; room temperature (18-22°C), 70°C and 85°C. The extracts were collected and measured gravimetrically and the results compared.

The number of extraction cycles per sample to obtain the most compounds was also tested. A single sample was sequentially extracted six times; each extraction cycle had its extracts collected separately and weighed to calculate the percentage of extracts per dry cell wall obtained per extractive cycle. The most economical number of extractions in terms of time and extracts obtained was selected.

Each sample of this *E. bosistoana* was extracted using the optimal extraction process determined in the previous section with three different solvents; ethanol, water and ethyl acetate. In each extraction 9.6g of dry milled wood was extracted.

Measuring Extractive Content

After extraction a solution of solvent and extractives remained. This solution was halved, one half of the solution was placed in a fume cupboard to allow the solvent to evaporate followed by an hour at 100°C in an oven, leaving the dry extractives. The dry extracts were weighed and divided by 4.8 (half of the 9.6 g dry wood sample extracted) to give the extractive content of the sample.

Mid Infrared Spectrometry

The dry extracts were examined after weighing using a FTIR spectrometer using the Attenuated Total Reflectance (ATR) sampling technique. The spectra allowed a comparison between individual samples to identify differences in their composition.

Fungicidal Properties

Preparation of Agar

The other halve of the extractive were used to test their fungicidal activity. The solution was added to 100 ml of distilled water. A solvent control was created containing the same amount of solvent. A water control was also created which only contained water. The sample contents were placed in a water bath at 90°C, until all of the solvent was evaporated, this was tested by a smell test. The volumes were replenished with distilled water to give 100ml per beaker. Malt agar was added to these solutions (4.8 g) and placed back into the water baths for a further 10 minutes to dissolve. Once dissolved the samples were placed in an autoclave for 10 minutes at 121°C for sterilization. The sterilized agar was distributed into 8 15ml (50mm diameter) petri dishes at equal volumes and left to set.

The concentration of the extracts in the agar was the same as that in the wood sample, with the extracts of 4.8g of wood dissolved into the 4.8 g of agar. Therefore the concentrations of extracts in the agars differ with the samples in relation to that in the wood.

Fungal Tests

Trametes versicolor (L.) (white rot) and *Gloeophyllum trabeum* (brown rot) were obtained from the International Collection of Microorganisms from Plants (ICMP) Landcare Research. Each of the fungi was introduced onto four petri dishes of each agar. This was carried out with a sterilized disposable pipette, the diameter of the pipette allowed a consistent amount of fungi to be transferred to each agar. Three separate cultures of the fungi were used to eliminate the possibility of using contaminated fungi. The petri dishes were then placed into an incubator at 25.2°C.

Each petri dish was measured on a 24 hour basis for a week or until the fungal body had reached the petri dish sides (50mm). Callipers were used to measure the diameter of the fungal body across two marked, perpendicular points (Figure 4).

Figure 4 - *T. vesrsicolor* growth upon an agar containing 8 year old ethanol extracts

Data Analysis

Initially all data was screened to remove obvious outliers from the data set. The dataset was also modified to remove all diameter values below 5 mm and above 40 mm. This removed the variability of the time taken for fungi to establish and eliminate the slower growth period once nearing the dish edge.

An average fungal body diameter was calculated at each measurement time for each dish. The growth rate was calculated on average for each dish on an hourly basis. The growth rates were compared between agars using F-tests. A number of linear models were created to predict average diameters with the variables of samples and solvents to determine their statistical significance. The data for the two fungi were kept separate. Comparison of the two fungi was provided by calculating their retarding ability i.e. the ratio of growth rates on agar with and without extracts.

Results

Method Development

Table 1 - Effect of extraction temperature on extractive yield from a 60 year old *E. bosistoana* sample using an ASE with 6 cycles and 5 minute static times

60 Year old sample	Temperature		
Solvent	**Room (18°C -22) °C**	**70°C**	**85°C**
Ethanol	12%	14%	NA
Ethyl Acetate	2%	3%	NA
Water	12%	14%	11%

Table 1 shows the influence of temperature on extractive yields for the 3 different solvents. Ethyl acetate was found to obtain a much lower percentage of extracts than ethanol or water at both room temperature and 70°C. Water and ethanol both performed equally for room temperature and 70°C. At a slightly higher temperature (85°C) water did not yield more water extracts.

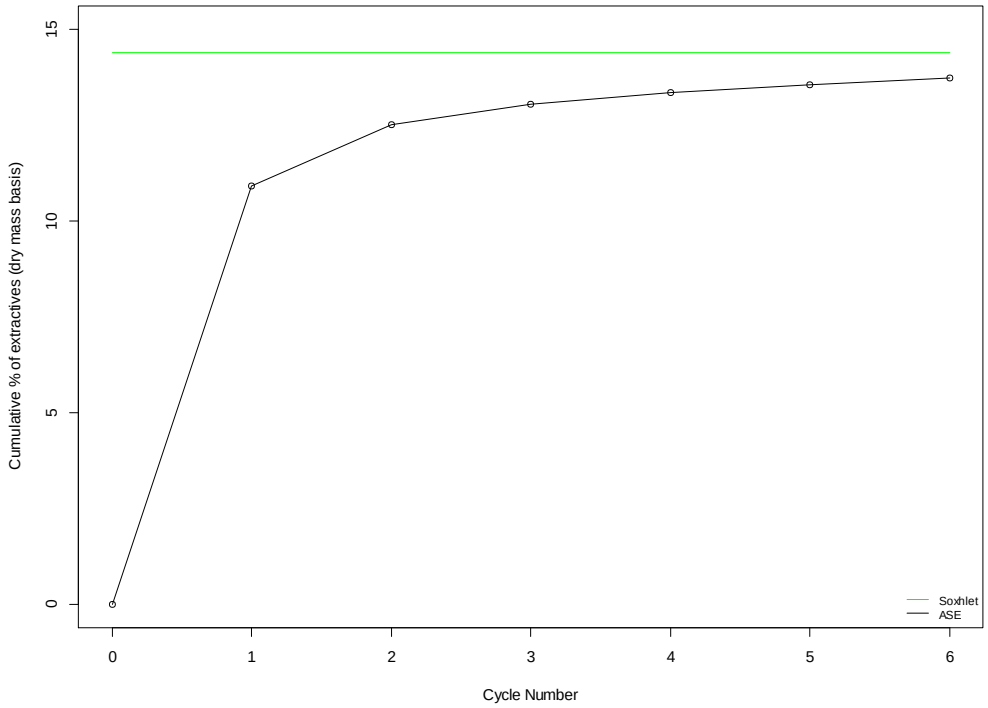

Figure 5 - Comparison of extractives obtained from a soxhlet extraction and numerous ASE cycles. This was performed on a 60 year old *E. bosistoana* sample with the ASE extracting at 70°C with ethanol. The soxhlet was also performed on a 60 year old sample and carried out for 9 hours

