Antimicrobial defence in the sapwood of *Eucalyptus nitens*: studies of the reaction zone

by

Karen May Barry

B. Sc. (Hons) University of Tasmania

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This thesis is dedicated to Ray Pearce,

a lover of trees, cacti and motorcycles.

DECLARATIONS

This thesis does not contain any material which has been accepted for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge, this thesis contains no material previously published or written by another person except where due acknowledgement is made.

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ABSTRACT

Decay arising from pruning wounds in plantation-grown *Eucalyptus* spp. is a concern for the Australian solid wood products industry, as the potential for decay spread within the tree is unknown at present. Mechanisms of antimicrobial defence in the xylem of *Eucalyptus nitens* were investigated. Key factors investigated within the host-fungal interaction included phenolics, moisture content, mineral content and anatomical alterations (tyloses and suberization).

A purple reaction zone was regularly observed at the sapwood interface of decay lesions developing from naturally-infected pruning wounds in plantation-grown *E. nitens*. Total phenols levels were increased substantially and vessel tyloses were abundant in the reaction zone. A log incubation experiment showed that after 9 months the reaction zone remained intact while sapwood became decayed. The reaction zone was significantly drier than healthy sapwood, with lower levels of potassium, and a lower pH than both sapwood and heartwood. These trends are more similar to conifer reaction zones than deciduous angiosperms. The mode of reaction zone defence appears to be a dynamic process (also similar to conifers) rather than a static process typical of deciduous angiosperms.

The initial stages of antimicrobial defence formation were also studied. Xylem defence responses occurring in young pot-grown *E. nitens* saplings were analysed within the first few weeks following stem wounding and inoculation with the decay fungus *Ganoderma adspersum* (which displayed relatively slow colonization of the wounded xylem). Events occurring most quickly after wounding and inoculation were tylose formation and phenol accumulation, which indicates that they are key defences. NMR imaging revealed that changes in xylem moisture content were not rapid after wounding.

Reaction zone extracts were inhibitory to test-fungi in thin-layer chromatography bioassays. Analysis of extracts by liquid chromatography – electrospray ionization mass spectrometry revealed that a diverse range of hydrolyzable tannins were present in *E. nitens* wood, including over thirty gallotannins, ellagitannins and phenols. A number of ellagitannins (particularly pedunculagin) were considerably more abundant in the

reaction zone than the healthy sapwood and may contribute to the effectiveness of the reaction zone as an antimicrobial barrier. The process of reaction zone formation may be an accentuation of processes that occur when heartwood forms. Heartwood extracts were qualitatively similar to reaction zone extracts but actual increases in ellagitannins were not as great.

Conditions such as season of wounding and the type of fungi which become established in wounds, appear to effect the degree of defence response. This was indicated by phenol levels. More extensive decay lesions were generally associated with more phenolic production. Sterile inoculations and weakly-aggressive fungi were associated with no or little reaction zone formation, while aggressive fungi elicited more discolouration and phenolic accumulation in advance of infection. This suggests that non-aggressive fungi are less tolerant of the inherent xylem microenvironment and active defence responses are not "required" or elicited.

Xylem defence responses appear to be relatively successful in *E. nitens*. This may be facilitated (at least in part) by a fast and considerable accumulation of phenolic compounds. Qualitative studies of phenolic compounds may be a useful indicator of defensive capacity.

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PREFACE

This PhD thesis is composed of 5 papers, which have been published or submitted for publication to refereed international journals. To enhance the structure of the thesis, the following changes have been made:

- References have been removed and aggregated to a single list of references at the end of the thesis;
- Abstracts and acknowledgements have been removed;
- Figures and tables have been renumbered according to the chapter;
- Cross-references to papers within the thesis have been changed to refer to chapter number.
- Cross-references to appendices have been made and expanded on where required.

Additional details and experiments that were not published (or were superceded) have been included as appendices. These follow appropriate chapters as supporting material and their context is briefly discussed within the appendix.

Publications arising from this project (including preliminary studies not discussed here) are as follows:

Refereed journal papers

Barry, K.M., Pearce, R.B. and Mohammed, C.L. 2000. Properties of reaction zones associated with decay from pruning wounds in plantation-grown *Eucalyptus nitens*. *Forest Pathology*. **30:** 233-245.

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Mohammed, C., Barry, K., Battaglia, M., Beadle, C., Eyles, A., Mollon, A., Pinkard, E. 2000. Pruning-associated stem defects in plantation *E. nitens* and *E. globulus* grown for sawlog and veneer in Tasmania, Australia. *The Future of Eucalypts for Wood Products.* International Union of Forestry Research Organisations. Launceston, Tasmania, Australia, 19-24th March. Proceedings pp. 357-364.

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biennial Australasian Plant Pathology Society Conference, Canberra, 27-30th September.

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Peacock, E. J., Barry, K. M. and Mohammed, C. L. 2000. A preliminary study of antimicrobial defence in *Eucalyptus nitens* using NMR Microscopy. *The Australian and New Zealand Society for Magnetic Resonance, ANZMAG 2000 Conference*, Mt Buller, Victoria, Australia, 13-17th February 2000, Poster 53.

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Technical reports

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Eucalypts and decay

Wood decay is a natural and necessary component of forest ecosystems, occurring mostly as a saprophytic process in the heartwood or of wood on the forest floor. Decay is one of the major factors that limit the life-span of eucalypts. Typically eucalypts live between 200-400 years (Jacobs, 1955). Trees with more durable woods have a longer life-span, for example oaks can live for 1500 years (Jacobs, 1955). In older, wet forest ecosystems in Tasmania, eucalypts are succeeded by slower-growing more durable trees such as *Nothofagus cunninghamii* (Gilbert, 1958).

Wood decay can be a major source of financial loss to the forest industry, especially for solid wood products. Decay columns reduce the merchantable volume, strength and appearance quality of logs, therefore attracting lower pricing due to downgrading.
In the shift away from native forest logging, the Australian forest industry is applying more intensive silvicultural regimes to timber production, involving thinning in native forests ("regrowth" forests) and the establishment of plantations. The Australian forest industry is committed to an Australia-wide target of an extra two million hectares of commercial plantations by the year 2020 (there are currently one million) with an added incentive of expanding plantations for the reduction of atmospheric CO_2. Current expectations are that most plantations will be used for pulpwood. Plantation management for sawlogs is more intensive than for pulpwood, and is therefore more economically sensitive to fluctuations in wood yield and quality. Specifications for acceptable levels of decay determine in which grade a sawlog will be classed. Current prices (2001) for sawlogs of veneer quality are approximately AUS$50/m^3 while first grade and second grade sawlog are $30 and $20 respectively (M. Wood, Forestry Tasmania, pers. comm.). Where sawlog is downgraded for pulpwood, it will attract prices of $12/m^3.

Eucalyptus nitens (Maiden) is the major species planted in Tasmania, with ca. 7000 hectares planted between 1991 and 1996 (Forestry Tasmania, 1998). As of July 1999,

over 17000 hectares of hardwood plantation was present in Tasmania, with 2500 hectares planted in the previous year (Forestry Tasmania, 1999). This increase was promoted by the Tasmanian Regional Forest Agreement in 1997. *E. nitens* is favoured for high quality timber production due to good growth rates on a variety of sites, frost tolerance, good form and wood quality suitable for pulp, sawn timber and veneer (Gerrand *et al.*, 1997). *E. nitens* is also planted outside Australia, including New Zealand (Lausberg *et al.*, 1995) and South Africa (Purnell, 1988).

Currently, intensive management practices including stand thinning and branch pruning are practiced within Tasmanian plantation forestry for sawlog production. Some eucalypt species favoured for plantation (such as *E. nitens*) do not naturally shed dead, lower branches. As knots will persist throughout the timber, green pruning is carried out to produce knot-free clearwood for sawlog and veneer products (Gerrand *et al.*, 1997). The size of the knotty core is reduced if pruning is completed early and current prescriptions involve first lift pruning at 3-4 years of age (ca. 6 m height; 8-10 cm DBH) to a height of 2.5 m (Pinkard *et al.*, 1999). Pruning of up to 50 % of canopy length is unlikely to affect height, diameter growth, stem taper or dominance in *E. nitens* (Pinkard and Beadle, 1998). Second and third lift prunes are completed at 4-5 years and 5-6 years respectively (Pinkard *et al.*, 1999). Form pruning (the removal of large or high-angle branches) is also often completed, with current prescriptions suggesting this procedure be done at 2 years age (Pinkard *et al.*, 1999). Thinning is necessary to maximize diameter growth of selected, high-quality final crop trees. This also minimizes the rotation length, which is between 25-40 years (Gerrand *et al*, 1997).

Both pruning and thinning operations result in wounding. Pruning provides an entry point for decay mainly through the branches themselves or through damage to the branch collar or stem. Previous studies of some *Eucalyptus* species have not reported major concerns for pruning-associated decay. Decay associated with pruned branches was rarely found in plantation-grown *E. regnans* (Glass and McKenzie, 1989) and pruned branches were no more likely to be the source of stem decay than unpruned branches for *E. delegatensis* grown in New Zealand (Gadgil and Bawden, 1981). As unpruned stands of *E. nitens* appear to be less susceptible to decay than some other species such as *E. regnans* (Yang and Waugh, 1996) it may be expected that decay would also be minimal as a result of pruning.

However, studies of eight-year-old *E. nitens* have indicated that the incidence of decay entry from pruning wounds may be high in *E. nitens* (Mohammed *et al.*, 1998). Branches which are of large diameter and living ("green") when pruned result in particularly high levels of decay, particularly on the wetter and warm sites (Mohammed *et al.*, 2000). When dead branches were pruned less decay resulted (Mohammed *et al.*, 2000), which is probably explained by the formation of a "protective zone" in the process of senescence (Jacobs, 1955). Branches must be pruned while living as pruning of dead branches results in the branch stub breaking internally and becoming drawn out of the stem with the bark. This will result in a kino-filled void which forms throughout the clearwood (Neilsen and Pinkard, 2000).

The internal spread of decay associated with pruning wounds in plantation eucalypt forests is not yet fully known (as the harvest age of current plantations has not been reached at present). It is vital to understand xylem defence processes in eucalypts and particularly the success of these defences in restricting decay to the knotty core. Wilkes (1982) stated that "...Australian forestry is possibly paying dearly for [the] lack of knowledge...[about stem decay]...as it is now evident that plantation and regrowth eucalypt stands contain extensive heartrot". In the mid-1980's John Wilkes conducted a range of studies of wood defences in some eucalypt species, but this information requires expansion and application to species that are currently relevant. Due to the increasing reliance of the forest industry on plantation forests, involving increasing capital investment and marginal profits, it is now even more critical to establish factors able to limit the spread of decay.

1.2 Xylem defence models

The ability of trees to "compartmentalize" infection (whether it be a decay or stain-related) is well known. The relative ability of trees to compartmentalize (ie. restrict extent of infection) differs with a variety of factors. These include species differences (Schmitt *et al.*, 1995; White *et al.*, 1999), and within species variation, including studies with different clones (Shigo *et al.*, 1977; Smith and Shortle, 1993a) and provenances (White *et al.*, 1999). Differences in compartmentalization ability have also been noted

for different sites (Smith and Shortle, 1993b) and with seasonal variation (Schmitt and Liese, 1992; White and Kile, 1993; Mohammed *et al.*, 2000).

The term compartmentalization is used to denote restriction of decay and can be used in a broad sense. For example, the limitation of some decay infections to the heartwood is a form of compartmentalization. That is, decay is excluded from the sapwood which constitutes living cells and is usually higher in moisture content. In fact, the original concept of the decay process was as a saprophytic infection of the heartwood (Boyce, 1961). However, decay can also occur in sapwood during a range of plant-fungal interactions. Studies of compartmentalization have been most frequently completed with wounded sapwood as a means of assessing the extent to which decay can develop from wounds. This provides important information for the forest industry.

Various models of compartmentalization and wood defence have been proposed in parallel since the 1960's. These include :

1. Compartmentalization Of Decay In Trees (CODIT) by Shigo and Marx (1977)
2. Dynamic reaction zones (Shain, 1967, 1971, 1979)
3. Microenvironmental restriction of decay (Boddy and Rayner, 1983; Rayner and Boddy, 1988).

These concepts have developed from a variety of studies and are still being reviewed and debated (Boddy and Rayner, 1983; Shigo, 1984; Rayner and Boddy, 1988; Blanchette, 1992; Pearce, 1996).

The CODIT model was proposed by Shigo and Marx (1977) and has created the widest impact out of the 3 models, particularly for arboriculture. This proposed the existence of four "walls" around the decay column. Wall 1 contains the axial spread of decay by the formation of vessel occlusions such as tyloses. Wall 2 is a tangential anatomical restriction, ie. growth rings with cells of reduced pitting, a higher lignin content and small lumens. Wall 3 is the radial restriction by the activities of parenchyma ray cells. These walls represent both inherent features of the wood (passive defence) and induced anatomical and chemical restrictions (active defence). Wall 4 represents alterations made in new tissue in response to wounding, known as a "barrier zone". This wall limits the outward expansion of decay to new wood and is formed by the vascular

cambium. The position of these walls is shown in Figure 1.1, using an example of decay entering from a pruning wound.

The original CODIT model proposed that the "walls" were static boundaries. Shain (1967, 1971) proposed that it was a more dynamic process after studying the interaction of *Heterobasidion annosum* (or *Fomes annosus*) infections of loblolly pine and Norway spruce. From these studies the concept of a "reaction zone" was developed (Shain, 1967, 1971, 1979). Two zones were reported including the reaction zone and transition zone (Shain, 1979). A continuous progression of these zones in advance of decay was envisaged. After these studies, the CODIT model was re-defined; including part 1 (the reaction zones) and part 2 (the barrier zone) (Shigo, 1984).

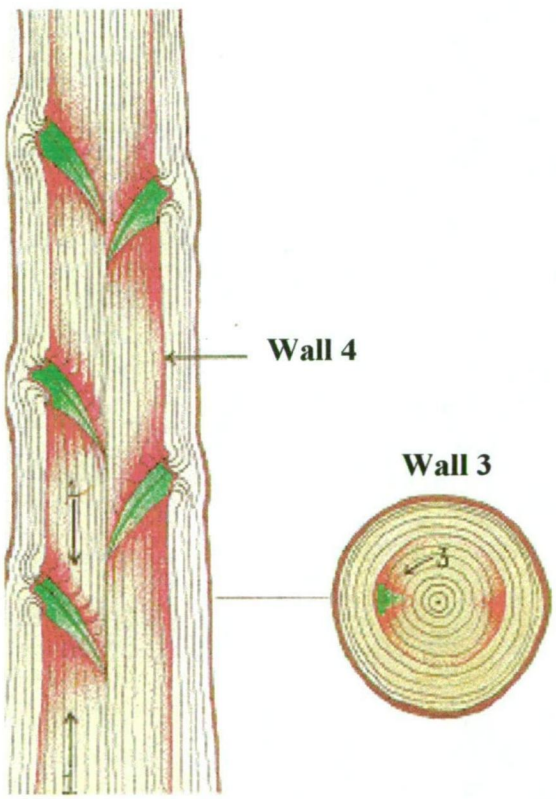


Figure 1.1. Patterns of defence in the expanded concept of tree decay, where infection (green) is restricted to the pruned branches by active defences (red) including a continuous barrier zone (wall 4). [From Shigo, 1979].

A number of studies have suggested that a succession of microorganisms is involved with the decay process (Shigo, 1967, 1972). Wounding experiments with *Acer rubrum* revealed that decay fungi commonly found associated with advanced decay were not capable of invading fresh wounds (Shigo, 1974). Shigo and Sharon (1968) divided this succession following wounding into three stages. The first stage involved cell death of xylem parenchyma and production of phenolic compounds resulting in discolouration. The access of air is considered necessary for discolouration, but microorganisms are not involved in this stage. The second stage develops if the phenolic compounds in the discoloured tissue can be overcome by microorganisms entering the wound. These may be bacteria and non-decay fungi that can utilize or detoxify the phenolic compounds. In advance of this interaction, phenolic compounds are then elicited again. The third stage involves the invasion of altered wood by decay fungi, which have the ability to decompose cell wall substances and result in "advanced" decay. However, decay fungi cannot supposedly advance ahead of the new region of phenolic accumulation. While wound colonization appears to occur by a succession of microorganisms in some interactions, it may be an over simplification of the decay process. Other studies indicate that decay can occur rapidly after wounding with no apparent microbial succession, for example by aggressive fungi such as *Chrondrostereum purpureum* in *Acer pseudoplatanus* (Pearce *et al.*, 1997; Pearce, 2000).

More recent studies have provided evidence that in angiosperms reactions zones are not dynamic but static in nature. When the reaction zone boundary is disrupted (for example by boring insects, or ray shakes) and circumvented, a volume of wood may be colonized with little or no expression of characteristic reaction zone responses, until ultimately a new reaction zone is established. Examples where reaction zone "relics" have been found include *Fagus sylvatica* (Rayner, 1986; Pearce, 1991) *Aesculus hippocastanum*, *Fraxinus excelsior* (Pearce, 1991). In wounding studies with *Acer saccharum* and *Acer rubrum*, the distribution of reaction zone relics indicated annual defeat and re-formation (Shortle and Smith, 1990). A number of recent studies have referred to defence responses occurring at the interface of infected wood as column boundary layers (Shortle and Smith, 1990) or column boundary layer reaction zones (Pearce, 1996) to avoid the concept of dynamic reaction zones.

Boddy and Rayner (1983) suggest that reaction zone barriers are breached when the moisture content behind them drops to a critical level. The exposure of xylem to air subsequent wood moisture content is an important factor that may determine fungal spread. Boddy and Rayner (1983) proposed that the very nature of functional sapwood as a microenvironment of high moisture content and lack of readily assimilable nutrients is able to explain the restriction of decay development. The walls of the CODIT model were interpreted as a means of maintaining hydraulic integrity, rather than as physical and chemical barriers to restrict or inhibit fungal growth (Rayner, 1986; Rayner and Boddy, 1988). The idea that xylem responses (such as tyloses and gums) provide a closed system to prevent desiccation was originally proposed in early studies of discoloured wood (e.g. Swarbrick, 1926). The "waterproofing" of cells by the deposition of suberin (which is hydrophobic) in the barrier zone supports this theory (Pearce and Rutherford, 1981). Rayner and Boddy (1988) state that even barrier zones cannot be considered a persistent obstruction to fungal growth, as observations can show the outward spread of the same fungal genotype across a barrier zone.

Boddy and Rayner (1983) suggested that wounding studies could potentially distort an understanding of the decay process. Various studies of attached branches of *Quercus robur* (Boddy and Rayner, 1981) and *Fraxinus excelsior* (Boddy *et al.*, 1987) have revealed the development of decay in the absence of major wounds, patterns of which can be related to xylem drying patterns. In *Fagus sylvatica* branches, fungal colonization appeared restricted to "fields" that were no longer active in water conduction (Chapela and Boddy, 1988). Some recent studies have disputed that moisture content is more important than active defence responses. This includes *in vitro* studies of *Cryptomerica japonica* wood, which found that fungal colonization of wood treated in order to kill living cells was much greater than when cells were alive (Yamada, 1998).

Pearce (1996) stated that the CODIT model and theories of microenvironmental restriction probably operate in concert. It is unwise to infer broad generalizations about decay restriction to all xylem-microbe interactions and each system may reveal different features. An important factor is the nature of the invading microorganism, for example, whether it is a saprophyte or pathogen. In the latter case decay may not be contained as the pathogen either advances more quickly than active defence processes or can tolerate

or degrade these responses. Rayner (1986) classified decay fungi into five behavioural classes including heart rots, unspecialized and specialized opportunism, active pathogenisis and desiccation tolerance. Records of fungi causing heart rot in the eucalypts have been compiled and over 70 percent of heart rots were associated with white rot fungi (Kile and Johnson, 2000). Fungi establishing in pruning wounds of *Eucalyptus delegatensis* have been assessed in New Zealand (Gadgil and Bawden, 1981). Very few studies have been completed of saprot in eucalypts in general.

In summary, a number of theories have been developed in order to explain wood decay compartmentalization. The relative importance of active defence processes versus passive, inherent features of the wood (such as microenvironment) remain unclear. It is necessary to study more host-pathogen systems and interpret them without the bias of a generalized concept. It is also necessary to develop the research for practical solutions to decay reduction in a forestry context.

1.3 Aims of this research

The aims of this research began as a general investigation of defence responses associated with decay from pruning wounds in *E. nitens*. A particular goal was to characterize the blue-purple "reaction zone" that was previously observed (Wardlaw and Neilsen, 1999). Research was therefore focussed on this zone and responses occurring in wood extant at the time of injury. A long-term aim of this study was to identify indicators which may reflect the relative ability of eucalypts (of a certain site, provenance or species) to be more or less successful in restricting decay.