Extractions with the ASE, with a static time of 5 minutes at 70°C did not remove all possible compounds. Figure 5 shows that after 2 cycles 91% of the total extracted matter was obtained and 87% compared to the soxhlet extraction. After 2 extraction cycles, subsequent cycles only marginally increased the total yield with ASE. In order to take advantage of the fast ASE extraction process, a 2 cycle per sample method was chosen for this study. The lower yield obtained is compensated by the significant reduction in extraction time from 9 hours (soxhlet) to 30 minutes (ASE). Comparable results are obtainable as long as the method is consistently applied.

Optimal extraction process:

- 2 cycles per sample
- 70°C extraction temperature
- 50% rinse
- 5 minute static time

Extractives in *E. bosistoana*

Quantity of Extracts

Table 2 – Extractive content (% of dry mass) of different *E. bosistoana* samples obtained with different solvents

Sample	Ethanol	Ethyl Acetate	Water
8YO	2.17%	0.07%	3.34%
60YOA	12.78%	2.52%	13.79%
60YOB	13.73%	2.57%	11.43%

The eight year old sample contained 3.34% water extracts of dry weight whereas the 60 year old samples had four times more water and ethanol soluble extracts present (~13%)(Table 2). This indicated a clear effect of the age on extractive content. Ethyl acetate was found to extract much smaller amounts of extractives than ethanol or water (~3%).

Mid Infrared Spectrometry – Type of Compounds

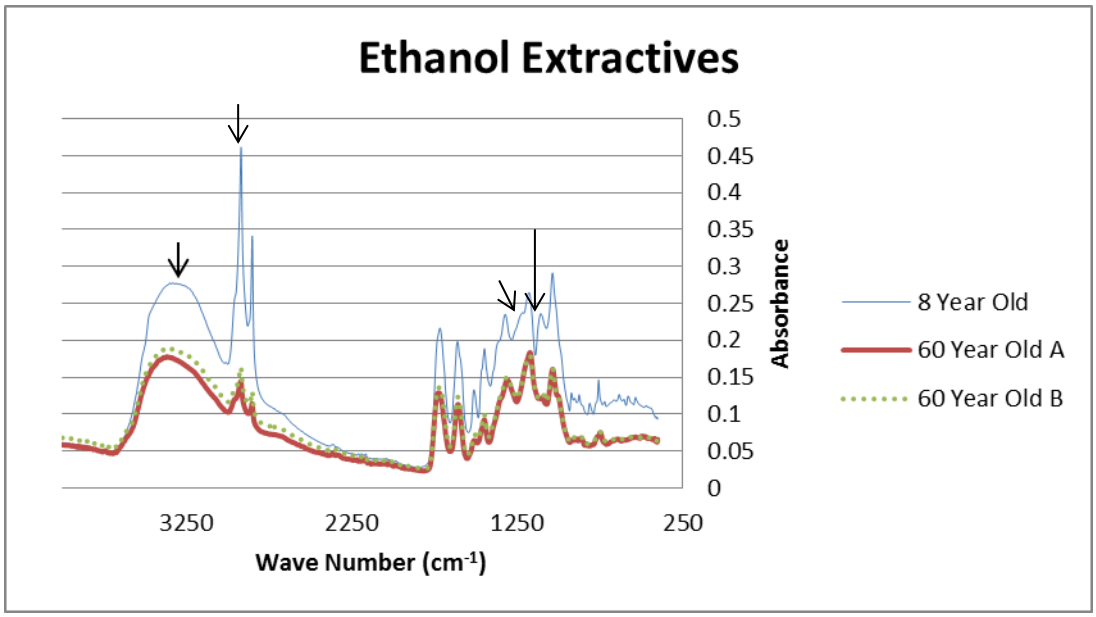

Figure 6 - Mid infrared spectra of ethanol extracts from *E. bosistoana* heartwood

Figure 6 shows the composition of extracts obtained from the samples. No qualitative difference was found for the two 60 year old samples. A higher ratio of hydro carbons (CH) (groups ~ 2800 cm $^{-1}$) to hydroxyl groups (~3300 cm^{-1}) was observed in the 8 year old samples in comparison to the 60 year old samples. Differences were also present in the 'fingerprint' region (~1250/1200 cm^{-1}), but assignment to functional groups is difficult here.

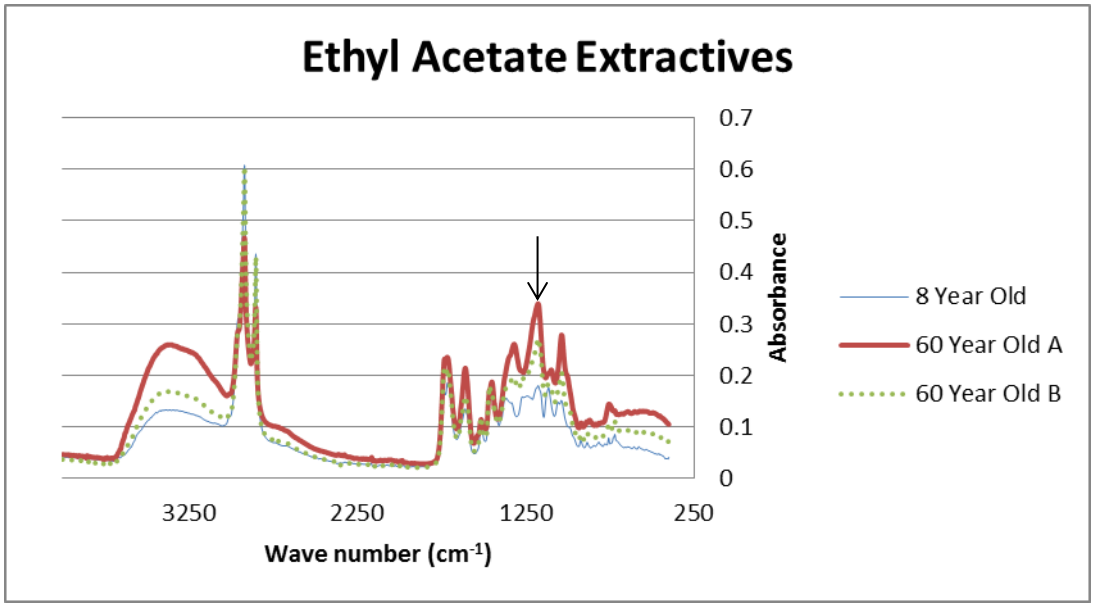

Figure 7 - Mid infrared spectra of ethyl acetate extracts from *E. bosistoana* heartwood

Ethyl acetate extracts have also been found to differ in composition between the old and young samples as observed for ethanol extracts. The variation was observed in the fingerprint region at ~1200 cm^{-1} (Figure 7).

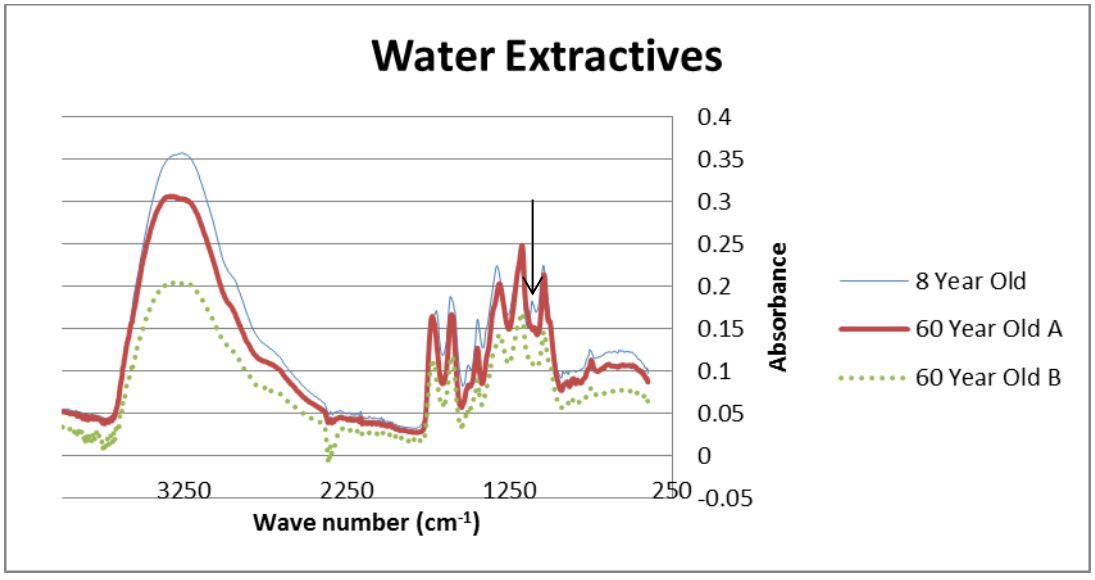

Figure 8 - Mid infrared spectra of water extracts from *E .bosistoana* heartwood

Water extracts also differ in their composition between age groups. A peak is observed at ~1100 cm^{-1} for the 8 year old sample was absent in the older samples.

Overall the different extracts of the two 60 year old samples have been found to be very similar, but there seems to be a genuine difference in compounds between the two age groups.

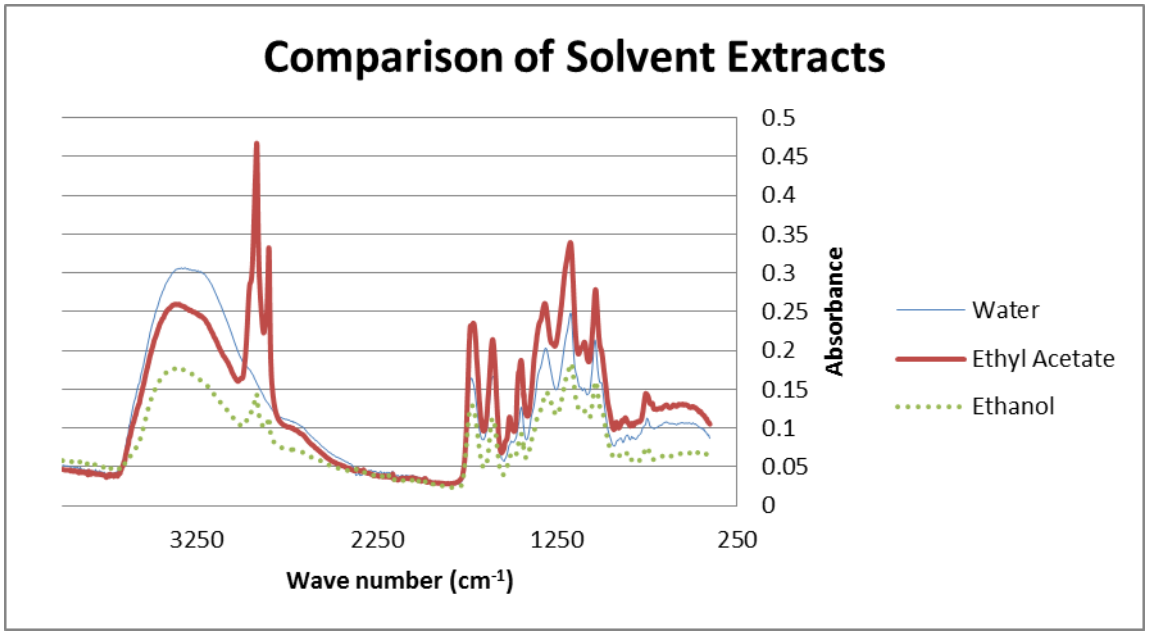

Figure 9- Mid infra-red spectra of the compounds removed from the 60 year old *E. bosistoana* sample with water, ethanol and ethyl acetate

An FTIR scan showed that the solvents extracted different compounds from the 60 year old sample (Figure 9). As expected with increasing hydrophilicity (water > ethanol >> ethyl acetate) the amount of hydroxyl groups in the extracts increased while the amount of aliphatic groups decreased (CH_2 ~2800 cm^{-1})