CHAPTER 2

PROPERTIES OF REACTION ZONES ASSOCIATED WITH DECAY FROM PRUNING WOUNDS IN PLANTATION-GROWN *EUCALYPTUS NITENS.*

2.1 INTRODUCTION

Eucalyptus nitens (Maiden) is a species preferred for plantation in Tasmania and pruning is required to produce high quality sawlogs (Gerrand *et al.*, 1997). However, the incidence of stem decay associated with pruned trees is high compared to unpruned trees (Mohammed *et al.*, 1998; Wardlaw and Neilsen, 1999). A full understanding of antimicrobial defence mechanisms in *E. nitens* sapwood is vital. The long term ability of the tree to resist stem decay spread will determine the extent of decay and consequential effect on wood quality.

Decay in sapwood is contained by a number of inherent features of the wood and by actively formed zones (Blanchette, 1992; Pearce, 1996). Barrier zones are formed by the cambium at the time of wounding and offer protection to new xylem (Shigo, 1984; Pearce and Woodward, 1986) and are considered the strongest of "walls" in the CODIT model (compartmentalization of decay in trees, Shigo and Marx, 1977). Reaction zones are formed in pre-existing xylem at the interface between infection and the healthy sapwood and present particularly resistant barriers. They are often coloured zones due to polyphenolic deposits, for example, they are green or orange in *Acer* spp. (Pearce and Woodward, 1986; Pearce, 1996) or brown in *Fraxinus excelsior* infected by *Inonotus hispidus* (Pearce, 1991). The formation of reaction zones (Shain, 1971) or column boundary layers (Shortle and Smith, 1990) as a defence to challenged sapwood appears to be a ubiquitous phenomenon in woody plants, apparent in both angiosperms and gymnosperms (Shain, 1971; Pearce, 1996).

While elicitation of phenolic compounds appears to be a universal and important component of defence (Kemp and Burden, 1986; Duchesne *et al.*, 1992; Yamada, 1992; Pearce, 1996), Boddy and Rayner (1983) propose that the wood microenvironment is of primary importance in determining decay restriction. Fungal metabolism is dependant

on suitable oxygen access, which is reduced by an increasing wood moisture content. The creation of a wound renders surrounding wood compromised due to the withdrawal of water and ingress of oxygen. The ability of the tree to restore the moisture content to normal levels may be crucial to restrict infection. In some angiosperms, the moisture content of the reaction zone is increased in comparison to the healthy sapwood (Pearce *et al.*, 1994; Pearce *et al.*, 1997). This may serve as an important defence mechanism.

Reaction zones and discoloured tissues are commonly associated with high mineral concentrations, particularly potassium, calcium, manganese and magnesium (Safford *et al.*, 1974; Shigo and Sharon, 1970; Yamada *et al.*, 1987; Grime and Pearce, 1995). Pearce *et al.* (1997) suggests that the increase in cation concentration may create an osmotic potential that would drive water accumulation in the reaction zone. Factors such as moisture and mineral content have often been studied in discoloured and decaying wood as indicators of infection (e.g. Hart, 1965; Hart, 1968; Wilkes, 1985) but less commonly as aspects of defence.

Mechanisms of sapwood defence (particularly the reaction zone) have been poorly studied in the large genus of *Eucalyptus*. A number of studies have found that barrier zones formed upon wounding are anatomically similar to other trees but form as macroscopic kino veins in some species (Wilkes, 1986; Tippett, 1986), which is not common in *E. nitens* (Yang and Waugh, 1996). Wilkes (1985) studied the wood properties of infected (discoloured) tissue resulting from naturally infected drill wounds in 40 year-old coppice stems of *E. bancroftii, E. dealbata, E. macrorhyncha* and *E. sideroxylon*. In these studies a "clear" marginal zone adjacent to the discoloured wood was described as staining intensely for phenolics and having a high abundance of tyloses. In drill-wounded 14-year old *E. maculata,* red-brown marginal tissues were described adjacent to infection (Mireku and Wilkes, 1988). In these cases, the marginal zone represents a zone of active sapwood response and can be interpreted as a reaction zone.

In previous studies of *E. nitens*, a distinct purple reaction zone has been regularly observed at the boundary between decay columns and healthy sapwood in young, fast-grown plantation trees that had been pruned (Wardlaw and Neilsen, 1999). These trees had been pruned up to 9 years prior to sampling and decay was retained within the wood present at the time of wounding (the knotty core). This suggests that the xylem defence

processes are effective in restricting decay spread. The present study aimed to characterize the main properties of this reaction zone (including moisture content, mineral levels and total phenol levels) to provide a focus for future studies.

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Approximately 30 *E. nitens* plantation trees aged between 5-8 years were felled from six sites in Tasmania. Four sites were in the North East including Evercreech plot 108A and 29C, Urana 24B and Payanna 102B. The two other sites included Flowerdale 37D in the North West and Hastings 28B in the South West. Site information is provided in Appendix 2.1. Trees at all sites had been winter pruned up to a height of 2.5 m (1st lift) between 1-5 years prior to harvest. Trees were harvested throughout two years from the range of sites and utilized for different experiments as material allowed. After felling, the pruned section of the tree was then cut into 0.5 m billets. Decay or discolouration indicative of a decay column within the billet could be seen on the cut billet face. In 21 of the 30 trees, one or more decay column(s) interfaced by a purple reaction zone were detected, while in the other trees decay was not present or only within the heartwood and therefore not sampled.

Stems were either further dissected in the field and sampled, or the stem lengths were stored in plastic bags and transported directly to the laboratory where they were stored at 4°C until dissected and sampled in the following 1-2 days. Reaction zone and decayed tissue were sampled in close proximity, while "healthy sapwood" was excised from the same stem height but at the furthest point away from the decay lesion as possible. For moisture content and mineral assays, replicate samples (up to 4) were obtained for each decay column. In some cases only single determinations were possible due to a small amount of reaction zone and decayed material.

2.2.2 Decay spread in excised logs

Six freshly felled *E. nitens* logs (ca. 50 cm length) from four different trees (2 from Urana and 2 from Evercreech 29C) with white-rot decay columns interfaced by reaction zones (in the sapwood) were selected. Both ends of the log were photographed. Logs

were placed in plastic bags, sealed and stored at room temperature for 9 months. After storage, several slices were sawn off each end and the subsequent decay spread was noted and photographed.

2.2.3 Morphology, anatomy and histochemistry

Material from selected trees was utilized. Transverse stem slices (ca. 1 cm thick) were stained with a solution of 0.2% dimethyl yellow (Aldrich Chemical Co., Sydney) in 80% ethanol to detect the heartwood-sapwood boundary. This stain is a pH indicator in the range of 2.9 (pink) to 4.0 (yellow). Some stem slices were also stained with a solution of 1% aqueous tetrazolium at 25 °C in the dark for 24 hours to detect "cell vitality" (Gramss, 1989).

Sections (10-30 μm) were cut using a sliding microtome from unfixed and unembedded wood blocks of both healthy sapwood and reaction zone tissue. For general wood anatomy 1% toluidine blue in a 0.2 M phosphate buffer (pH 6.5) was used. Phenols were detected with an 0.5% aqueous solution of the diazonium salt fast blue RR salt (Sigma Chemical Co., Sydney) as a red colouration, while the nitroso reaction indicated tannins as a brown-red colouration (Harris *et al.*, 1994). Sections were subjected to lignin extraction with chlorine dioxide and stained with Sudan IV to detect suberin (Pearce and Woodward 1986). In addition sections were examined for suberin with a Zeiss Axiovert fluorescence microscope using a mercury lamp. Sections were mounted in either water, phosphate buffer (pH 9.1) or stained with 1% phloroglucinol and a drop of concentrated HCl to quench the autofluorescence of lignin (Biggs, 1984).

2.2.4 Gravimetric determinations of moisture content

Samples from 12 decay columns were obtained from 9 trees. From 7 columns replicate samples were obtained, from the other 5 only single samples were possible. Blocks of wood were excised with a chisel (ca. 0.5 cm^3) and a fresh weight (FW) was determined immediately. Blocks were split to ensure only one tissue type was included in the sample. Where samples were excised in the field, they were placed in a pre-weighed air-tight vial and weighed as soon as possible. Samples were then dried at 85°C for three days and a dry weight (DW) was determined. Moisture content was expressed as % DW.

2.2.5 Mineral concentrations

Samples from 8 decay columns (6 trees) were prepared for analysis. Two replicate samples for each type of wood tissue were prepared for five decay columns. Only single samples from each tissue were possible for the other three. Blocks of wood (2-4 pieces adding to ca. 1.5 g FW) were excised with a chisel and dried at 85° C for three days. Samples were chipped and then ground through a 0.5 mm sieve. Acid digestion of the samples (ca. 500 mg DW) was carried out with 5 ml nitric acid at 100° C for at least an hour, followed by a further hour after addition of 1 ml H_2O_2. The fully digested solution was diluted to 50 ml with water. As Mg and Ca form oxides during atomic absorption spectroscopy, lanthanum chloride was added to samples analysed for Mg and Ca to act as a releasing agent. Sodium chloride was added to samples analysed for K to prevent ionization. Mineral concentrations (expressed as mg/kg DW) were obtained by atomic absorption spectroscopy with reference to appropriate standard curves by applying regression analysis.

2.2.6 Total phenol determinations

Wood samples were obtained from 3 decay columns (3 trees). Wood tissues were sampled with a chisel and shavings were extracted in 100% methanol for 24 hours at 4°C. The methanol was transferred and centrifuged to remove any remaining particulate matter. Total phenols were estimated by the Folin-Ciocalteu method as previously described by Bonello and Pearce (1993). For each methanol extract, 15 µl was diluted to 1500 µl water. To this, 750 µl of diluted Folin and Ciocalteu's reagent (Sigma Chemical Co., Sydney) was added and left for 3 minutes, followed by adding 750 µl of 1M aqueous Na_2CO_3. The solution was shaken and left for 1 hour before the absorbance was measured at 725 nm with a spectrophotometer. Concentrations of total phenols were calculated with reference to a gallic acid standard curve (2-40 µg/ml) by applying regression analysis and results were expressed as gallic acid equivalent (mg) per g FW.

2.2.7 Data analysis

Results were grouped together regardless of site or season of sampling to establish general differences between tissue type for each dependant variable analysed (moisture

content, each mineral and total phenols). Therefore each decay column was treated as one replicate. Where internal replicates were obtained, the average was first calculated. Data was subjected to analysis of variance (single factor) and where $P<0.05$, values of least significant difference (LSD) were calculated.

2.3 RESULTS

2.3.1 Morphology, anatomy and histochemistry

Decay columns resulting from pruning wounds were observed challenging the sapwood by two main routes. Decay was observed to develop through pruned branches into the heartwood, then extending radially outward to challenge the sapwood. Alternatively, decay escaped from the branch and extended axially (usually both above and below the branch) into sapwood and/or heartwood. In either case, reaction zones were found to be similar in appearance, being approximately 5 mm wide and typically blue-purple in colour (Fig. 2.1). Additional examples of reaction zones are displayed in Appendix 2.2. Reaction zones were found existing within the heartwood (Fig. A.2.2.1), at the heartwood-sapwood transition (Fig. A.2.2.2.), or in the outer sapwood (Fig. A.2.2.3). When reaction zones were within the heartwood, they not sampled for experiments in this study.

Staining of transverse slices of the stem with dimethyl yellow differentiated the heartwood (pale pink) from sapwood (yellow). In most cases the reaction zone stained intensely pink (Fig. 2.2), indicating the pH was lower than both the heartwood and sapwood, possibly about pH 3.0. Slices of wood stained with tetrazolium showed similar patterns to the pH stain, indicating activity only in sapwood cells.

E. nitens wood has distinct growth rings, the late wood being characterized by dense fibres and a lack of vessels. Vessels are in an oblique arrangement and average approximately 150 μm in diameter. Ray parenchyma are uniseriate and axial parenchyma are paratracheal. The occurrence of diffuse parenchyma was not obvious in this study but may be found in varying degrees in *E. nitens* (Ilic, 1997). Vessel-occluding tyloses were abundant in the reaction zone (Fig. 2.3) but not common in the healthy sapwood. Phenolics were detectable with the fast blue RR salt stain, evident in parenchyma cells (particularly rays, Appendix 2.3: Fig. A.2.3.1) and associated with

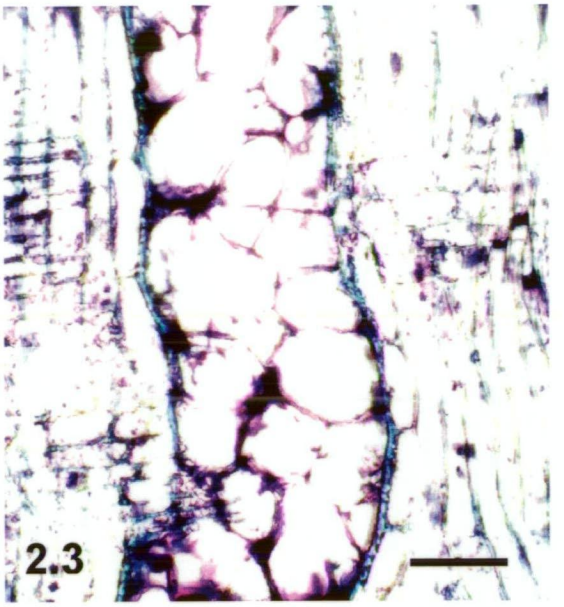
vessels. Phenols were not detectable in fibres. The nitroso reaction indicated tannins in the reaction zone, while tannins were weakly detected in the healthy sapwood. No evidence of suberin was detected in the reaction zone, by either staining with Sudan IV (Appendix 2.3: Fig. A.2.3.2) or using fluorescence microscopy (Appendix 2.3: Fig. A.2.3.3).

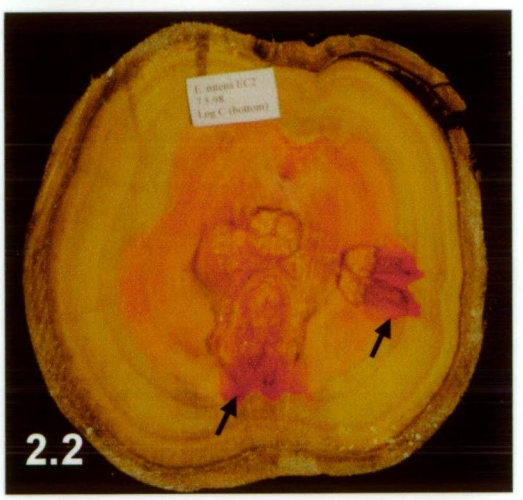
Between the reaction zone and decayed tissue, a band of brown discoloured tissue (2-3 mm wide) was present (Fig. 2.4). Occasionally a narrow (2-3 mm) white zone with dry appearance was observed at the interface between the reaction zone and healthy sapwood (Fig. 2.4). This white zone stained yellow with the pH stain as did healthy sapwood. The white zone stained more strongly for phenols with fast blue RR salt than the adjacent reaction zone. These phenols were associated with the parenchyma and tyloses.

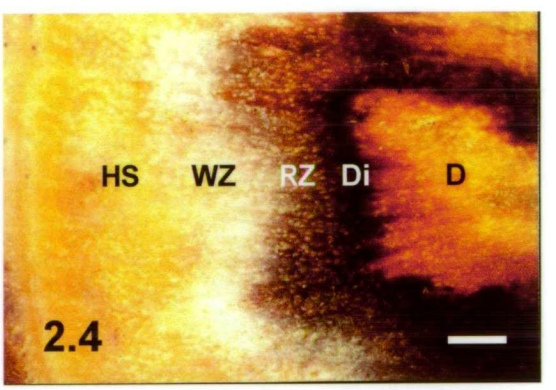
2.3.2 Decay development in excised logs

After 9 months storage, all log surfaces were covered with fungal and bacterial growth. Internal inspection of the wood following dissection showed that the bark and sapwood was extensively decayed and discoloured. Existing decay within the heartwood decay column had not progressed visibly, as shown by comparing Fig. 2.5a and 2.5b. However, some additional discolouration and flecks of decay were observed in heartwood (Fig. 2.5b). Importantly, all reaction zones remained intact (no visible degradation) after the logs had been stored for 9 months. Reaction zones retained their purple/blue colour except in two cases where they had become an orange/brown (Appendix 2.2: Fig. A.2.2.4).


2.1


2.3


2.2


HS
WZ
RZ
Di
D
2.4


E. nitens U2
7.5.98
Log B (bottom)
2.5a


2.5b

Figures 2.1-2.5. In all cases arrows indicate position of reaction zones.
2.1. Axial stem section through a branch that was previously pruned, showing the development of decay in the branch and into the heartwood and sapwood (Bar = 3 cm). **2.2.** Transverse surface of a stem with two decay columns challenging the sapwood. The dimethyl yellow stains intensely pink in the reaction zone indicating a lower pH than the sapwood which stains yellow. **2.3.** Radial longitudinal section stained with toluidine blue, showing tyloses filling a vessel in the reaction zone (Bar = 50 μm). **2.4.** Transverse surface of a stem showing decay wood (D), incipiently decayed (Di), reaction zone (RZ), white zone (WZ) and healthy sapwood (HS) (Bar = 5 mm). **2.5a.** Transverse surface of a stem segment prior to storage. **2.5b.** Transverse surface of the same stem after nine months storage, showing the extensive decay in the sapwood, yet intact reaction zones.

2.3.3 Moisture content

Moisture content was determined as an average from 12 decay columns (Fig. 2.6). Both the decayed wood and reaction zone tissue were significantly drier than the healthy sapwood. The decayed wood moisture levels were 38% of the intact sapwood, while the reaction zone average represented 80% of the healthy sapwood moisture content.

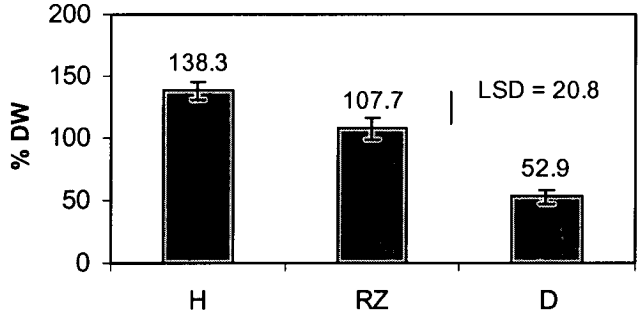


Figure 2.6. Moisture content determined gravimetrically for healthy sapwood (H), reaction zone (RZ) and decayed wood (D), expressed as % DW (± SE).

2.3.4 Mineral concentrations

Potassium concentrations were significantly decreased in both the reaction zone (ca. 3-fold) and decayed wood in comparison to the healthy sapwood (Fig. 2.7). Levels of calcium, manganese and magnesium were similar in the healthy sapwood and reaction zone, but increased (ca. two-fold) in the decayed wood. This increase was highly variable and only significant for calcium.

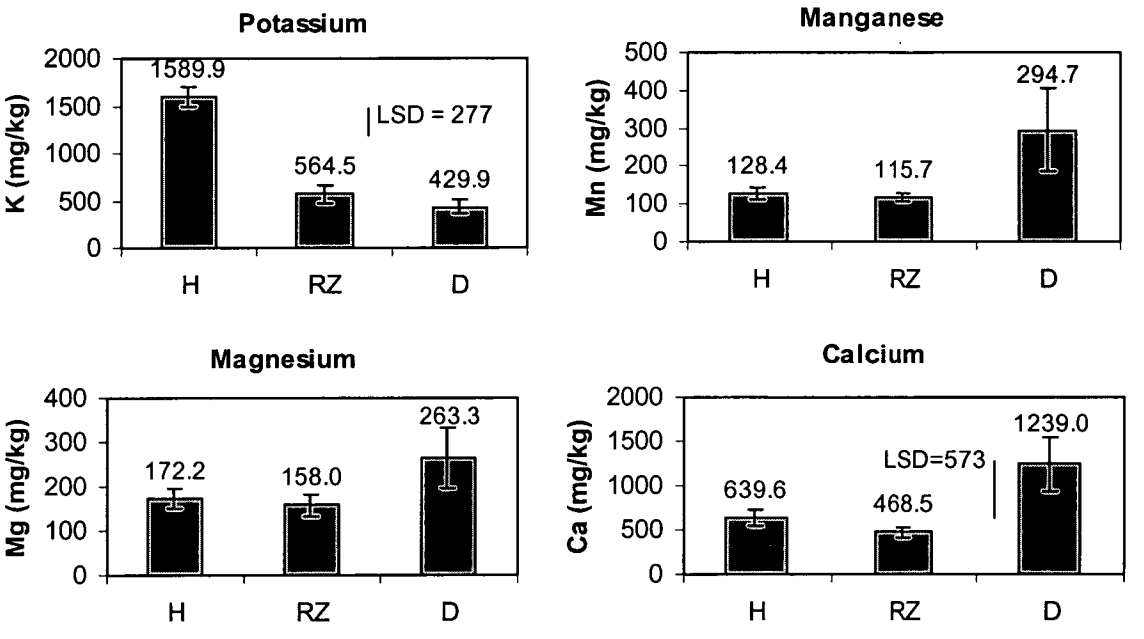


Figure 2.7. Mineral concentrations determined for healthy sapwood (H), reaction zone (RZ) and decayed wood (D), expressed as mg/kg DW (± SE).