Durability Testing

Bench Marking Fungi

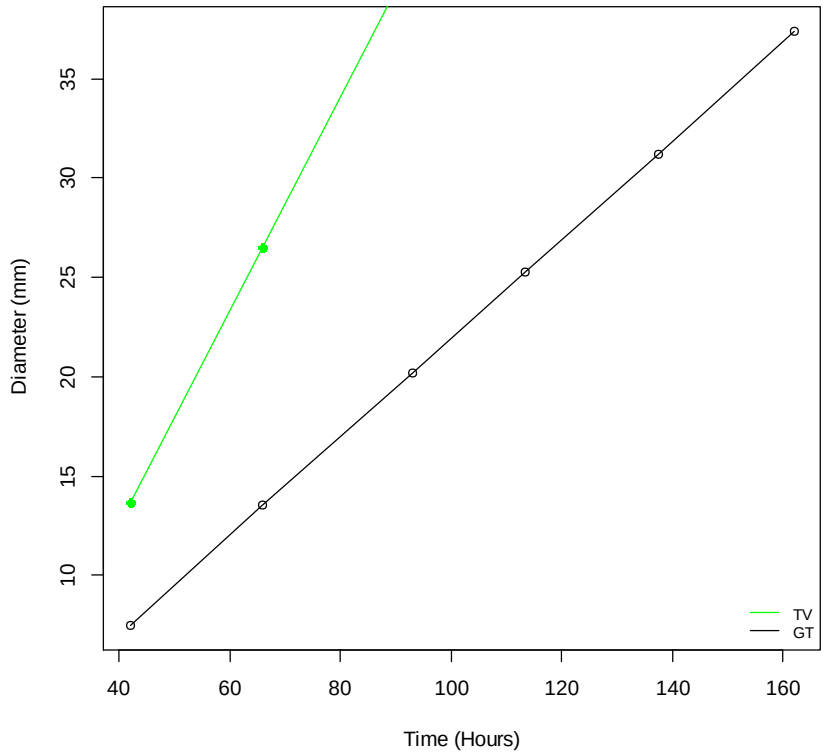

Figure 10 – Control growth rates of both fungi used, *Trametes versicolor* (TV), *Gloeophyllum trabeum* (GT) on malt agar at 25.2°C. The displayed growth rates are the average of 4 samples for both fungi

T. versicolor and *G. trabeum* were found to have different growth rates. *T. versicolor* grew at an average rate of 0.518 mm per hour whereas *G. trabeum* grew at a rate of 0.241 mm per hour at 25.2°C (Figure 10).

Fungal Tests

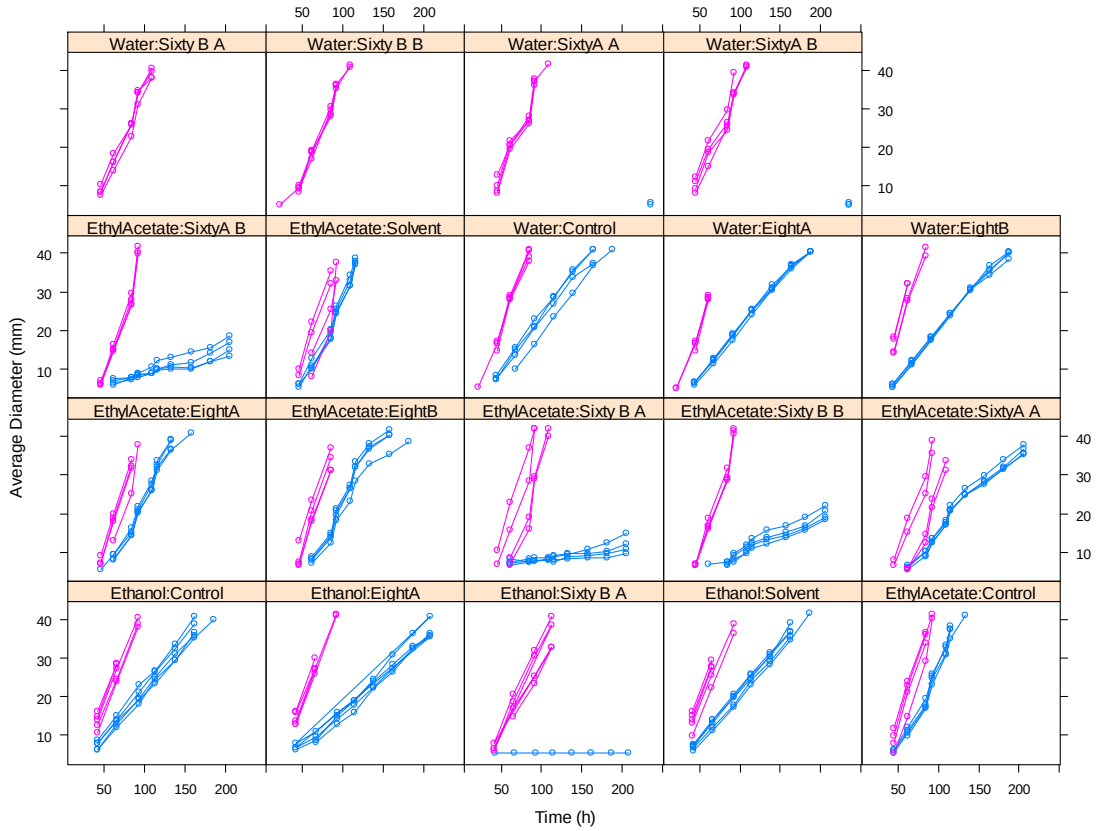

Figure 11 - Raw data of fungal growth upon agars containing different extracts (pink = *Trametes versicolor* (TV), blue = *Gloeophyllum trabeum* GT)

Figure 11 illustrates the growth rates of *G. trabeum* and *T. versicolor* on the agars containing different extracts. *T. versicolor* was observed to grow on nearly all agars whereas *G. trabeum* had not grown on all. *T. versicolor* had a faster growth rate than *G. trabeum* on all agars.

The ethanol results were collected at a later date than that of ethyl acetate and water experiments. Only one 8 year old sample and one 60 year old sample were measured giving fewer comparable results than that of ethyl acetate and water for where all samples were extracted in duplicate.

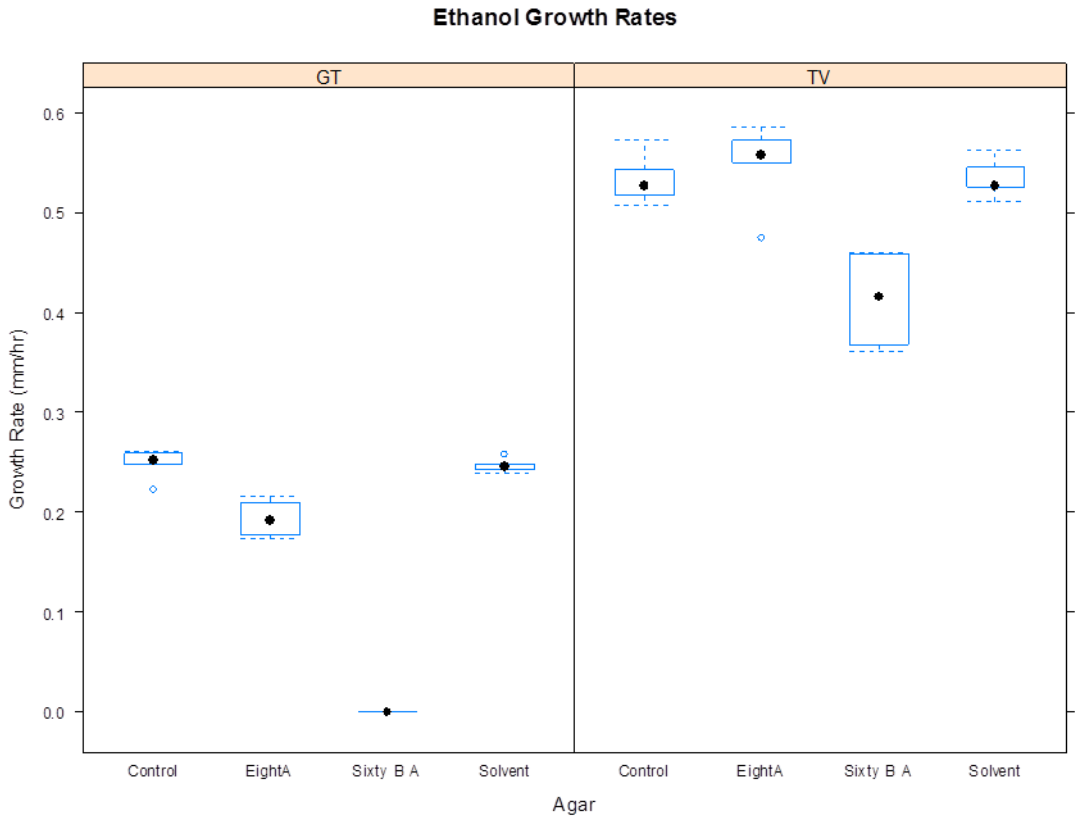

Figure 12 - Fungal growth rates of *T. versicolor* and *G. trabeum* on agar containing ethanol extractives of *E. bosistoana* heartwood

Ethanol was found to have no effect upon the growth rate of *G. trabeum* and *T. versicolor* fungi once evaporated, allowing the control and solvent control to be pooled (p=0.900 and 0.648 respectively) (Figure 12, Appendix 1,Appendix 2). However extractives obtained from the 8 year old sample were found to significantly reduce the growth rate of *G. trabeum* (p<0.001) but not *T. versicolor* (p=0.31) (Table 3, Table 4). *T. versicolor* in fact increased its growth rate when subject to 8 year old extractives. The sixty year old sample completely inhibited the growth of *G. trabeum* and reduced the growth rate of *T. versicolor* significantly (p<0.01) (Table 3 and Table 4).

Table 3 – Significance of growth rate for ethanol extracts of *E. bosistoana* samples, *Gloeophyllum trabeum*

Ethanol GT	Control	8YO
8YO	<0.001	1
60YO	<0.001	<0.001

Table 4 - Significance of growth rate for ethanol extracts of *E. bosistoana* samples, *Trametes versicolor*

Ethanol TV	Control	8YO
8YO	0.31	1
60YO	0.019702	0.0116

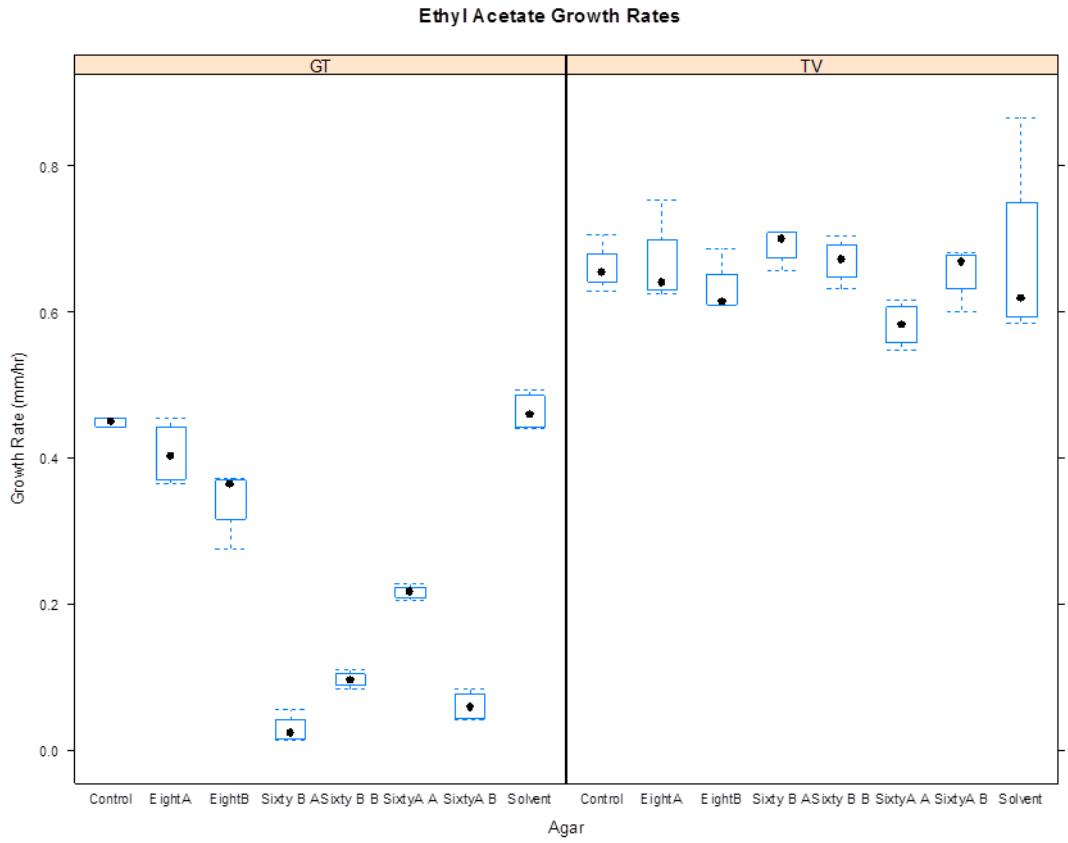

Figure 13 - Fungal growth rates of *T. versicolor* and *G. trabeum* on agar containing ethyl acetate extractives of *E. bosistoana* heartwood

Ethyl acetate was found to have no significant effect upon the growth rate of either fungi when added to the agar ($p<0.001$), allowing the control and solvent control data to be pooled (Appendix 1, Appendix 2). Ethyl acetate extractives had significant effects upon the growth rate of *G. trabeum* but not *T. versicolor*. Each agar containing an extractive of a sample had a significantly different effect upon *G. trabeum* in comparison to each other ($p<0.001$) (Table 5). *T. versicolor* growth rates were however all significantly the same for all extractives and controls (Table 6, Appendix 4).