Preliminary studies with proton-induced X-ray emission (PIXE) analysis supported these trends (Appendix 2.4). Data obtained using external beam analysis revealed large decreases in potassium in the reaction zone, but less alteration in manganese and calcium levels. Surprisingly, distribution maps of a range of minerals did not reflect any alterations across zones.

2.3.5 Total phenols

Total phenol levels of methanol extracts increased by six times in the reaction zone compared to healthy sapwood and decayed wood (Fig. 2.8). This increase was statistically significant. Reaction zone methanol extracts were a dark purple-plum colour while healthy sapwood was a pale yellow.

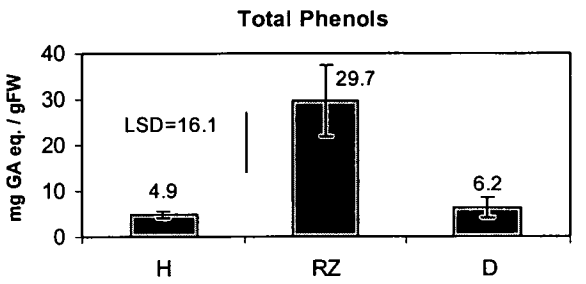


Figure 2.8. Total phenol concentrations determined for healthy sapwood (H), reaction zone (RZ) and decayed wood (D), expressed as mg of gallic acid equivalent per g FW wood tissue (± SE).

2.4 DISCUSSION

Compared to healthy sapwood, the *E. nitens* reaction zone was found to be significantly drier (reduced to 80% moisture content), with lower potassium levels (ca. three-fold) and a decreased pH. As will be discussed, these results suggest that the *E. nitens* reaction zone differs from many of the other angiosperms previously studied. The reaction zone phenolic content was significantly increased (ca. six-fold) compared with the healthy sapwood and this increase is comparable with reaction zone studies of other trees (Pearce, 1996).

Phenols were detectable in parenchyma cells (ray and axial) and associated with tylosed vessels in *E. nitens*, but were not apparent in fibres. This suggests that the *E. nitens* reaction zone does not provide a continuous barrier unlike many other angiosperms where all xylem cells in the reaction zone are infiltrated with polyphenolics (e.g. *Fraxinus excelsior*, Pearce, 1991; *Platanus x hispanica,* Schwarze and Fink, 1997). In this sense it would be difficult to explain the persistence of the *E. nitens* reaction zone. Polyphenolics of eucalyptus heartwood are distributed largely in the vessels and rays and to some extent in the fibres (Hillis, 1971). The fact that axial parenchyma are mainly paratracheal in *E. nitens* (that is, not well dispersed throughout the tissue) may explain the apparent lack of phenols in the fibres. Alternatively, phenolics may be present in the fibre cell wall capillaries and pits (Hillis, 1971) and provide a barrier to fungal penetration. Suberin was not detected in the *E. nitens* reaction zone, but has been detected in the *E. nitens* barrier zone by sudan IV staining (Barry, K.M., unpublished). While suberin is commonly found in the reaction zone of many trees, there are some

cases where suberin is also found in the barrier zone but not the reaction zone, including *Acer* spp. (Pearce, 1990).

Regardless of this apparent lack of properties common to many reaction zones, the *E. nitens* reaction zone was more resistant to decay than healthy sapwood in a log-store experiment. In this experiment, both the heartwood and reaction zone maintained their original condition, except for two cases where the reaction zone changed colour. This may indicate the beginning of colonization. Heartwood is more durable than sapwood in fallen timber due to impregnation with extractives (Hillis, 1987; Rayner and Boddy, 1988) and the durability of the reaction zone evidenced in this study may also repose on extractive content. To what extent the observed durability of the reaction zone in this experiment can be compared to processes within the living tree is difficult to fully ascertain.

The evidence of lower moisture content in the *Eucalyptus* reaction zone is intriguing. Previous studies have found that accumulation of water is more typical in the reaction zone of angiosperms (Pearce *et al.*, 1994, 1997). Increases in moisture content of discoloured sapwood have also been found to be typical of a number of angiosperms (Hart, 1965, 1968). Reaction zones that are drier than adjacent sapwood are more common to conifers (Shain, 1971;Yamada *et al.*, 1987, 1988) but the decrease is more accentuated than that found in *E. nitens*. Comparative data from a range of these studies is provided in Appendix 2.5.

Increases in calcium, manganese and magnesium levels have been evidenced in *E. nitens* decayed wood, but no increase of these minerals was found in the reaction zone. Levels of potassium were significantly lower in the reaction zone than the healthy sapwood, which is an unusual result. The typical trend for both angiosperms and gymnosperms is for total mineral content (ash content) to increase in the reaction zone and for levels to increase progressively in discoloured and then decayed wood (Shigo and Hillis, 1973; Wilkes, 1982; Pearce, 1996). Data from some of these studies is summarised in Appendix 2.5.

Alterations in mineral and moisture content in the discoloured tissues of the four *Eucalyptus* species studied by Wilkes (1985) are reasonably similar to those differences between the reaction zone and healthy sapwood in this study of *E. nitens*. That is, lower

or similar levels of cations (including decreases of potassium in two of the species) in the discoloured wood compared to clear sapwood were reported. The discoloured wood was decreased in moisture content by approximately 80% compared to the clear sapwood for three of the *Eucalyptus* species studied (Wilkes 1985). The similarity between the results for the discoloured and reaction zone tissues (from different *Eucalyptus* species) suggests that the process of both discolouration and defence in *Eucalyptus* is not characterized by high mineral or moisture increases, unlike other angiosperms. Heartwood formation in the *Eucalyptus* genus is commonly associated with substantially decreased nutrient and mineral levels (Van Den Driessche, 1984). Bamber (1985) states that the reabsorption of minerals into functional sapwood may be vital for *Eucalypts* which typically grow on nutrient poor soils. It is reasonable to suggest that a similar process occurs when the reaction zone is formed.

A number of factors (either directly or indirectly associated with defence) may influence wood moisture content distribution and measurement. In *E. nitens* the lower concentration of potassium may create an osmotic potential favouring movement of water out of the reaction zone. Pearce (1996) suggested that the accumulation of potassium and calcium ions in the *A. pseudoplatanus* reaction zone may create an osmotic potential facilitating the movement of water into the reaction zone. However, this link does not appear to be universal, as accumulation of potassium is also found in the reaction zone of Japanese cedar (Yamada *et al.*, 1987) and potassium and calcium accumulate in the reaction zone of Norway spruce (Shain, 1971) yet neither trees accumulate water in the reaction zone. The presence of extractives in the reaction zone may influence the moisture content determinations by increasing the sample dry weight. A relationship between increasing extractives content and decreasing moisture has been previously documented (Jankowsky and Galvao, 1979). The formation of tyloses may also influence the moisture content of the reaction zone by effectively blocking water flow through vessels.

A causal link is suggested between cation content and pH (Wilkes, 1982). In *E. nitens* the lower pH of the reaction zone is correlated with the lower potassium concentration. In contrast, in other genera reaction zone pH is typically increased compared to the healthy sapwood which is correlated with an increased cation content (Pearce, 1996). Other factors such as volatiles may also influence wood pH (Yamada *et al.*, 1987) and the presence of acids such as gallic acid in *Eucalyptus* contribute to the wood being

sufficiently acidic to corrode metal (Krilov and Lasander, 1988). As most decay fungi have pH optima for growth between 4 and 6 (Rayner and Boddy, 1988) a reaction zone pH above or below this range may constitute a barrier to fungal spread. Staining with dimethyl yellow revealed that the pH of the *E. nitens* reaction zone was as low as 3.

Coutts (1977) suggested that the withdrawal of water seen in conifers may be an essential part of the tree's resistance mechanism as it precedes or accompanies the formation of phenolic compounds required for defence. One similarity between *E. nitens* and many conifers is that they have high sapwood moisture contents. Average sapwood moisture content for a range of conifers has been calculated as 149 %, while only 83 % for angiosperms (Skaar, 1988). In plantation-grown *E. nitens* sapwood moisture determinations up to 180 % DW have been obtained in this study. There is some clear evidence that oxygen is required in the elicitation of phenolic compounds (Rayner and Boddy, 1988; R.B. Pearce, unpublished). Therefore it could be postulated that a decrease in moisture content in the transition zone and reaction zone serves the purpose of promoting necessary phenol production in trees with high moisture contents.

The white zone occasionally associated with *E. nitens* reaction zones is reminiscent of the transition zones that have been described in conifers (Shain, 1971; Coutts, 1976) and may provide the same function. The transition zone is thought to be the first stage in reaction zone formation, being a site of phenolic precursors for polyphenolic production, in a dynamic process (Shain, 1967, 1971, 1979). However, the transition zone described in conifers is typically more extensive, e.g. 10-20 cm beyond infection (Coutts, 1976) than the white zone described for *E. nitens* in this study. While transition zones have not been as well documented in angiosperms there is evidence that a similar process occurs. For example, a zone of apparently healthy sapwood in *A. pseudoplatanus* is concentrated with coumarins, which may be precursors for coumarinolignan polyphenols found in the reaction zone (Pearce, 1996, 1998). In the white zone of *E. nitens* histochemical staining indicated that phenols were more abundant than in the adjacent reaction zone. If the white zone observed in *E. nitens* does play a role similar to that proposed for the transition zone, the fact that it is not always evident may suggest that active reaction zone formation (or maintenance) only takes place occasionally in *E. nitens* and is not a continuous process. The fact that the reaction zone remained intact in an excised stem for 9 months indicates that the presence of

adjacent living cells may not be required for the maintenance of its integrity, at least on this time-scale.

Preliminary studies have revealed that microorganisms are less frequently isolated from reaction zone tissues in comparison to adjacent discoloured and decayed wood (Appendix 2.6), which supports their role as antimicrobial regions. The identification of decay fungi was not a goal of this study, but this knowledge will be required to gain a full understanding of the plant-pathogen interaction. This will be particularly important in assessing the ability of different decay fungi to overcome or penetrate the reaction zone. In the early stages of wound colonization, a greater range of microorganisms are likely to be present and plant defence responses may vary considerably (Pearce, 1996). It will be of interest to determine how defence responses vary in *E. nitens* in these early stages, particularly in cases where decay does not develop.

In summary, this study provides evidence that many properties of the *E. nitens* reaction zone present a different trend in comparison to other angiosperms previously studied. These properties may not be important in the sapwood defence of *E. nitens per se*. However, they may influence other factors, for example the reduction of moisture content may be required to elicit phenolic production. In *E. nitens* it is likely that the induction of phenolic compounds and tyloses are the most important aspects of induced sapwood defence and current studies aim to expand on these factors.

APPENDIX 2.1

Site information

Material used within Chapter 2 was from six sites in Tasmania, Australia (Table A.2.1.1).

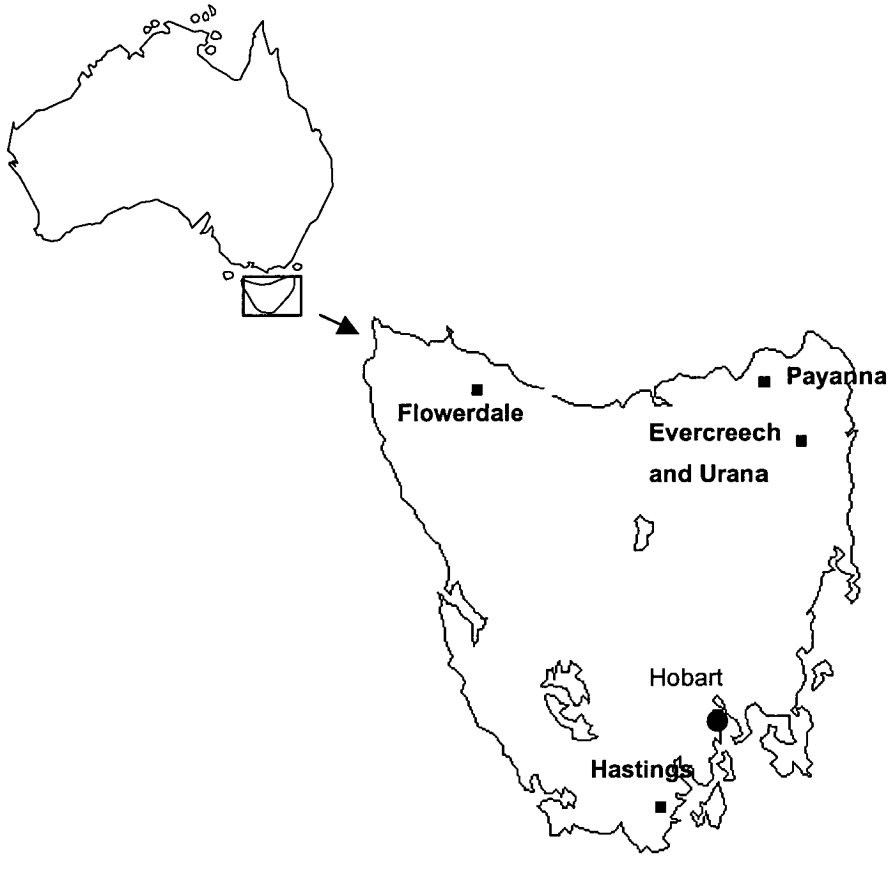


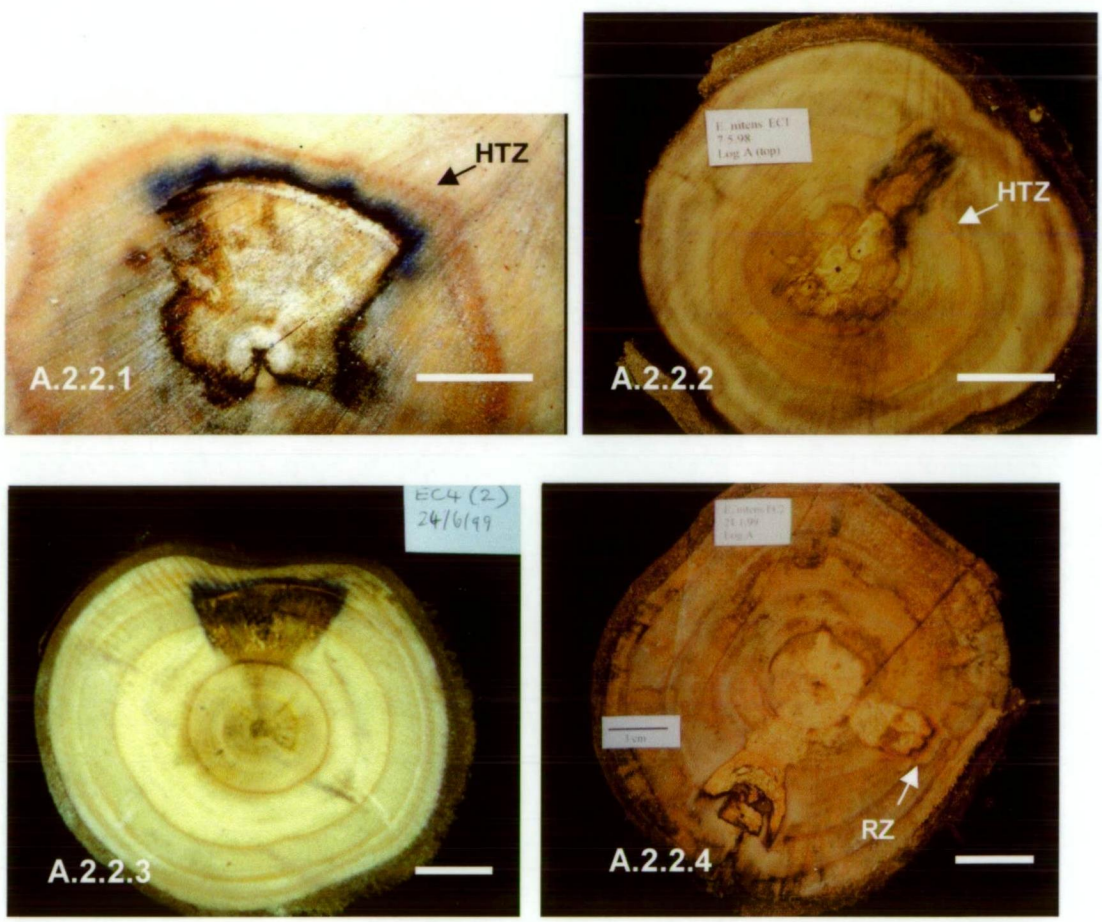
Table A.2.1.1. Details of six sites.

Site and coupe	Altitude (ASL)	Latitude	Longitude
Evercreech 108A	635 m	41°20' S	147°55' E
Evercreech 29C	670 m	41°22'S	147°56' E
Urana 24B	450 m	41°23' S	148°00' E
Payanna 102B	160 m	41°02' S	147°39' E
Flowerdale 37D	260 m	41°04' S	145°29' E
Hastings 28B	140 m	43°24' S	146°53' E

APPENDIX 2.2

Morphology of the reaction zone

Various patterns of compartmentalization were observed over the course of sampling, as mentioned in Chapter 2.



Figures A.2.2.1-A.2.2.4.Transverse surface of harvested stems of *E. nitens*. All scale bars approximately 5 cm.
A.2.2.1. Decay column from a pruning wound of a tree from Urana (site 24B) wounded 2 years prior to harvest. Purple reaction zone is within the heartwood (heartwood transition zone = HTZ). **A.2.2.2.** Wounds created 3 years prior to harvest; the reaction zone is beyond the sapwood/heartwood boundary (HTZ), but this has been "extended" around the decay column. **A.2.2.3.** Wounds created 4 years prior to harvest; the reaction zone is in the outer sapwood. **A.2.2.4.** After incubation in the log-store experiment, the reaction zone (RZ) turned orange/brown in colour but remained intact.

APPENDIX 2.3

Anatomy and histochemistry of the reaction zone

In reaction zone sections stained with fast blue RR salt (FBRRS), phenols were detected in the ray parenchyma (Fig. A.2.3.1). The stain sudan IV did not detect suberin in the reaction zone (Fig. A.2.3.3), however red deposits were detected in both healthy sapwood and reaction zone tissues by this method. Sections were also examined for suberin with fluorescence microscopy. Little fluorescence was detectable from the reaction zone (Fig. A.2.3.3), or the healthy sapwood. The bright blue typical of suberin under this method, was apparent at the edge of callus tissue surrounding wounds (Fig. A.2.3.4).

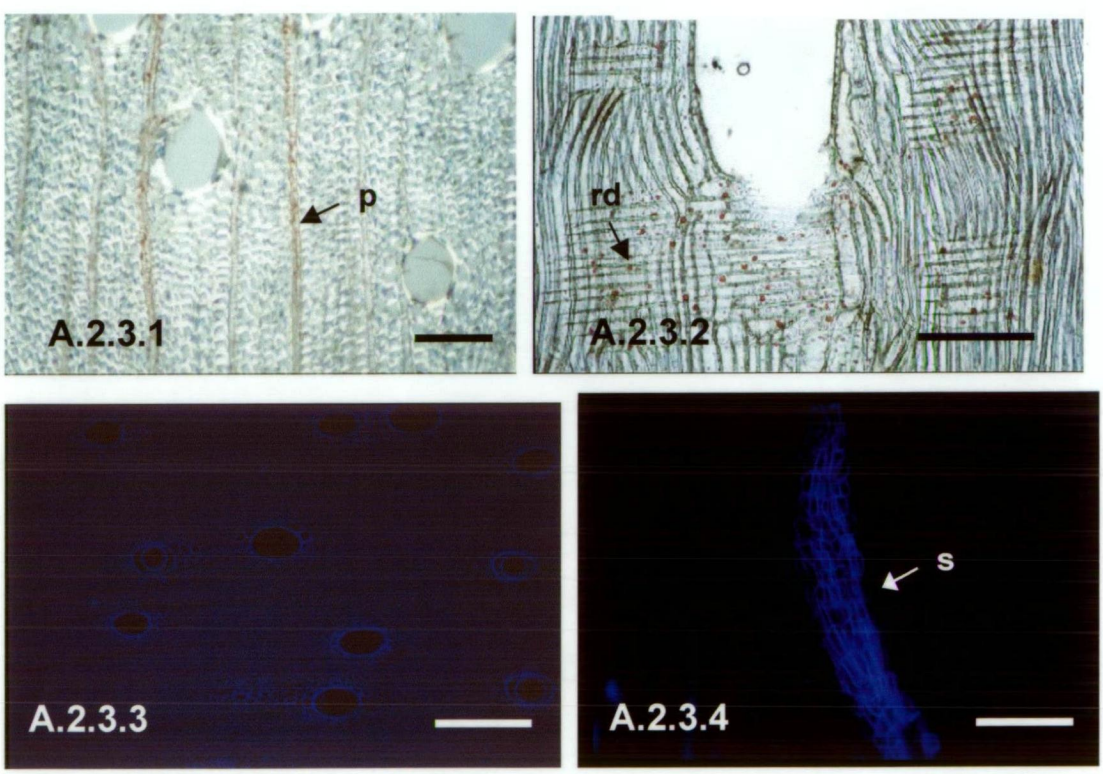


Figure A.2.3.1-A.2.3.4.
A.2.3.1. Transverse section of the reaction zone, stained with FBRRS for phenols (p), showing red colouration of the ray parenchyma (arrow). Scale bar = 100 μm. **A.2.3.2.** Radial longitudinal section in the reaction zone, extracted with chlorine dioxide and stained with sudan IV. Red deposits (rd) indicated. Scale bar = 80 μm. **A.2.3.3.** Weak fluorescence of the reaction zone region, stained with phloroglucinol-HCl (digitally brightened by 10%). Scale bar = 200 μm. **A.2.3.4.** Fluorescence microscopy of a radial longitudinal section of callus tissue surrounding a wound, stained with phloroglucinol-HCl; suberin (s) is indicated with an arrow. Scale bar = 150 μm.

APPENDIX 2.4

Proton-induced x-ray emission (PIXE) analysis

To investigate the distribution of a range of minerals in wood tissues, one wood sample was subjected to proton-induced x-ray emission (PIXE) analysis. Sections (ca. 15 μm) of wood comprising decay, reaction zone and healthy sapwood were prepared with a teflon-coated microtome knife. The prepared sections were analysed in the Oxford Scanning Proton Microprobe (SPM) using a beam of 3 MeV protons focused on fields up to 2,500 μm. This method has been fully detailed (Kramer *et al.*, 1997). Point averages (external beam) were calculated for specific tissues (healthy sapwood, reaction zone, and decay/reaction zone interface). These latter values were calculated for K, Ca and Mn only, as other elements were present at levels below the detection limit for all or some tissues. Concentrations were normalised to measurements of K determined from nitric acid digestion and flame photometry of wet wood samples. Results were calculated as mg/kg FW. This data is presented in Table A.2.4.1. A representative set of maps for elemental distribution across a wood section at the reaction zone and healthy sapwood interface indicates that there is little alteration in a range of minerals (Fig. A.2.4.1).

Table A.2.4.1

Concentration, Min. detectable limit for PIXE data calculated by GUPIX.
NB. "K mdl" is minimum detection limit, therefore if element K < K mdl the element was not detected (*).

Point averages (external beam)

Run	Ar K	Ar K mdl	K K	K mdl	Ca K	Ca K mdl	Ti K	Ti K mdl	V K	V K mdl	Cr K	Cr K mdl	Mn K	Mn K mdl	Fe K	Fe K mdl
Reaction zone	7272.00	299.90	423.00	273.80	893.00	130.60	*0.00	129.50	*53.00	57.10	*0.00	69.70	333.00	36.80	44.00	35.90
Healthy sapwood	5887.00	295.90	2114.00	242.20	1063.00	140.60	*0.00	112.70	*0.00	93.20	*0.00	69.30	601.00	31.40	*0.00	82.90
Decay/RZ interface	8815.00	330.60	1151.00	330.50	2696.00	156.10	*0.00	165.30	*6.00	80.80	*0.00	90.20	1075.00	38.40	*53.0	67.40

(continued)

Co K	Co K mdl	Ni K	Ni K mdl	Cu K	Cu K mdl	Zn K	Zn K mdl
0.00	40.70	*0.00	31.00	*0.00	46.00	*21.00	33.30
0.00	57.10	*0.00	39.60	*8.00	26.90	*0.00	44.00
48.00	32.20	*0.00	42.10	48.00	36.50	*0.00	54.00

Measurements of K(mg/g wet weight) determined by atomic absorption

Healthy wood	0.97
Reaction zone	0.78
Decayed wood	0.63
Healthy wood	0.93

External beam results **Normalised to Ar**

Run	K	Ca	Mn
reaction zone	0.06	0.12	0.05
healthy sapwood	1.99	0.18	0.10
decay/RZ interface	0.43	0.31	0.12

Normalised to K in healthy wood = 0.972mg/g

K	Ca	<m	
0.028	0.060	0.022	(mg/g)
0.972	0.088	0.050	
0.209	0.149	0.060	
28	60	22	(mg/kg)
972	88	50	
209	149	60	

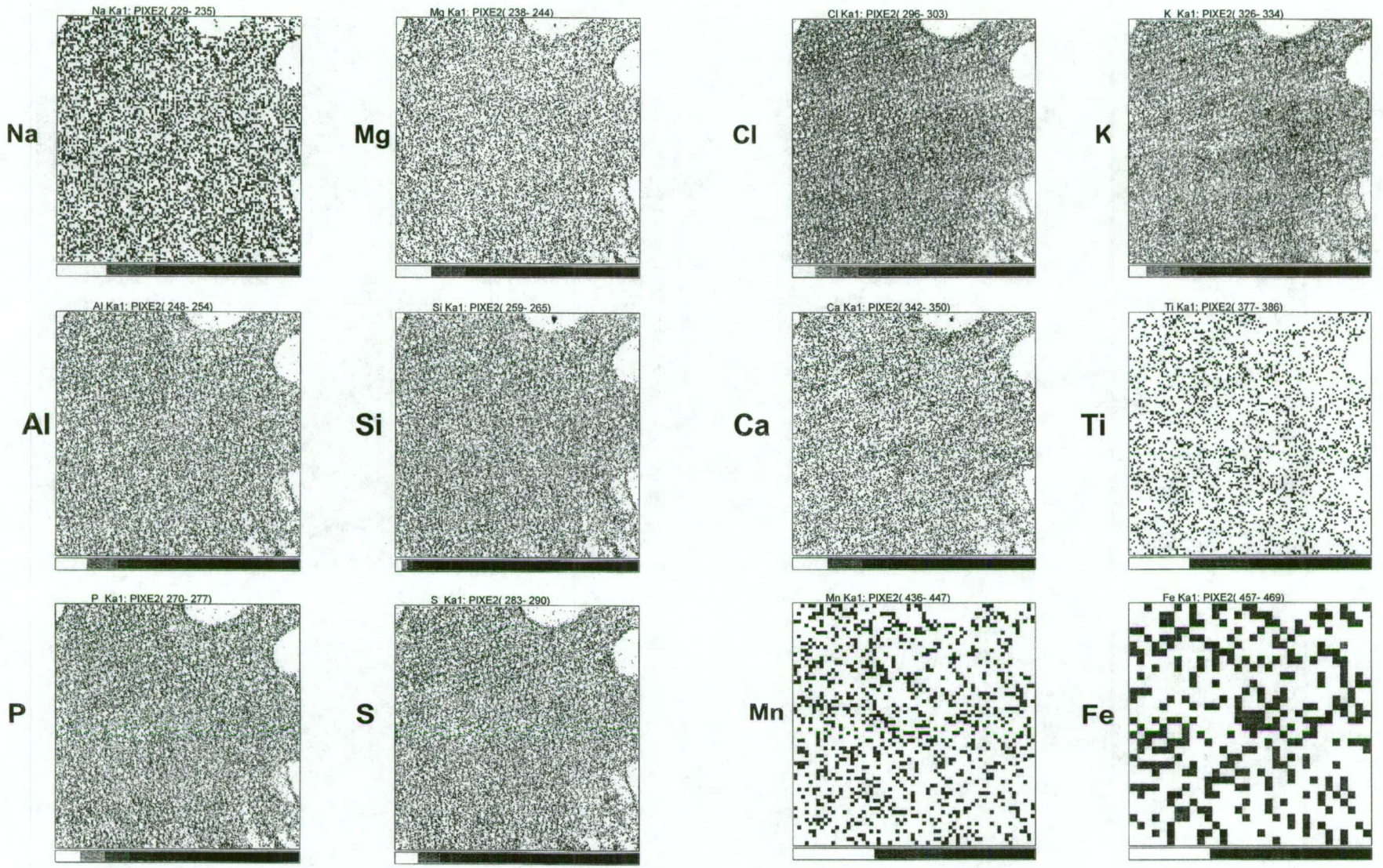


Figure A.2.4.1. PIXE elemental maps of a transverse section of *E. nitens* wood, comprising reaction zone and healthy sapwood interface.

APPENDIX 2.5 Comparison of moisture and mineral content of defence zones in range of trees (summary of literature)

Table A.2.5.1. Mean moisture and mineral content of tissues associated with woody host-pathogen interactions.

For clarity, actual mineral content has only been recorded for healthy sapwood and has then been recorded as either higher or lower than this for other tissues. Interpretation of descriptions of "discoloured tissues" from some studies was difficult. These tissues were either classed under "decay" if they appeared to be infected zones (and annotated by "Di"), or under reaction zone (also annotated as "Di") if they were described as non-infected. For studies by Hart (1968) the nature of the discoloured wood was not interpreted and has been recorded here as reaction zone, but may have been incipient decay (associated with microorganisms). For Wilkes' (1985) studies, the discoloured tissue was infected and had not been classified as a reaction zone. Kile and Wade (1975) examined discoloured tissue which was sound (classed as a reaction zone) as opposed to adjacent incipient decay.

Tree-pathogen system	Mean moisture content % DW			Mean mineral Content (K, Ca, Mn, Mg) ppm / DW (unless stated)			Source
	HS	RZ	D	HS	RZ	D	
Deciduous angiosperms							
Acer pseudoplatanus – Cryptostroma corticale	81	107	31	1000 5000 4000 NA	↑ ↑ ↑ ↑	↑ ↑ ↑ ↑	Pearce *et al.* 1997a; Grime and Pearce, 1995
Acer pseudoplatanus – Ustilina deusta	56	64	61	600 600 2 NA	ns	↑ ↑ ↑ ↑	Pearce *et al.*, 1997a; Grime and Pearce, 1995
Acer pseudoplatanus – Ganoderma adspersum	59	101	ns	NA	NA	NA	Pearce *et al.*, 1997a
Acer saccharum (7.5 yr.)* (Mn in ppm) [D=Di]	60	ns	56	0.06 0.11 24 0.05	ns	↑ ↑ ↑ ↑	Good *et al.*, 1955.
Acer saccharum – naturally infected controls** [D=Di]	NA	NA	NA	3.0 2.9 -0.2 1.8	↑ ↑ ↑ ↑	↑ ↑ ↑ ↑	Smith and Housten, 1994
*Acer saccharum – Ceratocystis virescens*** [D=Di]	NA	NA	NA	2.9 2.9 -0.3 1.9	↑ ↑ ↑ ↑	↑ ↑ ↑ ↑	Smith and Housten, 1994

Acer rubrum – naturally infected wounds*** [D=Di & De]	NA	NA	NA	186	218	32	39	ns	↑ ↑ ↑ ↑	Safford *et al.*, 1974
Fagus sylvatica – branch collar RZ	NA	NA	NA	1218	1272	192	906	↑ ↑ ↑ ↑	ns	Pearce, 2000
Maclura pomifera [RZ=Di] – naturally infected wounds	44	30	NS	NA	4600	3	700	NA ↑ ↑ ↑	ns	Hart, 1968
Malus sylvestris (orchard apple) – *Trametes versicolor* [RZ=Di]										
Strumer Pippen variety	110	108	76	1000	1500	3	280	↑ ↑ ± ↑	NA	Kile and Wade, 1975
Cleopatra variety	94	104	75	1400	1500	5	300	± ↑ ± ↑	NA	
Robinia pseudoacacia – naturally infected wounds [RZ=Di]	45	47	ns	1800	1700	8	190	↑ ↑ ± ↑	ns	Hart, 1968
Evergreen angiosperms										
*E. bancroftii**** [D=Di] – naturally infected stem wound	88	ns	72	910	200	85	200	ns	↓ ↑ ↓ ↑	Wilkes, 1985
*E. dealbata**** [D=Di] – naturally infected stem wound	55	ns	44	760	260	50	120	ns	± ↑ ↑ ↑	Wilkes, 1985
*E. macrorhyncha**** [D=Di] – naturally infected stem wound	73	ns	63	480	120	26	58	ns	↓ ↑ ↑ ↑	Wilkes, 1985
*E. sideroxylon**** [D=Di] – naturally infected stem wound	40	ns	39	740	330	90	77	ns	↑ ↑ ↑ ↑	Wilkes, 1985

Gymnosperms										
Cryptomeria japonica - ***associated with sugi bark borer after 2-5 yrs [D=Di]***	191	131	63	1020	1060	NA	167	↑ ↑ ↑ ↑	↑ ↑ ↑ ↑	Yamada *et al.*, 1987
Picea abies – Fomes annosus	130	80	45	745	535	20	70	↑ ↑ ↑ ↑	↑ ↑ ↑ ↑	Shain, 1971

% of dry weight, ** µmol/g, *** µg cm^{-3}

↑ increased compared to HS, ↓ decreased, ± about the same

ns = not sampled , NA = data not obtained.

APPENDIX 2.6

Isolation of microorganisms from wood tissues

Microorganisms were isolated from white-rot decay columns and adjacent regions, from billets freshly split with a sterile axe. After direct transfer to a sterile cabinet, a sterile chisel was used to excise small chips of wood from each tissue [including apparently healthy sapwood (HS), purple reaction zone (RZ), discoloured tissue surrounding the decay column (Di) and decayed tissue (De)]. These chips were then placed on 3% malt agar (and in one case a malt agar selective for basidiomycetes, described in Chapter 6). The cultural morphology and microscopic features of isolated microorganisms were examined after 7-12 days. They were then placed in either of three classes including decay fungi, non-decay fungi (including cultures typical of *Penicillium* spp. or *Trichoderma* spp.) or bacteria. In general, microbial isolations were less common from the reaction zone tissue, compared to surrounding tissues such as the discoloured wood (Table A.2.6.1).

Table A.2.6.1. Isolation of microorganisms from wood tissues of a number of trees.

Site / tree number/ position of decay	Tissue	Number of isolate type / chips			
		"Decay" fungi	"Non-decay" fungi	Bacteria	No isolates
Round Hill / 1 / Just escaping branch	HS	-	1/4	-	3/4
	RZ	-	-	-	4/4
	Di	2/4	-	1/4	1/4
	De	4/4	-	-	-
Evercreech 29C / 1 / large decay column from pruning wound at butt of tree	HS	2/10	-	4/10	4/10
	RZ	2/10	1/10	-	7/10
	De	10/10	-	-	-
Evercreech 29C / 2 /small decay column from pruning wound	HS	-	6/10	2/10	2/10
	RZ	-	3/10	3/10	4/10
	De	-	-	-	10/10
Evercreech 29C / 3 / older decay column in butt of tree (+)	RZ	4/4 [A]	-	-	
	De	4/4 [B]	-	-	
Evercreech 29C / 9 / decay column from branch 15 cm from base	HS	-	-	-	6/6
	H'wood	-	-	-	5/5
	RZ (h'wood)	2/6	-	-	4/6
	De	1/4	-	-	3/4
Evercreech 29C / 9 / further up decay column as above (36 cm from base)	HS	6/10	-	-	4/10
	RZ (4 mm)#	1/5	-	-	4/5
	RZ (3 mm)	-	-	-	7/7
	RZ (2 mm)	-	-	-	5/5
	RZ (1 mm)	4/5	-	-	1/5
	Di	4/7	-	-	3/7
	De	3/5	-	-	2/7

(+) malt agar selective for basidiomycetes (in all other cases, normal malt agar).

[A, B] denotes different fungal isolates from each tissue, which were subsequently sub-cultured and used for experimental inoculations (see Chapter 6).

distance (mm) from the brown discoloured wood (ie. RZ ca. 4 mm wide).

CHAPTER 3

INITIAL DEFENCE RESPONSES IN SAPWOOD OF *EUCALYPTUS NITENS* FOLLOWING WOUNDING AND FUNGAL INOCULATION

3.1 INTRODUCTION

The response of living sapwood to wounding and subsequent fungal infection is complex. The divide between wound- and infection-related responses remains unclear (Pearce, 1996) and infection often encompasses a range of microorganisms (Boddy and Rayner, 1983) suggested to be in a successional manner (Shigo, 1967, 1972). The secondary xylem represents a hostile environment (e.g. lignified cell walls and low oxygen access) and when this is compromised by wounding active responses are elicited. The active processes occurring shortly after wounding (ie. the first hours or days) may determine the ability of the tree to restrict the long-term spread of decay resulting from infection (Johansson and Stenlid, 1985; Pearce, 1996; Pearce, 1998; Asiegbu *et al.*, 1998, Pearce, 2000). Increased oxygen access following wounding has been linked to the production of antimicrobial phenolics (Coutts, 1977; Rayner and Boddy, 1988) while changes in vessel osmotic potential can lead to the formation of tyloses (Murmanis, 1975). These two defence responses appear ubiquitous among woody plants (Yamada, 1992; Pearce, 1996). Other putative defence responses including cellular suberization (Pearce, 1990) and elevated moisture levels (Pearce *et al.*, 1994; Pearce *et al.*, 1997a) vary with specific tree-pathogen interactions.

Pearce (1996) proposed a model of factors contributing to host-pathogen interactions in living angiosperm sapwood, based mainly on studies of *Acer* spp. This included drying of xylem tissues resulting from wounding, followed by accumulation of phytoalexins within 24 hours and accumulation of water at the lesion margins within 4-7 days. Later stages involved the formation of a more permanent boundary including insoluble polyphenolics and/or suberin. The current study aims to determine whether these key events are typical of other angiosperms, such as the evergreen eucalypts.

Recent studies of distinct *E. nitens* reaction zones (formed in response to decay associated with naturally-infected pruning wounds) found a number of differences in

various properties compared to the reaction zones reported from other angiosperms (Chapter 2). For example potassium levels, pH and water content were lower in the *E. nitens* reaction zone than healthy sapwood. These traits are typically increased in reaction zone tissue of other angiosperms. These differences may be reflected in the initial stages of host-pathogen interaction. Attributes of the reaction zone that were common to other angiosperms included significantly higher phenol levels than the healthy sapwood and abundant tyloses (Chapter 2). Concurrent studies have recently revealed the nature of these phenols, which are dominated by hydrolyzable tannins (Chapter 4).

Destructive methods of determining wood moisture content are possible (ie. gravimetric methods) and are used routinely (Coutts, 1977; Rayner and Boddy, 1988; Pearce *et al.*, 1997a). However, this method is limited to relatively large wood blocks where immediate water loss upon preparation is reduced in significance. To sensitively study the changes in xylem moisture content following wounding and infection, a non-destructive method is essential. The use of nuclear magnetic resonance imaging (NMR imaging or MRI) for the study of intact saplings has provided a very sensitive means of studying moisture levels. The potential to use NMR to image water in whole plants has been investigated in the last two decades [e.g. Van As and Schaafssma, 1984; Brown *et al.*, 1986; Connelly *et al.*, 1987, Lohman and Ratcliffe, 1988). Pearce and co-workers (Pearce *et al.*, 1994; Pearce *et al.*, 1997a) have shown that NMR imaging can be used to study woody tissues with decay lesions using both stem segments and intact *Acer pseudoplatanus* trees.

Eucalyptus nitens is a commercially important hardwood plantation species in Tasmania. This paper is the first description of initial alterations and defence responses associated with stem wounding and infection in the *Eucalyptus* genus. Pot-grown *E. nitens* saplings were notch-wounded and challenged with *Ganoderma adspersum*. While the NMR imaging studies reported are relatively preliminary, results show that a non-invasive approach to monitor sensitive changes in moisture content can be informative.

3.2 MATERIALS AND METHODS

3.2.1 Plant and fungal material

Pot-grown *E. nitens* saplings between 10 - 11 months of age were used. *E. nitens* seed (Toorongo provenance) was provided by Forestry Tasmania (Perth, Tasmania). Seedlings were raised in a glasshouse and transferred to 17 cm diameter pots filled with commercial potting mix, subject to frequent liquid fertilizer and daily watering. Pot-grown trees were maintained in a poly-tunnel at the University of Birmingham, United Kingdom. Experiments began in August 1998 (summer) when the trees were over 1 m in height with abundant side branches. Secondary xylem in the saplings was well developed by this stage and stems were up to 2 cm in diameter.

Cultures of *Ganoderma adspersum* (Schulz.) were maintained on 3% malt agar in the dark at 25 °C.

3.2.2 Experimental design

Different sets of trees were used to facilitate a range of studies of initial defence responses. Fifteen trees were used for microscopic examination and phenol analysis at a range of times following wounding and inoculation. For moisture content studies 8 trees (plus one for preliminary examination) were utilized also at a range of times since wounding and inoculation. An additional tree was utilized for studies of potassium concentration at one time point since wounding and inoculation.