Table 5- Significance of growth rate for ethyl acetate extracts of *E. bosistoana* samples, *Gloeophyllum trabeum*

Ethyl Acetate GT	Control	8YO	60YO A A	60 YO A B	60 YO B A	60 YO B B
8YO	<0.001	1				
60YO A A	<0.001	<0.001	1			
60 YO A B	<0.001	<0.001	<0.001	1		
60 YO B A	<0.001	<0.001	<0.001	<0.001	1	
60 YO B B	<0.001	<0.001	<0.001	<0.001	<0.001	1

Table 6 - Significance of growth rate for ethyl acetate extracts of *E. bosistoana* samples, *Trametes versicolor*

Ethyl Acetate TV	Control	8YO
8YO	0.778	1
60YO	0.549034	0.404

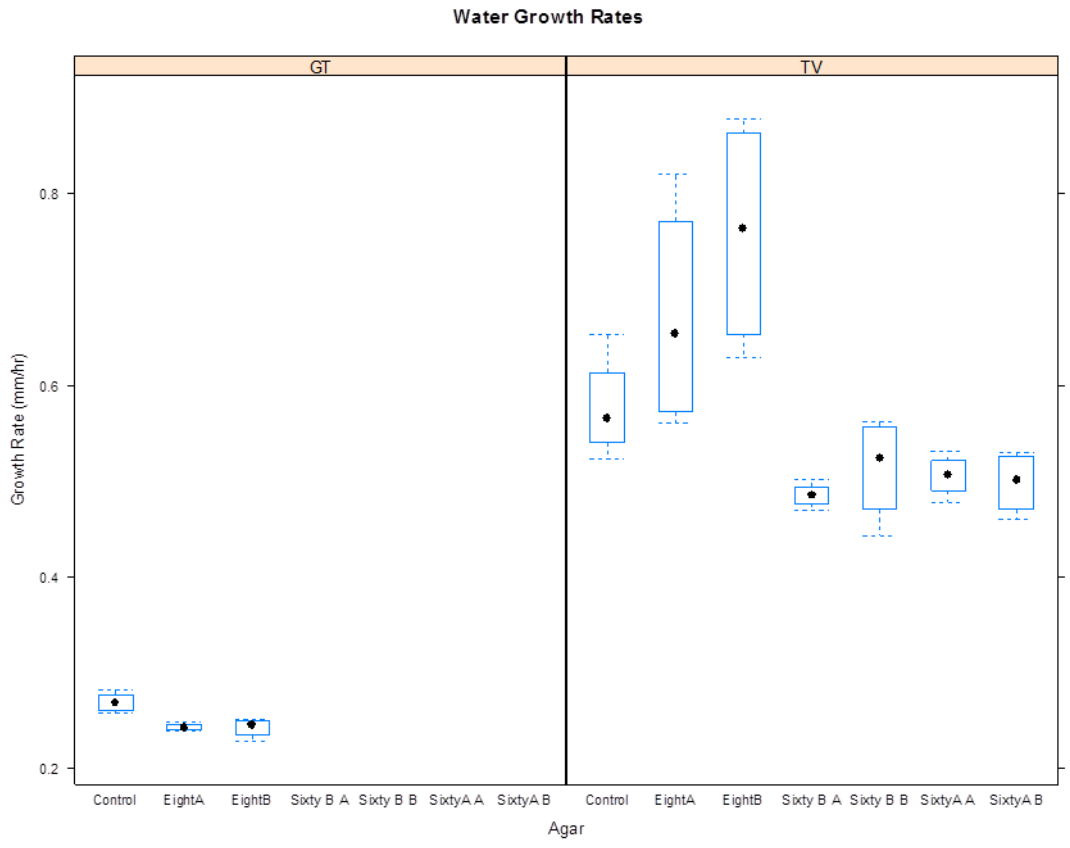

Figure 14 - Fungal growth rates of *T. versicolor* and *G. trabeum* on agar containing water extractives of *E. bosistoana* heartwood

Of the water extracts, the 8 year old sample did not significantly affect the growth rate of *G. trabeum* or *T. versicolor* ($p = 0.0923$ and $p=0.363$, respectively) (Table 7 and Table 8). A non-significant increase in growth rate and a high variability was observed for 8 year old *T. versicolor* trials. The 60 year old water extractives however caused a significant reduction of growth rate for *G. trabeum* and *T. versicolor* ($p<0.001$ and $p=0.0276$ respectively) (Table 7 and Table 8). The growth of *G. trabeum* was completely inhibited by the 60 year old samples (Figure 14).

There is a consistency between the extractive agar growth rates for both GT and TV indicating consistency of the method used (Figure 14).

The 8 year old extracts agar growing *T. versicolor* has a higher rate of variability, due to a small number of measurements caused by the fast growth rate before the fungal bodies reached the edge of the petri dish (2 measurements)

Table 7 – Significance of growth rate for water extracts of *E. bosistoana* samples, *Gloeophyllum trabeum*

Water GT	Control	8YO
8YO	0.092685	1
60YO	<0.001	<0.001

Table 8- Significance of growth rate for water extracts of *E. bosistoana* samples, *Trametes versicolor*

Water TV	Control	8YO
8YO	0.363086	1
60YO	0.027579	0.00155

Table 9 - Growth retardation/enhancement effect of each agar in comparison to the control (pure agar) growth rate for *Gloeophyllum trabeum*. Variance for growth rates were calculated according to Kendall, Stuart, Ord, and O'Hagan (1994)

	Water		Ethanol		Ethyl Acetate	
	Relative Growth Rate	Variance	Relative Growth Rate	Variance	Relative Growth Rate	Variance
Control	100%	0%	100%	1%	100%	0%
Solvent	NA	NA	99%	0%	103%	0%
8 YO A	90%	1%	78%	7%	91%	1%
8 YO B	90%	1%	NA	NA	77%	1%
60 YO (A) A	0%	0%	NA	NA	48%	0%
60 YO (A) B	0%	0%	NA	NA	14%	0%
60 YO (B) A	0%	0%	0%	0%	22%	0%
60 YO (B) B	0%	0%	NA	NA	7%	0%

*G. trabeum'*s growth rate was retarded by the extractives obtained by each of the solvents for both the 8 year old and 60 year old samples (Table 9). The 60 year old sample extractives tended to have much higher retardation ability than that of the 8 year old sample extractives, completely inhibiting the growth with water and ethanol extracts.