3.2.3 Wounding and inoculation

Pot-grown saplings were notch-wounded with a small chisel, creating a wound 6 mm wide, 18 mm long and approximately 4-5 mm deep. Rectangular pieces of *G. adspersum* culture (3-5 weeks old) were adpressed to the wound and sealed with parafilm.

3.2.4 Anatomical, histochemical and phenol analyses

Fourteen 10-month old *E. nitens* were subject to 2 inoculated wounds each. The first wound was ca. 15 cm above ground level on the stem and the second wound was ca. 30 cm above ground level on the opposite side of the stem. One un-wounded tree was destructively analysed as a control, using stem material from within the first 40 cm of stem. Destructive sampling of two trees (comprising 4 wounds) took place at 7 time intervals (12 hours, 24 hours, 3 days, 7 days, 14 days, 21 days and 30 days). For each plant at each time interval, the stem was dissected and a segment (ca. 10 cm) containing the wound was cut through axially. The extent of discolouration and decay associated with the inoculated wound was noted immediately.

Wood blocks for sectioning were removed from an axially-cut stem half of one wound per tree. One piece was prepared from the middle of the wound to 1 cm above, and a second piece from the middle to 1 cm below the wound. Transverse and radial longitudinal sections (20 – 60 μm) were cut using a sledge microtome. Wood blocks were sectioned without fixation or embedding and examined on the day of harvest. For general wood anatomy, 1% toluidine blue in a 0.2 M phosphate buffer (pH 6.5) was used. Fast blue RR salt was freshly prepared (FBRRS, 0.5% aqueous, Sigma Chemical Co.) and utilized to stain phenolics, resulting in a red colouration. To detect suberin, sections were stained with phloroglucinol-HCl and observed with fluorescence microscopy (Pearce and Woodward, 1986). To visualise viable parenchyma cells, sections were placed in a buffered solution of nitro-blue tetrazolium (1 mg NBT in 1 ml phosphate buffer, pH 7.1) added to equal parts of NADH (7 mg in 1 ml buffer) for 3 hours in the dark (Pearce and Woodward, 1986). NAD diaphorase activity is indicated by a purple colour. This was also useful to detect fungal hyphae. Fungal isolation from challenged sapwood was carried out by excising wood chips from xylem of decayed appearance at the 21 and 30 day stage (where enough decay was visually present). Wood chips were incubated at 25 °C in the dark on 3% malt agar. Isolated fungi were subsequently sub-cultured and *G. adspersum* was identified based on morphological characteristics.

The other half of each wound was utilized immediately for chemical extraction. Reaction zone tissue was sampled from within 2 cm above and below the wound,

adjacent to apparently infected or decayed tissue. Healthy sapwood was also sampled from a position on the opposite side of the stem, at least 5 cm below the centre of the wound. Between 20-100 mg (FW) of thinly-sliced wood material was obtained with a small chisel and then extracted twice in 500 µl of 100% methanol for 24 hours at 4 °C. Extracts were then pooled to yield a 1 ml extract and particulate matter was removed by centrifugation. Extracts were kept at –20 °C in the dark and analysed during the following weeks.

Total phenols were determined by the Folin-Ciocalteu method, as previously described (Bonello and Pearce, 1993). Absorbance of the solutions was measured at 725 nm with a spectrophotometer (LKB Biochrom Ultrospec II E). Concentrations of total phenols were calculated with reference to a gallic acid standard curve (2-40 µg/ml) after regression analysis and expressed as gallic acid equivalent (mg gFW^{-1}).

Extracts were also analysed by HPLC. The system consisted of two LDC Constametric III pumps (LCD Ltd., Stone, Staffordshire, UK) an LDC Spectromonitor III spectrophotometer and a Spectra-Physics SP4270 Integrator. A C18 Spherisorb ODS analytical column (4.6 x 250 mm) was used with a C18 Spherisorb ODS guard column (4.6 x 10 mm). The solvent program was a linear gradient from 100% solvent A (MeOH: H2O: acetic acid, 5:92:3) to 100% solvent B (100% MeOH) over 40 minutes with a further 4 minutes at 100% B before returning to the initial conditions. Compounds were detected at 260 nm, and external gallic acid standards (5-10 µg) were used to obtain semi-quantitative data for particular peaks. Peak height was measured, as the chromatographic separation did not allow accurate measurement of peak area.

3.2.5 Moisture content studies (NMR imaging and gravimetric)

As *E. nitens* had not been subject to NMR imaging before, a pilot study was carried out using a tree with a 42-day-old inoculated wound. The system initially tested for imaging *E. nitens* trees has been previously described by Pearce and co-workers (Pearce *et al.*, 1997a). This included a Bruker MSL 100 imaging spectrometer and a U-shaped probe. In order to attach the U-shaped probe to the *E. nitens* stems a branch had to be removed, which may have altered the water status of the stem near the wound site. As this was not desirable, a probe design that could accommodate abundant side branches was designed

for the main studies. Trial images were performed with both these systems to gain a comparison of T_1 and T_2 images of the 42-day-old wound.

The signal generated from NMR imaging studies largely corresponds to the "mobile" protons of water in the sample. Data is first acquired as a "spin-echo" in which a number of factors contribute to the image contrast, including the abundance of protons as well as factors relating to the molecular mobility of those protons. That is, the time the protons take to return to equilibrium after a radio-frequency pulse (T_1 relaxation) and the time taken to go "out of phase" with the magnetic field (T_2 relaxation) will increase with greater mobility and effect the signal. Data for T_1 and/or T_2 relaxation times can be obtained, enabling data relating closely to proton abundance (M_0 data) to be calculated. While obtaining M_0 data was the aim of this study, analysis of T_1 and T_2 relaxation times can provide additional information about the nature of the sample.

Seven trees were notch-wounded and inoculated as described above (approximately 35 cm from the base of the tree). Stems from different individual trees were imaged at 7 stages after wounding (within 1 hour, 24 hours and 4, 7,15, 22 and 72 days). An unwounded stem was imaged 35 cm from the base of the tree as a control. Imaging was done at the Herchel Smith Laboratory, Addenbroke's Hospital, Cambridge. All images were acquired at room temperature using a 2.35 Tesla, 31 cm horizontal bore superconducting magnet connected to a Bruker BMT (Bruker Medzintechnik Biospec II, Karlsruhe, Germany) imaging console. A 15 cm internal diameter gradient set built 'in-house' which produces ca. 0.1 Tm-1 gradient fields was used, together with a cylindrical eight strut birdcage probe (internal diameter 9.4 cm) operated in the quadrature mode to transmit and receive the signal. The probe was firmly positioned around the stem using foam inserts, with the wound directly in the centre of the probe.

Transverse and longitudinal pilot images were acquired using a spin-echo imaging sequence with inter-echo time of 20 ms. These pilot images were used to align the desired imaging plane longitudinally through the wound. The quantification of MR parameters was achieved by varying the number of echoes in the multi-echo Carr Purcell Meiboom Gill MRI sequence (Meiboom and Gill, 1958). The T_2 values were calculated from images with different TE (inter-echo) values (eight images, inter echo time 20 ms, repetition time (TR) 3 seconds) using the following equation;

$$M_{xy} = M_0 e^{\left(-\frac{1}{T_2}\right)} + C$$

where M_{xy} is the magnetisation at a particular pixel; C is the estimate of the average noise in the images; M_0 and T_2 are the estimates required for starting the iterative curve-fitting process based on the Levenberg-Marquardt method (Marquardt, 1963). The actual M_0 value was quantified by back-projection of the T_2 decay curve to where it intersected the y-axis at time zero.

Images had a field of view (FOV) of 7cm, imaging slice thickness of 2 mm, 256 frequency encoding points in read direction (along the stem) and 128 phase encoding points across the stem. A FOV extension factor of two in the read direction to prevent aliasing gave images 512 by 128 pixels that were processed to give images 256 by 256 pixels with a resolution of 273 μm (7 cm divided 256) per pixel. With four averages, the acquisition time was ca. 26 minutes (128 phase encode steps * 4 averages * TR) per image.

M_0 and T_2 values were calculated as the average of a circular area covering about 40 pixels from the M_0 and T_2 maps using 'Cmrview' software. These circular areas were chosen to correspond to prominent areas of contrast and to match the position of samples taken for gravimetric determinations. The M_0 and T_2 values were calculated three times and a standard error was determined for each area. As this standard error was typically a factor of 10^3 lower than the M_0 average, it has not been presented in the results. Unfortunately, the signal to noise ratio was not sufficient to enable accurate M_0 values to be determined from 2 of the trees. To facilitate comparison of tissue moisture content from tree to tree the ratio of reaction zone to the corresponding healthy sapwood on the opposite side of the stem was calculated for M_0 and gravimetric data.

Trees were dissected as soon as possible after imaging for gravimetric determinations of moisture content. Bark was removed and blocks of wood (0.8 cm^3 minimum) were excised with a chisel from positions around the wound and in corresponding positions on the other side of the stem. For the stems with 22- and 72-day-old wounds, two smaller blocks were excised from above and below the wound, in an attempt to include only material related to areas of bright contrast evidenced by NMR imaging. For the control tree, 4 blocks were excised around the centre of the image area. Fresh weight was determined immediately. Samples were then dried at 85°C for three days and a dry weight (DW) determined. Moisture content was expressed as % DW, as single determinations.

As a complementary study, samples from additional *E. nitens* saplings were used to determine moisture content as a proportion of saturated sample weight. Two trees were harvested, 8 samples (ca. 2 x 1 x 1 cm) of healthy sapwood were prepared and the fresh weight obtained immediately. For infiltration the samples were immersed in water until they sank and were then placed in a vacuum chamber for 5 hours. Samples were removed from the water, quickly surface dried by blotting and weighed to gain the saturated weight (SW). Samples were then dried at 85°C for three days and a dry weight determined. Moisture content was expressed as % DW and % SW.

3.2.6 Potassium concentration

A single tree was wounded with a 6 mm drill bit at eight positions on the stem, creating wounds approximately 6 mm deep. Each wound was inoculated with a plug of *G. adspersum* agar culture and sealed with parafilm. Small drill wounds were used instead of notch wounds to reduce structural weakening of the stem. After 55 days the tree was harvested and 3 wounds were sampled. Plant material (60-150 mg FW) was excised from positions corresponding to the reaction zone (above and below, representing two "replicates") and from the opposite side of the stem, representing healthy sapwood. Fresh weight was determined and samples were placed in 5 ml 1M nitric acid, heated to 80°C for 24 hours and then allowed to digest for 2-3 days. Plant material was then removed and the solution diluted 20-fold. The samples were analysed by flame photometry to measure K^+. Readings were related to a standard curve of K Cl (0–80 μM) by regression analysis.

3.3 RESULTS

3.3.1 Morphology, anatomy and histochemistry

Infection of inoculated wounds by *G. adspersum* progressed relatively slowly (Table 3.1). Within the first month, discolouration and decay proceeded in a triangular shape axially above and below the wound and did not extend transversely. Visible evidence of decay was not apparent in samples studied until 14 days after wounding and inoculation, however fungal hyphae were observable within vessels in samples studied after 7 days (Table 3.1). In samples studied after 14 days, hyphae were detectable in vessels and

Table 3.1. Observation of infection and defence responses in wounded and inoculated *E. nitens*.

	12 hrs	24 hrs	3 days	7 days	14 days	21 days	30 days
Infection							
Decay (axial extent)	-	-	-	-	1-2 mm	2-3 mm	1-5 mm
Hyphae	-	-	-	+V	+++ VA	+++ VA	++++VA
Responses							
Cell death	+	+	+	++	+++	+++	++++
Discolouration	C	C	C, OX	C, X	Pale	Pale	Varied
Phenols	-	+	++	++	+++	+++	+++
Tyloses	-	tr	+	++	+++	+++	+++
Callus	-	-	-	+	+	+++	++++
Suberin							-

C= cambial discolouration, OX = outer xylem, X = xylem discolouration, V= vessels, VA= vessels and other axial cells, tr = traces, + signifies extent of response.

other axial cells (Fig. 3.1). Hyphae appeared to colonize xylem cells which were not living (ie. did not stain with NBT). *G. adspersum* was re-isolated as the dominant fungi from wood-chips of decayed tissue from the 21- and 30-day old wounds. Some contaminant fungi (apparently non-hymenomytaceous, e.g. *Trichoderma* spp.) and bacteria were also isolated from wood-chips. In some sections from wounds of different ages, clamp connections could be seen, confirming that it was basidiomycete hyphae.

Brown/red discolouration was first evidenced in the cambial region (Table 3.1). Discolouration of the outer xylem was evident for wounds analysed after 3 days, extending at the most to 3 mm from the edge of one wound. Discolouration around wounds tended to be orange/brown in colour at the interface of infection. Evidence of tylose formation (as unlignified material within vessels) and phenol accumulation were first observed in samples studied 24 hours after wounding and inoculation, becoming more extensive in older wounds. Phenol accumulation was observed both in association with vessel tyloses and in axial parenchyma. General accumulation of phenols was apparent with FBRRS up to 18 mm from the wound in samples studied after 14 days. Discolouration was less evident in wounds analysed between 14 and 30 days following wounding and inoculation, compared to the younger wounds. Although discolouration was pale, histochemical detection revealed a prominent accumulation of phenols at the interface of the infected wood (Fig. 3.2) with a general accumulation of phenols

detected axial and adjacent to this, greater than on the opposite side of the stem. Callus tissue began to form by 7 days after wounding and inoculation.

Samples studied after 30 days showed some variation in colonization and response. That is, for both individual trees, one wound was considerably infected (ca. 5 mm axially) while the second wound on the same tree was infected much less (ca. 1-2 mm axially). At this stage an area of water-soaked appearance ahead of the decay was evident in some cases which was not observed in previous stages. Suberin was not detected by fluorescence microscopy in sections of the 30-day-old wounds.

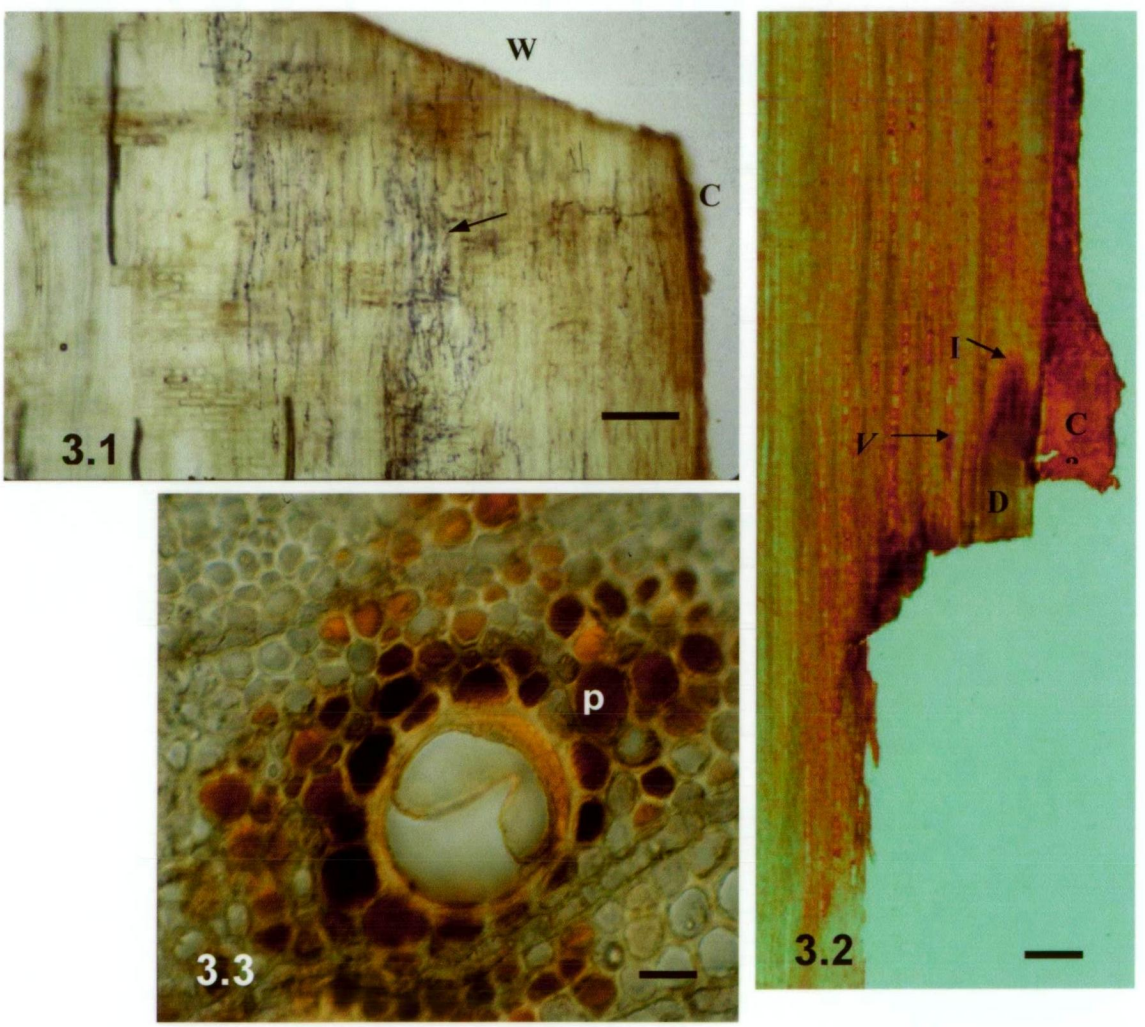


Figure 3.1. Radial longitudinal section directly below a 14-day-old inoculated wound (W), stained with NBT. Living fungal mycelium stains intensely purple (arrow) within vessels, parenchyma and fibres (weakly stained). C = cambium. Scale bar = 500 μm. **3.2.** Radial longitudinal section below a 21-day-old inoculated wound, stained with FBRRS. A discrete region of phenols is detectable at the interface (I) of decay column (D) and un-infected wood. General accumulation of phenolics surrounds this area, particularly associated with vessel tyloses. Callus tissue (Ca) near the wound stains strongly for phenolics. Scale bar = 2 mm. **3.3.** Transverse section above a 42-day-old inoculated wound, unstained. Polyphenolics (p) are deposited in the axial parenchyma. Scale bar = 30 μm.

The pattern of decay and discolouration of two trees with older inoculated wounds (which were used for NMR imaging) was observed. Above and below the single wound analysed 42 days after wounding and inoculation, a barrier zone and new xylem tissue was formed and infection appeared limited to xylem present at the time of wounding. Callus was extensive but the wound was still open. As this tree was not subject to destruction for gravimetric determinations of moisture content, sectioning was possible. Deposition of polyphenolic material in axial parenchyma was evident without histochemical staining (Fig 3.3). The single wound analysed after 72 days had almost completely callused over and a barrier zone and new xylem were also present. Decay spread axially from the wound to 3-4 mm and also transversely to 1-2 mm. A prominent reaction zone had formed axially, including both orange and brown zones.

3.3.2 Analysis of phenolic compounds

The levels of total phenols in the healthy sapwood samples remained essentially constant while the levels in the wood surrounding the wound (RZ) increased linearly to a peak at 21 days (Fig. 3.4). This peak represents a 4-fold increase in phenolics on average. After 21 days the total phenol levels began to decrease. HPLC analysis of the methanol extracts revealed that qualitative changes in phenols occurred within the time-scale of the experiment (Fig. 3.5). Major peaks in the chromatograms have been

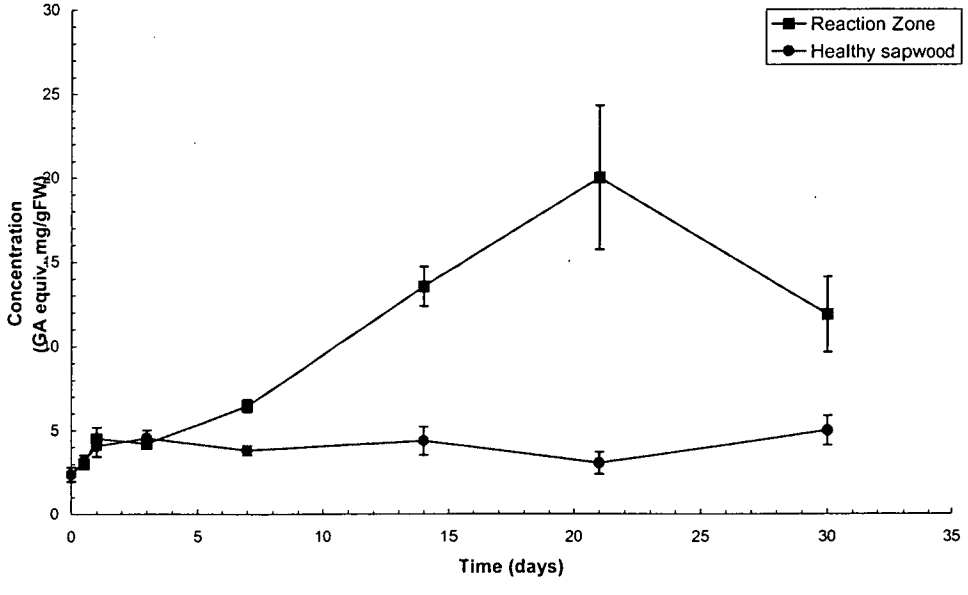


Figure 3.4. Total phenol levels (mg GA equivalent gFW^{-1} ± SE) determined from methanol extracts (4 replicates from 2 trees) of reaction zone and healthy sapwood over time following wounding and inoculation.