T. versicolor however was affected less by the same extractives than G. *trabeum* (Table 10). Agars which *G. trabeum* was incapable of growth (i.e. 60YO A A, 60YO A B, 60YO B A, 60YO B A, water and ethanol) *T. versicolor* was still able to sustain an average growth rate equivalent of ~80% of the control growth rates. *T. versicolor's* growth rates increased with the addition of some extracts (mainly 8 year old extracts and ethyl acetate extracts) (Table 10).

Table 10 - Growth retardation/enhancement effect of each agar in comparison to the control (pure agar) growth rate for *Trametes versicolor*. Variance for growth rates were calculated according to Kendall et al. (1994)

TV	Water		Ethanol		Ethyl Acetate	
	Comparable Growth Rate	Variance	Comparable Growth Rate	Variance	Comparable Growth Rate	Variance
Control	100%	2%	100%	0%	100%	0%
Solvent	NA	NA	100%	0%	102%	4%
8 YO A	117%	6%	103%	1%	101%	1%
8 YO B	131%	6%	NA	NA	96%	1%
60 YO (A) A	88%	1%	NA	NA	88%	0%
60 YO (A) B	86%	1%	NA	NA	99%	1%
60 YO (B) A	84%	1%	77%	0%	105%	0%
60 YO (B) B	89%	2%	NA	NA	101%	0%

Discussion

When analysing the growth rates of the fungi it must be kept in mind that agar contains a higher nutritional value than wood cell walls. This may enable fungi to grow in the presence of compounds which often inhibit their growth in-situ.

Solvent Differences

Extracts removed with ethanol from *E. bosistoana* were found to be the most fungicidal extractives in comparison to water or ethyl acetate extractives, for both fungi (Table 9,Table 10). Rudman and DaCosta (1961) found a similar result with methanol obtaining the most fungicidal extractives from various other eucalypt species. Ethanol is of a very similar chemical structure to methanol therefore is expected to extract similar compounds; however ethanol is less toxic. Water extracts also displayed a high fungicidal capacity as found in Anderson (1961) for *Sequoia sempervirens*. Extractives are very species specific therefore this finding may not be necessarily transferable to *E. bosistoana*. Ethanol seems to be the preferable choice of solvent for *E. bosistoana*.

Ethanol and water extracted similar percentages of extracts from each sample. However mid infrared spectrometry confirmed that the type of compounds obtained varied between the two solvents. This is consistent with research done by Rudman (1962). Ethanol is both polar and non-polar whereas water is a polar substance. This means that ethanol is capable of extracting not only the polar compounds but also some non-polar compounds. Water can only extract mostly polar compounds. This was observed in the IR spectra (Figure 9) with the signal of aliphatic groups at 2800cm^{-1} absent in the water extract. Much less was extracted by the non-polar ethyl acetate, which can be contributed to by 2 factor; 1) there are less non-polar compounds in the heartwood of *E. bosistoana,* 2) the non-polar ethyl acetate does not swell the wood cell wall making extraction different.

Differences in Properties with Age

Quantity of Extractives

Significantly more extracts were found in the samples of the old tree than the young tree (Table 2). This occurred with all three solvents used for extraction. These findings are consistent with data from other species (Gartner et al., 1999; Klumpers et al., 1994; Rudman, 1965; Wilkes, 1983). Klumpers et al. (1994) showed that the outer heart wood of an older tree of *Quercus robur* and *Quercus petraea* contained a higher total amount of extractives than the inner heartwood. However Klumpers et al. (1994) did not use the samples from a young tree, meaning the inner heartwood (formed at a young age) could have endured aging over long period of time, potentially changing its chemical composition simultaneously. Morita, Yatagai, and Fujita (1995) found that methanol extracts tended to increase from the pith (formed at a younger age) toward the outer heartwood (formed at older age) of Yakusugi (*Cryptomeria japonica* D. Don). This report focused on the heartwood of a young 8 year old tree and the heartwood of an older 60 years old tree, removing the effect of aging on the heartwood established at a younger age. To the best of my knowledge no studies have been published on the heartwood of young trees. The results are consistent in terms of quantity of extracts present but not necessarily comparable.

Type of Extractives

Mid infrared spectrometry of both age class extractives revealed a difference in the composition of compounds. 8 year old *E. bosistoana* extracts were found to contain a higher proportion of aliphatic groups (CH) relative to hydroxyl groups (OH) in the ethanol extracts. This indicates that juvenile heartwood differs from mature heartwood in consensus with wood characteristics like Micro Fibril Angle (MFA). This is consistent with work by Klumpers et al. (1994) who observed an increasing number of water soluble extracts towards the outer heartwood in *Quercus*.

Durability

It was found that durability of *E. bosistoana* was higher for the sample form the old tree. These findings are in accordance with Rudman (1965) whose study showed for some *eucalypts* the effect of fungal decay decreased with the increased distance from the pith (Figure 1). Wood located further from the pith is formed when the cambium is of an older age than that near the pith (therefore treated as an older sample). Several papers report on the difference between the inner heartwood and outer heartwood of an individual stem of an older tree, but not between separate individuals of different age.

Implications for Extraction and Fungal Test Methods

Optimising an extraction method with an Accelerated Solvent Extraction system (ASE) was found to reduce the extraction time in comparison to traditional soxhlet methods. An ASE performs extractions at high pressure allowing solvents to be used at a higher temperature than its room pressure boiling point and this increases diffusion rates to release compounds faster. The time taken of extractions has been reduced from 24 hours (Anderson, 1961) down to a total of 30 minutes per extraction.

Testing fungicidal effects on agar mixed with extracts has reduced the experiment time to 4 days for both *T. versicolor* and *G. trabeum*. Anderson (1961) would wait for 10 weeks before the effect of the fungi was measured by mass loss of wooden blocks.

The optimal ASE method and identifying that ethanol extracts of *E. bosistoana* are of highest fungicidal activity can be used in future research to characterize the natural durability of this species. Further fractionation with other solvents may help refine the exact key compounds displaying fungicidal activity.