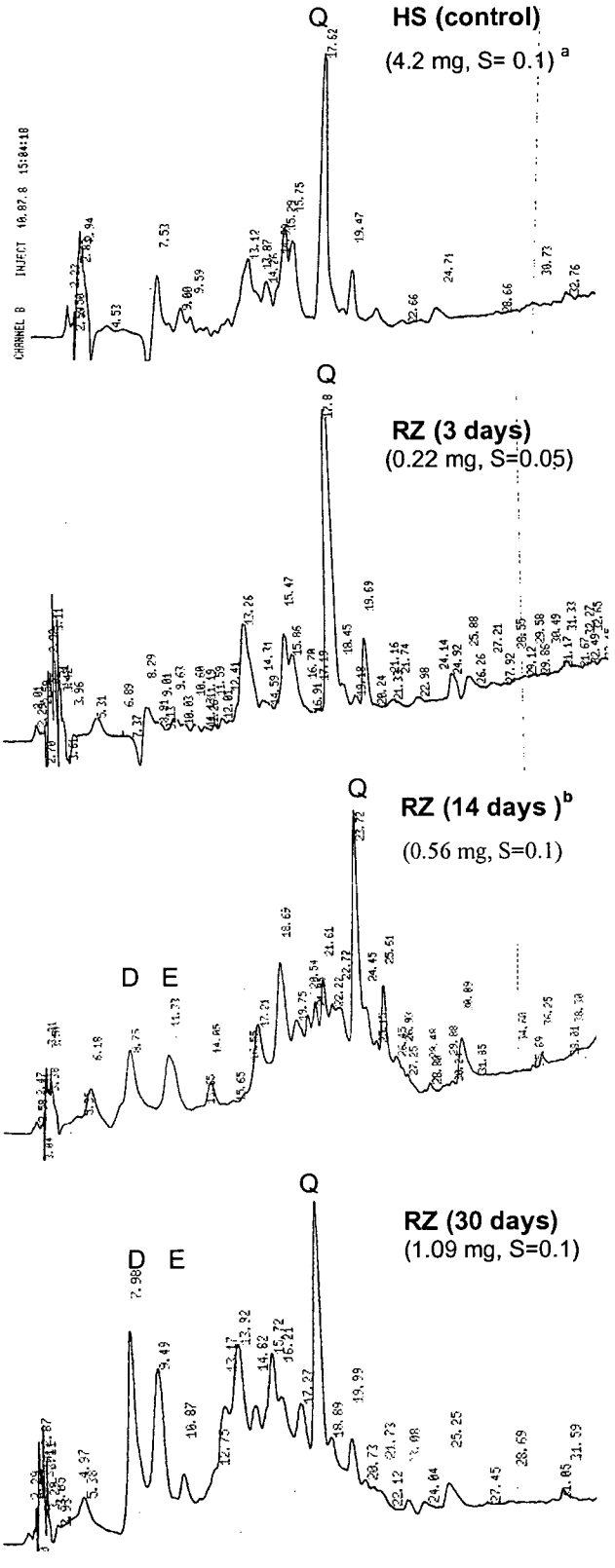


Figure 3.5. Representative HPLC chromatograms (detected at 260 nm) of methanol extracts from *E. nitens* healthy sapwood (HS) and reaction zone (RZ) at progressive stages (3, 14 and 30 days) after wounding and inoculation. [a] injections not quantitatively equivalent (mg FW extracted per 20 µl injection, S= detection sensitivity); [b] retention time in 14 days extract is late due to technical problems.

labelled alphabetically. Comparison of chromatograms shows that many more compounds were detected in the developing reaction zone compared to healthy sapwood. This includes peaks D and E, and a range of other peaks which cannot be fully distinguished due to relatively poor separation.

With the use of an external gallic acid standard, semi-quantification was acquired for selected prominent peaks (Fig. 3.6). Peak Q showed a dramatic increase in concentration (3.5-fold) within the first 24 hours after wounding and challenge and then remained at a similar level for the experimental period. Peaks D and E increased steadily until 21 days, following the trend found for the total phenol levels (Fig. 3.4). Comparison of retention times to samples subsequently studied with liquid chromatography – mass spectrometry (LC-MS) allows extrapolation of the nature of these compounds (Chapter 4). Peak Q is a tetra-galloylglucose isomer, while peak D and E relate to two anomeric peaks of pedunculagin and their concentrations have therefore been combined (Fig. 3.6). The changes in concentration of peak D and E (Fig. 3.6, plotted separately) are similar, supporting the conclusion that they are related peaks.

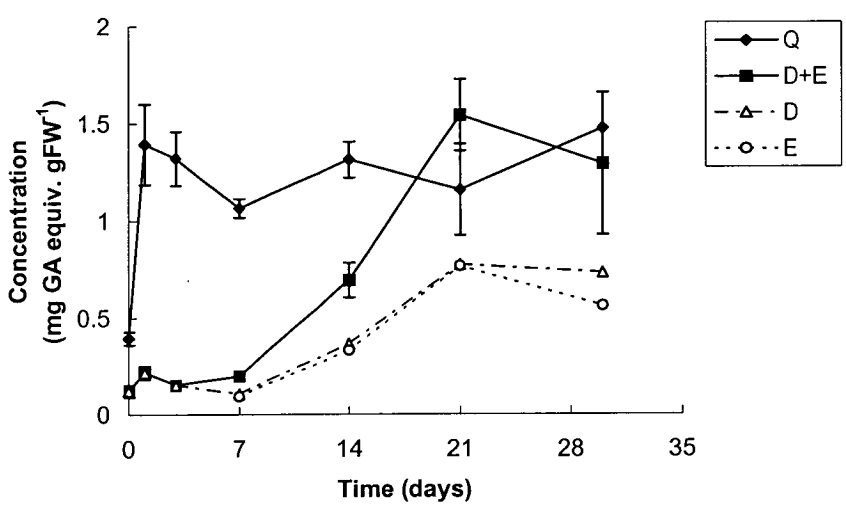


Figure 3.6. Concentration of individual HPLC peaks (mg GA equivalent gFW^{-1} ± SE) over time following wounding and inoculation.

3.3.3 Potassium concentration

The determinations of K^+ concentration for three 55-day-old wounds from one tree are shown in Table 3.2. Apart from wound 1, K^+ was lower in the reaction zone compared to the healthy sapwood. On average the level of K^+ is lower in the reaction zone, although this is not statistically significant (t-test, $P>0.2$).

Table 3.2. Concentration of K+ (μM $mgFW^{-1}$ ± SE) in healthy sapwood and reaction zone samples determined from 3 wounds on 1 *E. nitens* tree (2 replicates per wound).

Wound	Healthy Sapwood	Reaction Zone
1	7.91 ± 0.09	8.09 ± 0.34
2	7.38 ± 0.87	5.74 ± 0.16
3	7.28 ± 0.54	5.51 ± 0.39
Average	7.52 ± 0.43	6.45 ± 0.53

3.3.4 Moisture content

The preliminary imaging of a 42-day-old *E. nitens* wound with the MSL imaging spectrometer showed that regions of bright contrast were visible both below and above the wound. To determine the effect of proton relaxation times on the M_0 images, T_1 and T_2 derived maps were obtained. All images were similar in appearance, reflecting little distortion of M_0 from the T_1 and T_2 relaxation factors. Larger alterations in T_2 values across the image (and higher absolute values) were found by Pearce and co-workers in studies of sycamore (Pearce *et al.*, 1994; Pearce *et al.*, 1997a). Lower T_2 values with a smaller range were present in *E. nitens*. This data is summarized in Table 3.3.

Table 3.3. Comparison of proton relaxation times from healthy sapwood and reaction zone tissue of *E. nitens* and *A. pseudoplatanus*.

Plant Material	T_1 (m s)	T_2 (m s)	Reference
E. nitens, juvenile (11 months old)			
Healthy sapwood	~250	40-60	Present study
Reaction zone	~750	50-100	
A. pseudoplatanus, juvenile (3-4 yrs old)			
Healthy sapwood	700-1200	60-140	Pearce *et al.* (1997a)
Reaction zone	700-1000	30-100	
A. pseudoplatanus, older coppice stems			
Healthy sapwood	500-700	35-120	Pearce *et al.* (1997a)
Reaction zone	250-700	30-60	

Images of the 1st echo, M_0 map and T_2 map were obtained for each tree in the time-course experiment. Due to background noise in the M_0 and T_2 maps, the 1st echo maps are presented of the sequence of NMR images from wounds of different ages (Fig. 3.7 A-H). Because of the uniformity of T_2 values, the contrast in the 1st echo and M_0 map is virtually identical (Fig. 3.8). M_0 values cannot be cross-referenced from tree to tree, but are comparable within the same tree.

Image of the control tree (Fig. 3.7A) indicates that the outer xylem tissue is highest in moisture content, compared to the older xylem and central pith. The bark is barely detectable and therefore reasonably dry. Axial striations in the xylem probably relate to the distribution of vessels and axial parenchyma The gravimetric and M_0 results from this tree show considerable internal variability that does not correspond exactly between the two methods. This is probably a function of the difference in total volume measured by each method, as M_0 values were calculated from an area of 40 pixels from a 2 mm slice, while blocks excised for gravimetric studies were considerably bigger than this.

The results for the tree imaged within 1 hour of wounding and inoculation showed that the contrast on the wounded side of the stem was lower, observable as a general area (Fig. 3.7B). Gravimetric measurements also showed a decreased moisture content (ratio 0.84-0.87) on the wounded side of the stem (Fig. 3.7B, Table 3.4). M_0 results for the stem analysed 24 hours after wounding (Fig. 3.7C) indicate that moisture content is unchanged, although gravimetric results indicate a drop in moisture below the wound. Results for the stem analysed 4 days after wounding (Fig. 3.7D) are confounding, with little change in moisture content determined gravimetrically but decreases and increases in M_0 above and below the wound respectively.

Analysis of a stem 7 days after wounding revealed a distinct dry zone observable above and below the inoculated wound. Although M_0 values could not be calculated for this tree because of the low signal to noise ratio, a substantial drop in moisture content around the wound (ratio 0.76-0.80) of normal xylem levels was indicated by the gravimetric results (Table 3.4). This drying was not as prominent around the wounds at later stages; the tree imaged with a 15 day old wound (Fig. 3.7F) showed a decrease in M_0 above and below the wound, which was evidenced to a lesser extent by gravimetric data (Table 3.4).

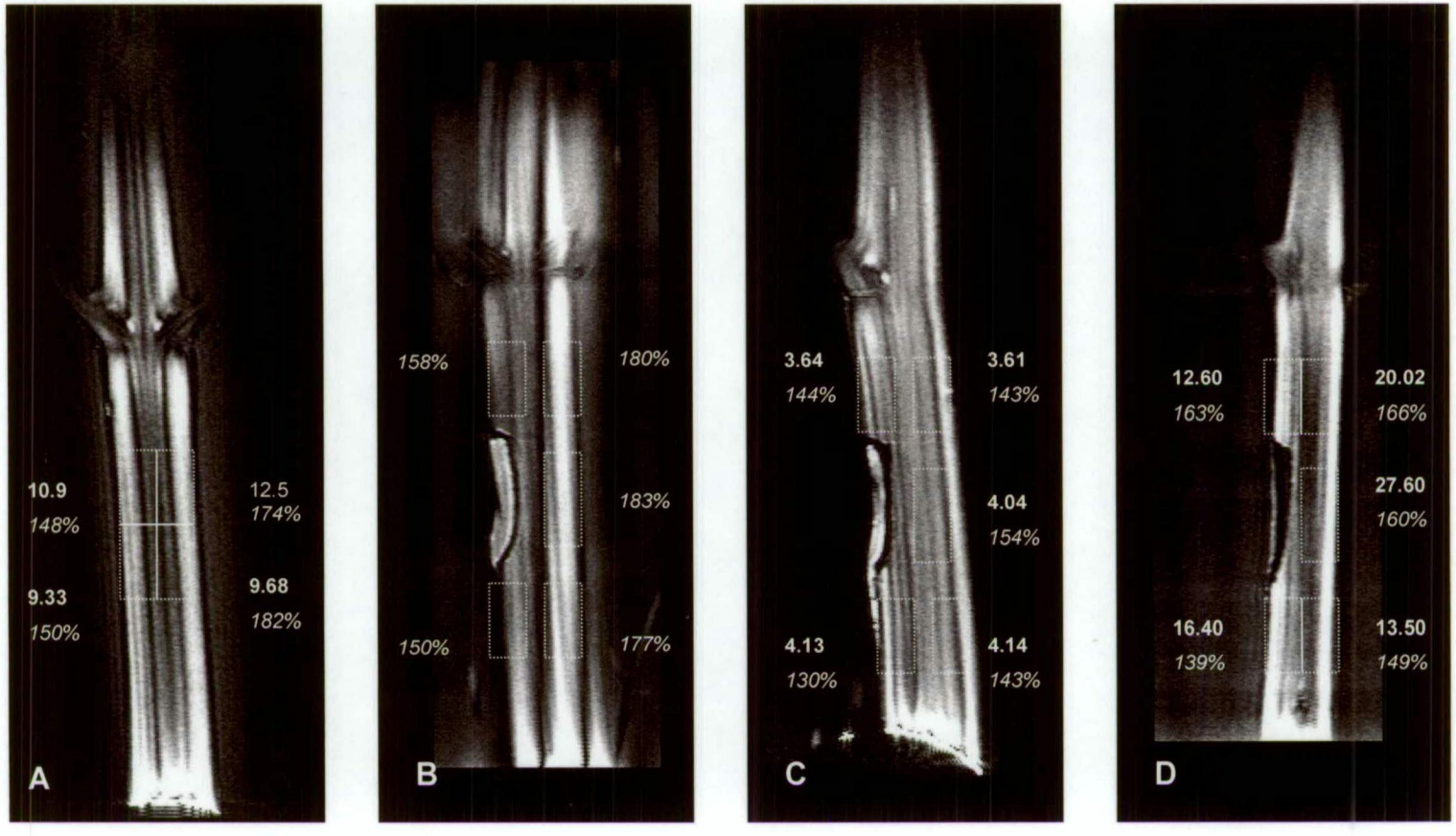


Figure 3.7. NMR images (1st echo) for each intact pot-grown *E. nitens* sapling: control (A) and various stages following wounding and inoculation with *G. adspersum*, within 1 hour (B), 24 hours (C) and 4 days (D). Boxes super-imposed on images (dashed lines) represent approximate position of wood samples excised for gravimetric determinations of moisture content, with results expressed in italics (single determinations). M_0 values (calculated at approximately the centre of these regions) are expressed in bold.

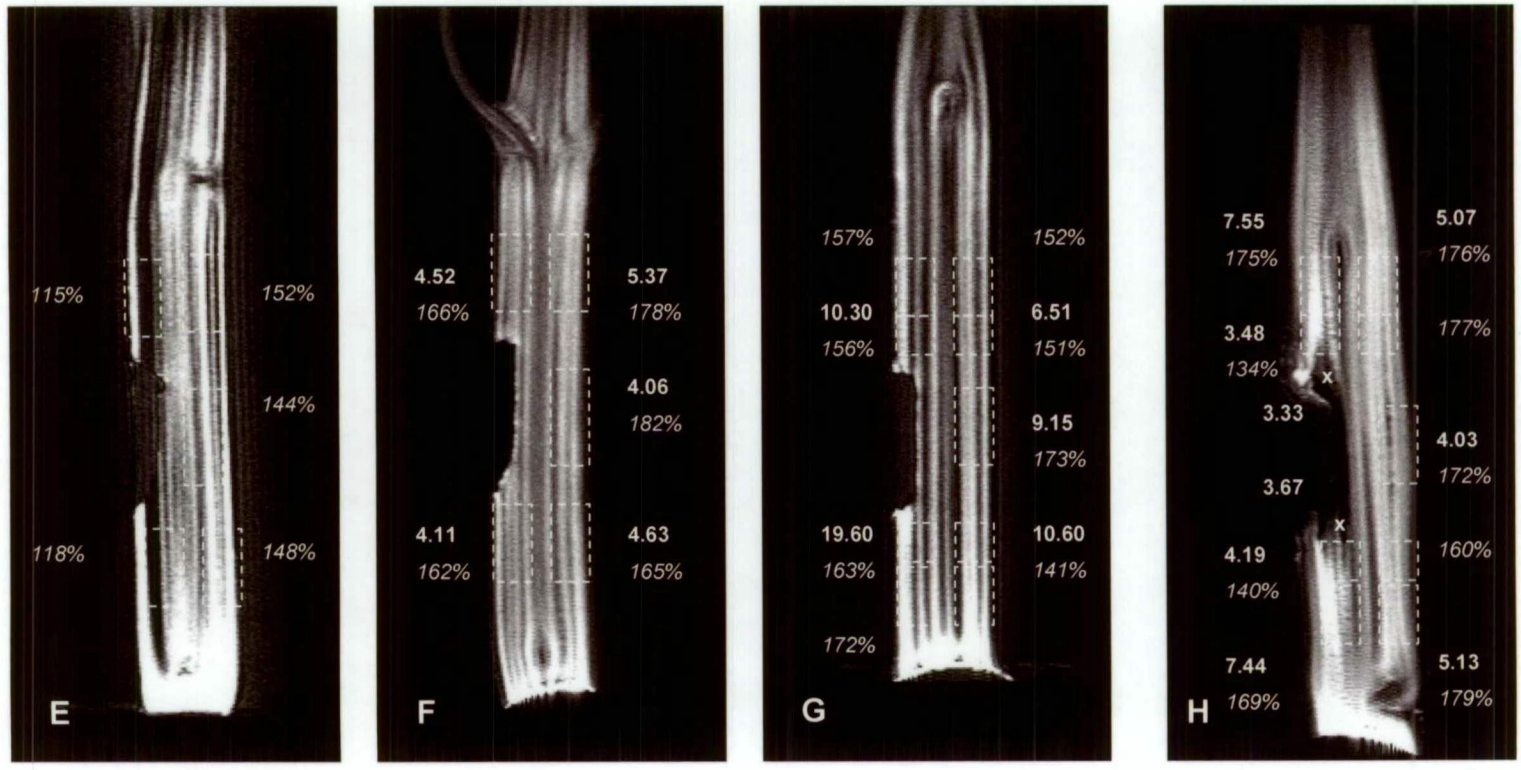


Figure 3.7 continued. 1st echo maps of *E. nitens* saplings at later stages after wounding and inoculation with *G. adspersum*: 7 days (E), 15 days (F), 22 days (G), and 72 days (H). Additional points where M_0 values were determined and marked with "x".

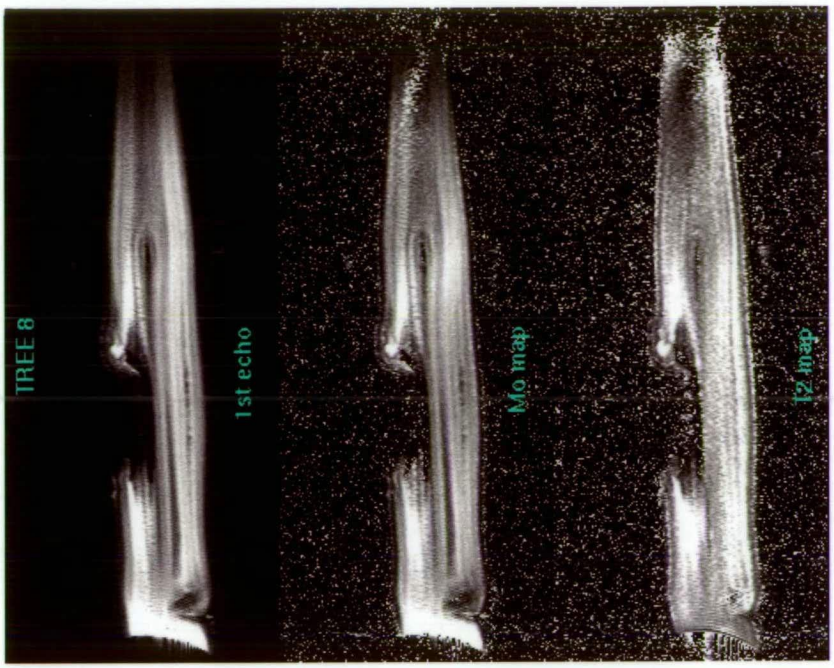


Figure 3.8. NMR images from an *E. nitens* sapling around a 72-day-old inoculated wound, including 1st echo, M_0 map and T_2 map.