Implications for Using Different Aged *E .bosistoana*

The fungicidal activity of *E. bosistoana* extractives is greater from older individuals, therefore it is more likely older trees are more naturally durable to fungal decay than younger trees. However this does not imply that young E. *bosistoana* timber is non-durable, but it is less than older trees.

To ensure high quality/durability from short rotation *E. bosistoana* plantations a breeding program seems necessary to improve durability. Further characterisation of the individual compounds present appears to be informative as the extracts differ between the young and old trees.

As a result, future studies of the natural durability of *E. bosistoana* should be carried out on young trees.

Limitations

There were a number of limitations which were encountered when carrying out the methods discussed.

The number of samples used for this research project was one of the fundamental flaws. For this project one 8 year old sample and two sixty year old samples of unknown origin/history were the only samples used. In doing so it was assumed that these samples were representative of their entire age class which they may not be. The samples however were useful to develop the extraction and assessment of methodology.

The optimal temperature for growth of each fungus was not taken into account. The fungi were however both subject to a constant 25.2°C, a randomly selected temperature. This temperature was more suited to *T. versicolor* which may be the reason behind its faster growth rate in comparison to *G. trabeum* which requires higher temperatures. However by keeping the temperature constant the results were still comparable

To assess fungicidal activity of extracts one needs to have constant concentration in the agar, not varied between samples. We replicated the extractive content of the samples in the agar, therefore measuring the natural durability of the wood. By keeping the percentage of extracts constant in the agar the fungicidal properties of

the different compounds could be investigated. There was however insufficient time to carry this analysis out.

Conclusions

The optimal extraction process was found to use an Accelerated Solvent Extraction system with the following specifications for extraction:

- 2 cycles per sample
- 70°C extraction temperature
- 50% rinse
- 5 minute cycle

Ethanol was found to obtain the extractives of the highest fungicidal properties (causing natural durability). Water extracted compounds of high fungicidal capacity; however ethanol was able to extract polar compounds as well as non-polar compounds from the *E. bosistoana,* shown by MIR spectra's.

60 year old trees were found to have a higher fungicidal capability than 8 year old trees of *E. bosistoana*. However it was not identified whether this was due to the higher amounts of extractives located in older samples (12% to 3%) or the difference in compounds present within them.

This study investigated the fungicidal capabilities of *E. bosistoana* timber as the amount percentage of extracts added to the agar varied to replicate that in the samples. To measure the fungicidal activity of the extractives, a constant percentage of extractives must be added to the agar.

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Appendices

Appendix 1 *Growth rates G. trabeum on each of agar and its significance in comparison to the control.*

GT	Water		Ethanol		Ethyl Acetate	
	Growth Rate	P Value	Growth Rate	P Value	Growth Rate	P Value
Control	0.257	<0.001	0.244	<0.001	0.440	<0.001
Solvent	NA	NA	0.243	0.900	0.423	0.506
Eight A	0.243	0.194	0.191	0.871	0.390	0.041
Eight B	0.243	0.187	NA	NA	0.325	0.000
Sixty (A) A	0.000	<0.001	NA	NA	0.216	<0.001
Sixty (A) B	0.000	<0.001	NA	NA	0.061	<0.001
Sixty (B) A	0.000	<0.001	0.000	<0.001	0.029	<0.001
Sixty (B) B	0.000	<0.001	NA	NA	0.097	<0.001

Appendix 2 *Growth rates T. versicolor on each of agar and its significance in comparison to the control.*

TV	Water		Ethanol		Ethyl Acetate	
	Growth Rate	P Value	Growth Rate	P Value	Growth Rate	P Value
Control	0.568	<0.001	0.506	<0.001	0.654	<0.001
Solvent	NA	NA	0.484	0.648	0.541	0.261
Eight A	0.586	0.671	0.536	0.437	0.608	0.508
Eight B	0.628	0.326	NA	NA	0.631	0.749
Sixty (A) A	0.504	0.152	NA	NA	0.448	0.068
Sixty (A) B	0.484	0.056	NA	NA	0.663	0.890
Sixty (B) A	0.486	0.021	0.407	0.040	0.557	0.389
Sixty (B) B	0.483	0.018	NA	NA	0.673	0.748

Appendix 3 *Significance of G. versicolor growth rates between ethyl acetate agars*

Ethyl Acetate GT	Solvent	Eight A	Eight B	Sixty (A) A	Sixty (A) B	Sixty (B) A	Sixty (B) B
Solvent	1						
Eight A	0.008	1					
Eight B	0.000	0.026	1				
Sixty (A) A	<0.001	<0.001	<0.001	1			
Sixty (A) B	<0.001	<0.001	<0.001	<0.001	1		
Sixty (B) A	<0.001	<0.001	<0.001	<0.001	<0.001	1	
Sixty (B) B	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1

Appendix 4 *Significance of T. versicolor growth rates between ethyl acetate agars*

Ethyl Acetate TV	Solvent	Eight A	Eight B	Sixty (A) A	Sixty (A) B	Sixty (B) A	Sixty (B) B
Solvent	1						
Eight A	0.531	1					
Eight B	0.402	0.736	1				
Sixty (A) A	0.505	0.196	0.147	1			
Sixty (A) B	0.210	0.418	0.647	0.047	1		
Sixty (B) A	0.905	0.684	0.558	0.396	0.325	1	
Sixty (B) B	0.159	0.297	0.506	0.034	0.860	0.271	1

Appendix 5 *Significance of T. versicolor growth rates between water agars*

Water TV	Eight A	Eight B	Sixty (A) A	Sixty (A) B	Sixty (B) A	Sixty (B) B
Eight A	1					
Eight B	0.571	1				
Sixty (A) A	0.175	0.089	1			
Sixty (A) B	0.092	0.045	0.651	1		
Sixty (B) A	0.040	0.020	0.622	0.973	1	
Sixty (B) B	0.036	0.019	0.575	0.969	0.932	1

Appendix 6 *Significance of G. trabeum growth rates between water agars*

Water GT	Eight A	Eight B	Sixty (A) A	Sixty (A) B	Sixty (B) A	Sixty (B) B
Eight A	1					
Eight B	0.942	1				
Sixty (A) A	<0.001	<0.001	1			
Sixty (A) B	<0.001	<0.001	1	1		
Sixty (B) A	<0.001	<0.001	1	1	1	
Sixty (B) B	<0.001	<0.001	1	1	1	1