Table 3.4. Comparison of water content in wounded and inoculated *E. nitens*, expressed as a ratio.

Sample	Ratio of water content (ie. wound-side value related to un-wounded side of stem)						
	1 hr	24 hrs	4 days	7 days	15 days	22 days	72 days
M_0							
Above wound	NA	1.01	0.63	NA	0.84	1.16[a]	0.74[a]
						1.58	1.49
Below wound	NA	0.99	1.21	NA	0.89	1.84[a]	0.81[a]
						NA	1.45
Gravimetric							
Above wound	0.87	1.01	0.98	0.76	0.93	1.03[a]	0.75[a]
						1.03	0.99
Below wound	0.84	0.91	0.93	0.80	0.98	1.16[a]	0.88[a]
						NA	0.94

[a] sample closest to wound.

Around the 22-day-old wound (Fig. 3.7G) some evidence of moisture content increase is apparent from both the M_0 values and gravimetric data, particularly

below. Figure 3.7H shows that areas of bright contrast are observable above and below the wound after 72 days (which was also present by 42 days). These areas correspond with the reaction zone and M_0 values are greater (ratio up to 1.49) than the adjacent healthy sapwood. Gravimetric determinations of moisture content did not detect these increases. The progress of decay around the wound is evidenced by a dark region in the images, with low M_0 and gravimetric values adjacent to the wound.

Complementary studies of *E. nitens* xylem moisture content as a percentage of saturated weight revealed an average of 55.35 %SW (± 0.58 SE) which related to an average of 179.76 %DW (±3.16 SE).

3.4 DISCUSSION

G. adspersum colonized *E. nitens* stem wounds to a limited extent and therefore represented a non-aggressive fungal-plant interaction. Responses to wounding and inoculation included parenchyma cell death, phenolic production beginning within 24 hours, tyloses formation and slight alterations in potassium levels and water content. Upon wounding, the withdrawal of water from severed xylem cells and ingress of oxygen may instigate a number of these responses (Rayner and Boddy, 1988; Pearce *et al.*, 1994). The extent of colonization may then determine the degree of development of these responses. That is, the fact that the inoculated *E. nitens* 21- and 30-day old wounds showed considerable variation in colonization and discolouration (Table 3.1) and phenolics content (Fig. 3.4 and 3.6) indicates that xylem responses to wounding may be secondary to responses to fungal colonization. Why some wounds became more colonized than others is difficult to determine.

The rapid accumulation of compounds detected by total phenol assays (Fig. 3.4) and HPLC (Fig. 3.5 and 3.6) suggest that phenolics are particularly important in *E. nitens* sapwood defence. Hydrolyzable tannins may be effective to halt microbial growth by binding proteins such as fungal enzymes (Field and Lettinga, 1992, Kawamoto *et al.*, 1997). Alternatively the ability of tannins to bind metals (Mila *et al.*, 1996) or act as antioxidants (Hagerman *et al.*, 1998) may restrict fungal metabolism. Levels of tetra-galloylglucose were increased by 3.5-fold within 24 hours of wounding and challenge while pedunculagin levels increased by 12-fold over 21 days (Fig. 3.6).

Increases were not evidenced by total phenol assays until 3 days after wounding and inoculation (Fig. 3.4). Pedunculagin has been found increased in the reaction zone of plantation-grown *E. nitens* up to 50-fold compared to healthy sapwood (Chapter 4). As ellagitannins are formed from gallotannins (Gross, 1992; Helm *et al.*, 2000) the rapid accumulation of tetra-galloylglucose would provide an immediate boost to the biosynthetic pathway. This may explain the slower accumulation of pedunculagin.

NMR imaging has allowed a non-invasive study of xylem moisture content in wounded and inoculated *E. nitens*. In the interpretation of these images a number of factors must be considered, including the effects of magnetic susceptibility (Pearce *et al.*, 1997a). That is, samples with a high moisture content (such as *E. nitens*) will result in an increase of image contrast at water-oxygen interfaces. Results obtained present a number of differences compared to a time-course experiment completed for *Acer pseudoplatanus* (Pearce, 1998) in which drying of xylem was apparent in the first 2 days, followed by re-wetting between the period 5 and 39 days. In *E. nitens* there was NMR imaging evidence for substantial drying 7 days after wounding (Fig. 3.7E). This was supported by gravimetric analysis, which indicated that the decrease in moisture was to 76% of normal xylem levels. As retraction of water and ingress of oxygen is suggested to determine the extent of compromised xylem accessible for fungal metabolism (Rayner and Boddy, 1988) the lack of evidence for quick and extensive drying may in part explain the limited xylem colonization by *G. adspersum*. It could be postulated that due to the high moisture content of the *E. nitens* saplings, a dry level critical for colonization takes longer to result than other hardwood trees such as sycamore. Although *E. nitens* may have a high moisture content when based on dry weight, determinations based on saturated weight reveal that even at a moisture content of 170 % DW the wood is far from saturated. That is, the experiments involving water-infiltrated healthy sapwood samples revealed that typical moisture contents were 55% when based on saturated weight.

Although drying does not seem to be dramatic, the withdrawal of water from vessels following wounding must be sufficient to instigate the accumulation of phenols and formation of tyloses (however, elicitation processes by fungi may also prove to induce these factors). Phenol biosynthesis appears to require the presence of oxygen (Coutts, 1977; Rayner and Boddy, 1988; R.B. Pearce, unpublished) and the accumulation of phenols is detected in *E. nitens* from 24 hours after wounding. The

formation of tyloses is also observable shortly after wounding, with evidence for formation within 24 hours in *E. nitens* (Table 3.1). Necesany (1973) found that tyloses formation in *Eucalyptus botryoides* sapwood increased with exposure to air. In *E. nitens* it was found that most tyloses development had occurred by 14 days (Table 3.1). Tyloses may be effective to block the vessels and prevent any further drying, allowing a return to equilibrium by apoplastic diffusion, as evidenced by M_0 and gravimetric data for the 15 day wound. In the discussion of these results, it is important to consider that individual trees were imaged in this experiment, and the progress of infection and defence responses may have varied in different trees at different stages.

Water soaking was evident in advance of infection in *E.* nitens by at least 30 days. NMR imaging detected an accumulation of water in the trees with inoculated wounds aged 22, 42 and 72 days, but this was not represented by results from gravimetric measurements. This suggests the increase was not great enough to be detected by gravimetric methods, in contrast to sycamore. M_0 values from the 72-day-old wound (Fig. 3.7H, Table 3.4) showed that this accumulation is approximately 1.5 times that of the healthy sapwood on the opposite side of the stem. As mentioned, magnetic susceptibility may accentuate this accumulation. Pearce *et al.* (1997a) found that the M_0 value in *Acer pseudoplatanus* infected with *Ustilina deusta* or *Ganoderma adspersum* (both non-aggressive fungi) was between 2.9 – 3.7 times higher, while the increase measured gravimetrically was only 1.1 – 1.7 times. As the difference between M_0 values in *E. nitens* is not to this extent it can be concluded that water accumulation is not as prominent as in *Acer pseudoplatanus.* A conservative evaluation of the data suggests that moisture content returns to typical or slightly higher levels in the compromised *E. nitens* xylem.

It appears that a different system of response occurs in *E. nitens* compared to *A. pseudoplatanus*. Firstly, large increases in potassium and calcium ions occur in the *A. pseudoplatanus* reaction zone (Grime and Pearce, 1995) which may osmotically drive the accumulation of water to the site. As a general decrease in potassium ions has been evidenced in the *E. nitens* reaction zone (and decreases in K^+, Ca^{2+}, Mn^{2+} and Mg^{2+} in previous studies – Chapter 2) this could not account for the moisture accumulation detected. Other means of increasing moisture content include increases of solubilized sugars or sugar alcohols in the osmoticum. This could be related to the

accumulation of hydrolyzable tannins (glucose based phenols) in the reaction zone. More likely, the production of tyloses and polyphenolic deposits reduce water flow resulting in a physiological "backwater" where water accumulates. This is more easily related to accumulation below the wound than above as bulk water flow in the xylem occurs axially through vessels, however diffusion also occurs through living cells such as ray parenchyma (Zimmerman, 1983) which may direct water above the wound.

Further studies are required to reveal whether the moisture content status of the reaction zone remains slightly elevated over time or declines to become drier than healthy sapwood as has been found for older reaction zones studied in plantation-grown trees (Chapter 2). However, comparison of these studies may be complicated as the current study involves younger trees and challenge of outer sapwood by a non-native fungus. Studies with *Acer pseudoplatanus* (Pearce *et al.*, 1994) have shown that differences in fungal aggression lead to dramatic differences in the expression of defence responses including accumulation of water. Regardless of differences in moisture content status, comparison of HPLC chromatographs between the *E. nitens* reaction zones from different studies suggest that the major compounds induced in both studies are similar (see Chapter 4).

In summary, this study has outlined the induction of key defence responses in *E. nitens* xylem in the early stages following wounding and inoculation. Moisture accumulation was evidenced in the reaction zone by NMR imaging, but may be a result of the physiological changes associated with wounding and infection rather than as an active defence as it was not observed until later in the interaction. Active defences such as rapid accumulation of phenolics are likely to be much more important in restricting the initial spread of the pathogen. The relative importance of active defences and microenvironment for fungal restriction was further investigated in an *in-vitro* experiment (Appendix 3.1). This showed that fungal invasion was restricted by active defences (requiring living cells), but poorly inhibited when the tissue was non-living.

APPENDIX 3.1

Stem segment challenge with variables including vitality and oxygen access

Plant material used was as for Chapter 3 (this experiment was completed at the University of Birmingham, UK). This experiment involved using stem segments to investigate the necessity for living cells in defence responses, and the effect of oxygen access.

Four treatments were designed (with three replicates) including the variables of stem vitality (autoclaved or living) and oxygen access (end of stem segment sealed or unsealed). Four internode segments (between 5-12 cm long) were excised from each of 3 *E. nitens* trees and the position from the tree was recorded. Half of the 12 segments were immediately placed in a sealed beaker and autoclaved, resulting in cell death. The weight of stem segments before and after autoclaving revealed that autoclaving did not appreciably reduce the fresh weight of the segments (less than 1%) and it is assumed that no significant amount of water was lost. All stem segments (living or autoclaved) were then challenged with *G. adspersum* by placing a piece of culture on one cut end and wrapping with parafilm. The other end of the segment was then either left open or sealed with parafilm to restrict oxygen access. Stem segments were then incubated at 25°C.

Challenged stem segments were analysed after 1 week. The extent of *G. adspersum* colonization was assessed visually and by isolation from a strip of wood excised from the stem, which was placed onto 3% malt agar and incubated at 25°C in the dark. For phenolic analysis, material was sampled from directly adjacent to the apparent infection at the inoculated end and from the middle of the stem segment. Wood shavings were extracted in 2 equal volumes of methanol and total phenol content was determined as above.

After 1 week, colonization of autoclaved stem segments by *G. adspersum* progressed rapidly, to a much greater extent than the living stems (Fig. A.3.1.1). As the length of the stem segments averaged 7.5 cm, the colonization relates to a substantial proportion of the segment, in many cases 100%. In living stems there was little visible evidence of colonization, however isolations indicated that *G. adspersum* was present.

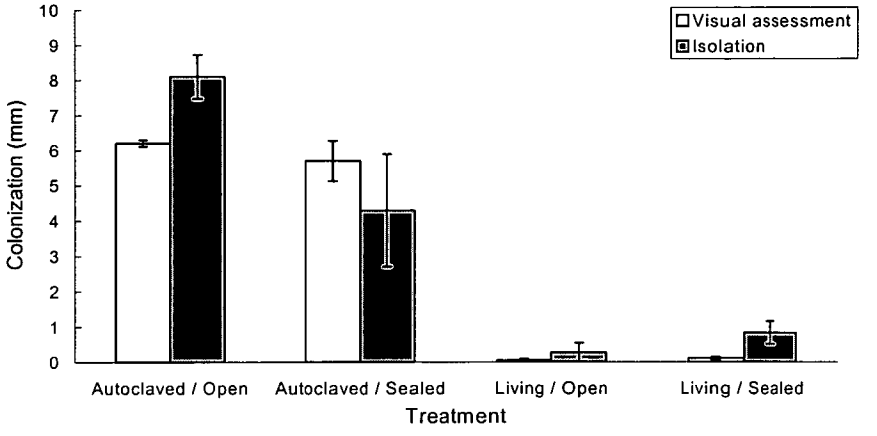


Figure A.3.1.1. Colonization of *E. nitens* stem segments (four treatments) challenged with *G. adspersum* for 1 week, assessed both visually and by isolation of the challenge fungus. n =3.

Total phenol contents determined for the living stems (Fig.A.3.1.2) shows that there were higher levels in the extracts from the middle of the stem segments compared to the area around infection at the inoculated end. In both cases this increase was significant ($P<0.001$). An increase in total phenol levels was also evident in the open treatment compared to the sealed treatment. However, this was not statistically significant (ie. $0.05>P>0.02$ based on "middle" data).

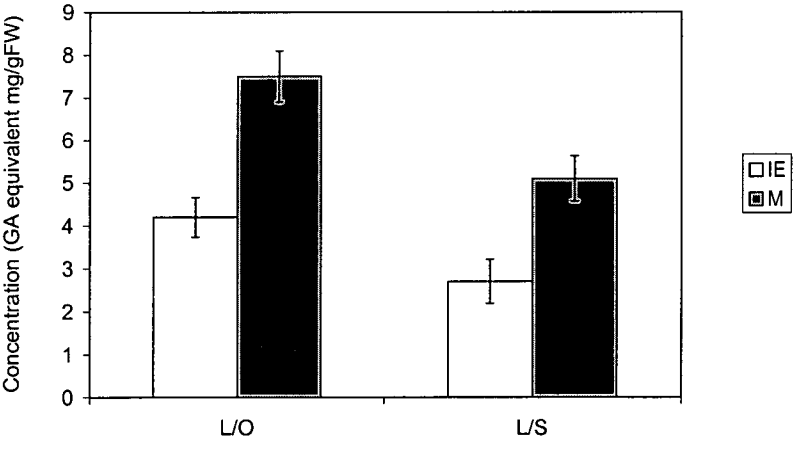


Figure A.3.1.2. Concentration of total phenols (mg GA equivalent gFW^{-1} ± SE) from "living" stem segments under two treatments and challenged by *G. adspersum*, from the inoculated end or the middle of the stem. n = 3.

The process of the autoclaving treatment in this experiment may have caused ultrastructural changes, which also attributed to the "dead" segments being colonized more rapidly. Similar experiments using autoclaved and freeze-treated *Cryptomeria japonica* logs showed that reaction zone were not formed as they were in fresh logs

or intact trees (Yamada, 1998). Freezing treatments may also be disruptive to cellular structure, and does not necessarily create a sterile environment. For example, bacteria can be isolated from freeze-treated wood (Bauch *et al.*, 1985).

Irradiation treatments were later investigated as a means to both sterilize the wood and kill xylem cells, with less disruption than autoclaving or freezing. X-ray irradiation was trialled for *E. nitens* stems with a radiotherapy accelerator (Clinac 600C, Royal Hobart Hospital) but even at 100 Grays of x-ray (7,800 mV) cells remained alive (determined by staining sections with nitro-blue tetrazolium, as used in Chapter 3). It is expected that gamma irradiation may be successful.

CHAPTER 4

IDENTIFICATION OF HYDROLYZABLE TANNINS IN THE REACTION ZONE OF *EUCALYPTUS NITENS* WOOD, BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - ELECTROSPRAY IONIZATION MASS SPECTROMETRY.

4.1 INTRODUCTION

The term hydrolyzable tannin refers to both ellagitannins and gallotannins. Ellagitannins are esters of "hexahydroxydiphenoyl" groups (HHDP) with a sugar core (usually glucose) and often contain galloyl groups. Gallotannins are a sugar substituted only with galloyl groups. For reviews of hydrolyzable tannins see Haslam (1989) and Okuda *et al.* (1995).

A role in antimicrobial defence has often been implicated for hydrolyzable tannins; for example, durable eucalypt and oak heartwoods are rich in ellagitannins (Scalbert, 1992). Hillis (1987) states that gallotannins are found in the heartwood of *Castanea* and some *Quercus* species, while large amounts of ellagitannins exist in the heartwoods of some *Eucalyptus* and *Quercus* species. Approximately twenty ellagitannins have been reported from the heartwood of *Eucalyptus* species (e.g. Seikel and Hillis, 1970; Hillis and Yazaki, 1973; Hart and Hillis, 1974; Yazaki *et al.*, 1993). Other studies have noted the presence of numerous ellagitannins in eucalyptus branch wood but have not identified individual compounds (Conde *et al.*, 1995; Cadahia *et al.*, 1997). Some heartwood ellagitannins are antifungal in bioassays, including ellagitannin D-6 and D-13 in *Eucalyptus* species (Hart and Hillis, 1974). Tannin toxicity to microorganisms has been explained by the characteristic ability of tannins to bind proteins (Field and Lettinga, 1992; Kawamoto *et al.*, 1997) or metals (Mila *et al.*, 1996) and their antioxidant nature (Okamura *et al.*, 1993; Hagerman *et al.*, 1998).

Reaction zones are a major component of active antimicrobial defence in the secondary xylem of woody plants (Shain, 1979; Pearce, 1996). The reaction zone forms at the interface between living sapwood and fungal infection, and is able to

restrict or slow fungal decay. This function has been attributed to the deposition of phenolics and polyphenolics, as well as microenvironmental factors (Boddy and Rayner, 1983; Yamada *et al.*, 1988; Pearce, 1996). The reaction zone in *Eucalyptus nitens* is purple-blue in colour, is rich in phenols and is resistant to colonization by decay fungi *in vitro* (Chapter 2). The composition of phenolics in the reaction zone has never been studied in any *Eucalyptus spp.*

The study of hydrolyzable tannins has developed substantially in the past two decades (Haslam, 1989; Okuda *et al.*, 1995). Molecular weight information has been obtained by fast-atom bombardment mass spectrometry of purified compounds (Okuda *et al.*, 1995). The majority of tannins are beyond the range of gas chromatographic analysis and high performance liquid chromatography (HPLC) has been necessary for complex tannin extracts. Combined HPLC-mass spectrometry (MS) has recently provided a powerful tool in the analysis of polyphenolics from crude and purified extracts by detecting negative ions produced by electrospray ionization (ESI) (Nawwar *et al.*, 1997; Puech *et al.*, 1999). However, little MS-MS detail of hydrolyzable tannins has yet been provided.

This study describes the application of HPLC-(ESI)MS to elucidate the major components of the complex extract from the *E. nitens* reaction zone. With reference to authentic standards of phenols, gallotannins and ellagitannins, eleven compounds have been unequivocally identified in crude extracts.

4.2 MATERIALS AND METHODS

4.2.1 Sample preparation

A number of *E. nitens* plantation trees between 5-7 years old were felled from various sites in Tasmania. Those with decay columns interfaced with reaction zones were sampled. Extracts were prepared from fresh wood by obtaining chisel shavings (100 - 500 mg) of the reaction zone and healthy sapwood tissue. Samples were extracted with 70% aqueous acetone and left to extract for 24 hours in the dark at 4°C. The extract was then transferred to a microcentrifuge tube and centrifuged to remove solids before appropriate dilution for LC-MS. Samples were stored at -20°C and analysed as soon as possible (within a week). Injections of 2 µl sample were

made, with pairs of extracts at equivalent concentration (based on g of wood tissue) where semi-quantification was determined. Typical yields gained (as a percentage of fresh weight) were approximately 6% for reaction zone extracts and 1.5% for healthy sapwood extracts.

4.2.2 Authentic standards

Tannin standards used included pedunculagin, pentagalloylglucose, corilagin, ellagitannins D-6 and D-13, 1,2,6-Tri-O-galloyl-ß-D-glucose, 1,2,3,6-Tetra-O-galloyl-ß-D-glucose, tellimagrandin I and II, casuarictin, casuarinin, rugosin A and B (generous gifts from both T. Yoshida and Y. Yazaki). Phenols included gallic acid, ellagic acid and catechin (Sigma Chemical Co.). Standards were weighed on a six-point balance and dissolved in 95% aqueous acetone at concentrations of 0.10 mg per 100 µl. Standards were taken to dryness after use under a stream of nitrogen at room temperature, stored at –20°C and later reconstituted when required.

4.2.3 HPLC-(ESI)MS

HPLC separations were carried out on a Waters Alliance 2690 using a Waters Nova Pak C18 column (150mm x 3.9mm). The mobile phase consisted of solvent A – water/acetic acid (98:2) and solvent B – methanol/acetic acid (98:2); initial conditions were 5% solvent B and 95% solvent A, then a linear gradient to 54% solvent B and 46% solvent A over 40 minutes with a flow rate of 0.8 ml/min, before returning to initial conditions, with 12 minutes re-equilibration between samples. Compounds were detected with a Waters 996 Photo-Diode Array detector, monitoring the range of 240 – 400 nm at a resolution of 1.2 nm.

Mass spectrometry was carried out on a Finnigan LCQ (San Jose, USA) with an electrospray ion source, using LCQ Navigator Version 1.2 software. The instrument was operated in negative ion mode, scanning from m/z 125 to 1500, with an AGC target value of $2x10^7$ and maximum ion injection time of 100ms. Operating conditions: sheath gas 90psi, aux gas 50psi, ESI needle voltage 4.5kV, capillary temperature 270, capillary voltage –30V. Data-dependent MS-MS spectra were routinely acquired from the most intense ion in the spectrum, with a default collision energy of 30% and a peak isolation width of 3 amu.

4.3 RESULTS AND DISCUSSION

4.3.1 Methodology and interpretation

The polar nature and complexity of the *E. nitens* reaction zone extracts limited successful HPLC resolution (Appendix 4.1). Difficulties in obtaining good HPLC separation of wood tannins is evidenced from previous studies (e.g. Scalbert *et al.*, 1988; Yazaki *et al.*, 1993). However long and time-consuming HPLC programs (e.g. 80 minutes) can be employed to improve separation (Cadahia *et al.*, 1997). Trials of a range of HPLC programs did not substantially improve separation of the *E. nitens* extracts, precluding peak identification based on retention times and UV spectra alone.

Fig. 4.1 (a, b) shows that the HPLC chromatogram detected with UV (280nm) for the reaction zone is more complex than the healthy sapwood. Fig. 4.2 (a, b) presents the corresponding total ion chromatograms from the LC-MS analyses for these extracts, which indicate that the mass range used (ie. up to *m/z* 1500) suitably includes the compounds detected by UV as similar peaks are observed. Mass spectrometry revealed that many compounds in the extract co-elute and that peaks cannot be interpreted by UV detection alone with current HPLC systems.

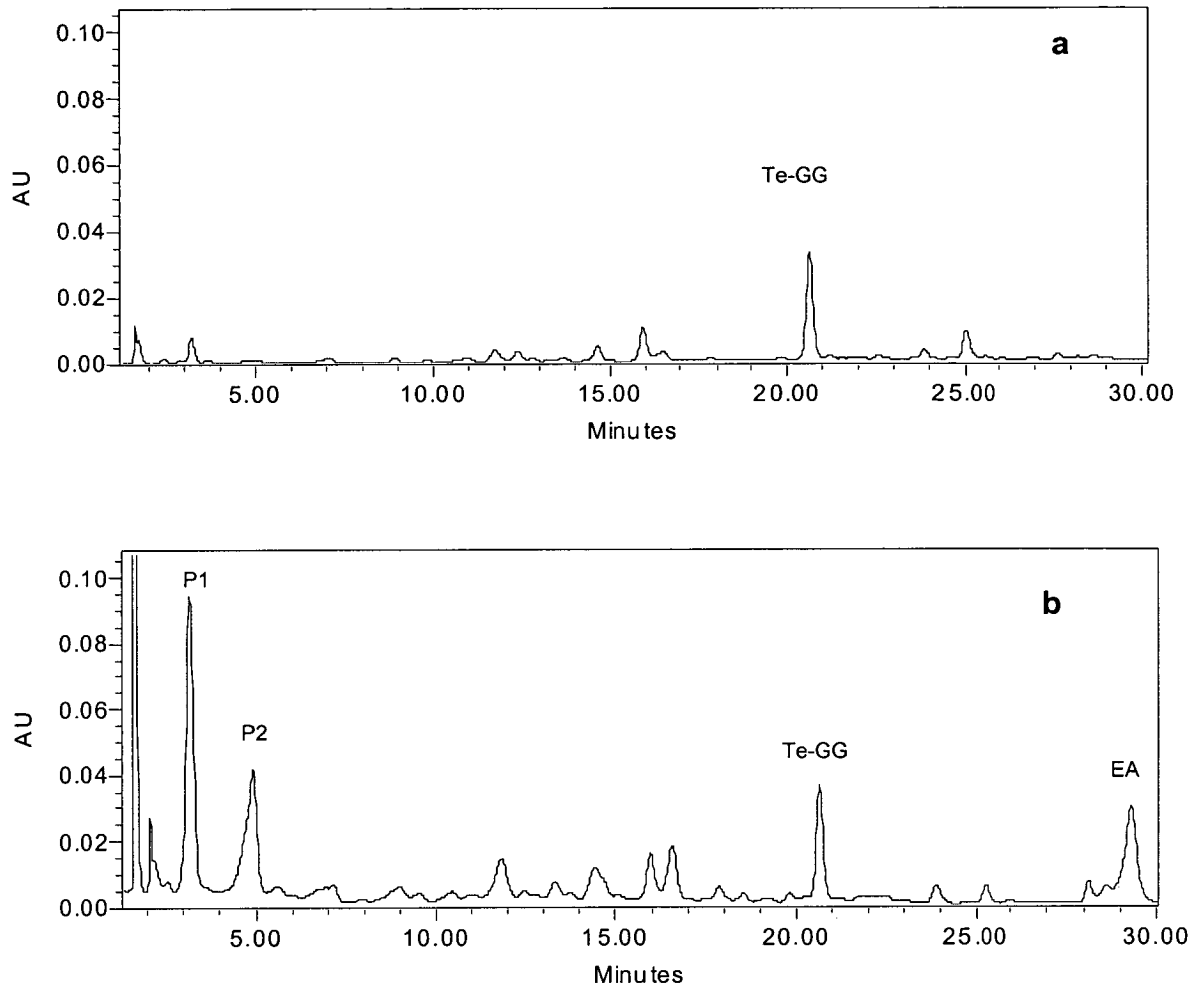


Figure 4.1a-b. HPLC chromatograms of 70 % acetone wood extracts detected at 280 nm: a) healthy sapwood; b) reaction zone. P1 and P2: anomers of pedunculagin, Te-GG: tetragalloylglucose, EA: ellagic acid. NB: acetone co-elutes with P1.

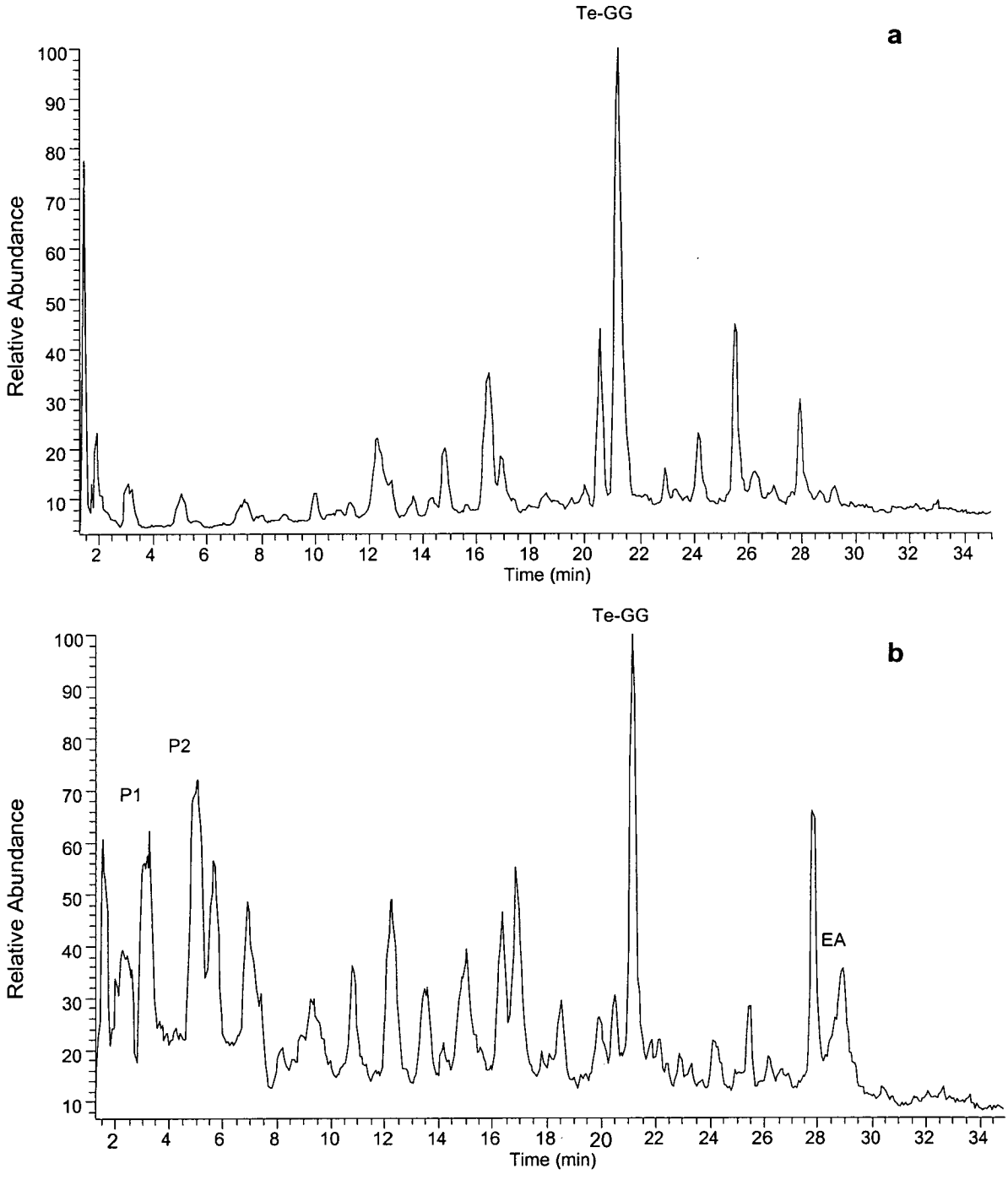


Figure 4.2a-b. MS chromatogram of 70 % acetone wood extracts: a) healthy sapwood; b) reaction zone. Refer to Fig. 4.1 for labels.

To gain an overview of the molecular weight range of the major compounds present in each extract, a summed mass spectrum was taken between *m/z* 125 and 1500 (Fig. 4.3 a, b). Of the major ions present, tannin and phenolic standards with molecular weights corresponding to these were obtained where possible. The retention times and negative ion electrospray MS characteristics of these standards were then determined. With reference to these standards, compounds were unequivocally identified based on matching mass chromatograms generated for specific $[M-H]^-$ ions (e.g. Fig. 4.4) mass spectra of the $[M-H]^-$ ions at specific retention times (e.g. Fig. 4.5) and subsequently MS-MS spectra of daughter ions from a selected parent ion (e.g. Fig. 4.6a). The assignment of numerous other peaks as positional isomers (e.g. various di-, tri- and tetra- galloylglucoses) was also possible.

Furthermore, the degree of substitution of HHDP and galloyl groups on unknowns could be assigned. Interpretation of MS-MS data from first principles indicated the presence of galloyl groups (losses of 152 and 170 from the $[M-H]^-$ ion), and/or HHDP groups (loss of 302 from the $[M-H]^-$ ion and the presence of an ion at *m/z* 301). Loss of 44 from the $[M-H]^-$ ion was characteristic of a free carboxyl (e.g. on a trisgalloyl group) and abundant losses of 18 (water) from the $[M-H]^-$ ion was characteristic of *C*-glucosidic ellagitannins.

The characteristic trait of two chromatographic peaks for ellagitannins forming anomer mixtures was also a feature for identification (Hatano *et al.*, 1988). That is, a free anomeric hydroxyl group at C1 of the glucose core of an ellagitannin molecule results in two chromatographic peaks. Addition of sodium borohydride to aqueous extracts reduces tannins with anomeric hydroxyls, resulting in a single peak (Hatano *et al.*, 1988) which also presents a tool for identification. Another characteristic of the electrospray mass spectra of most tannins is that doubly-charged ions (ie. $[M-2H]^{2-}$) are also formed (e.g. Fig. 4.5).

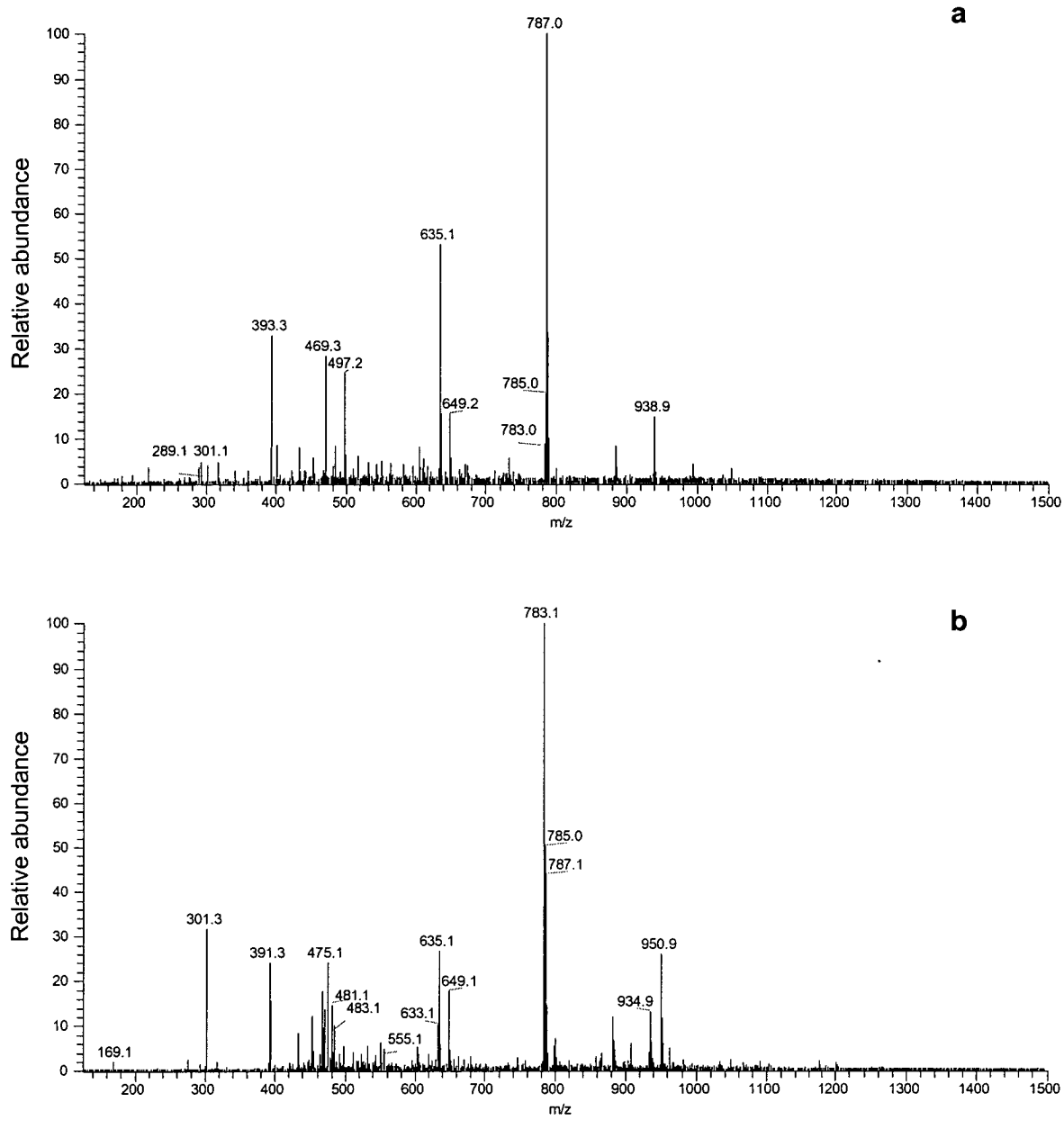


Figure 4.3a-b. Summed mass spectra in the range of *m/z* 125 – 1500 for 70 % acetone wood extracts: a) healthy sapwood; b) reaction zone.

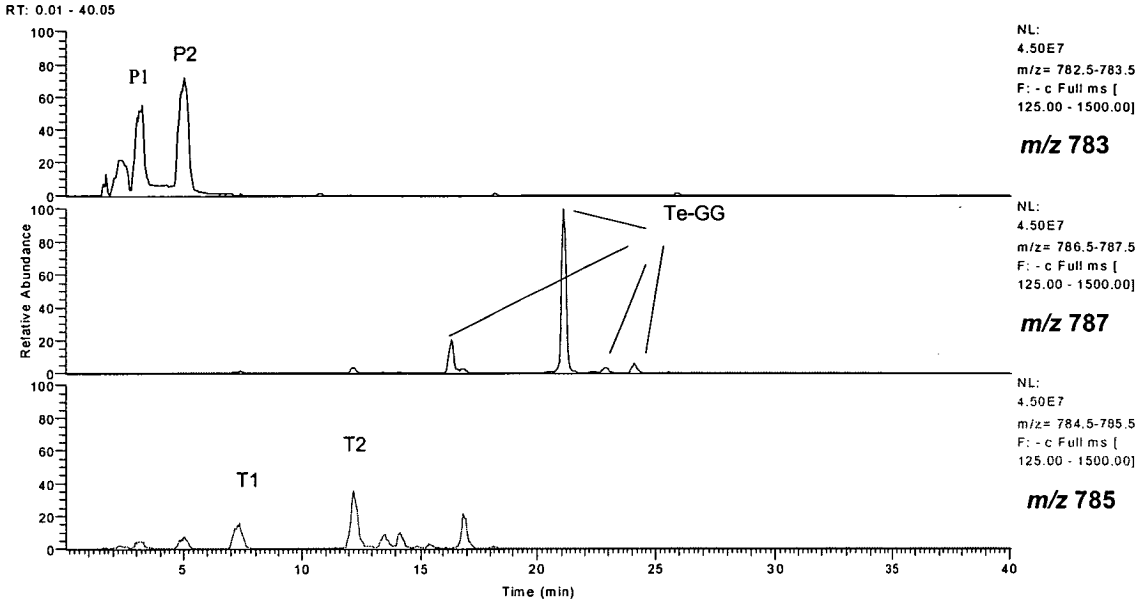


Figure 4.4. Mass chromatograms of three selected $[M-H]^-$ ions in reaction zone extract; P1 and P2: pedunculagin anomers, Te-GG: tetra-*O*-galloyl-*β*-D-glucose isomers, T1 and T2: tellimagrandin I anomers.

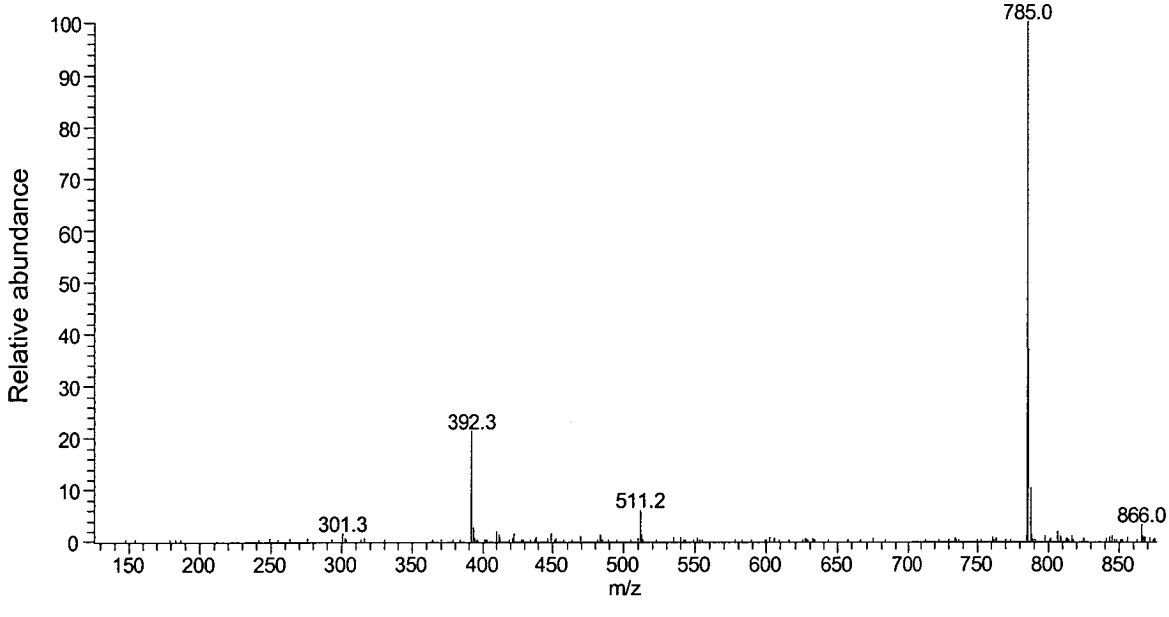


Figure 4.5. Mass spectrum for tellimagrandin I from the reaction zone extract; $[M-H]^-$ = 785, $[M-2H]^{2-}$ = 392.

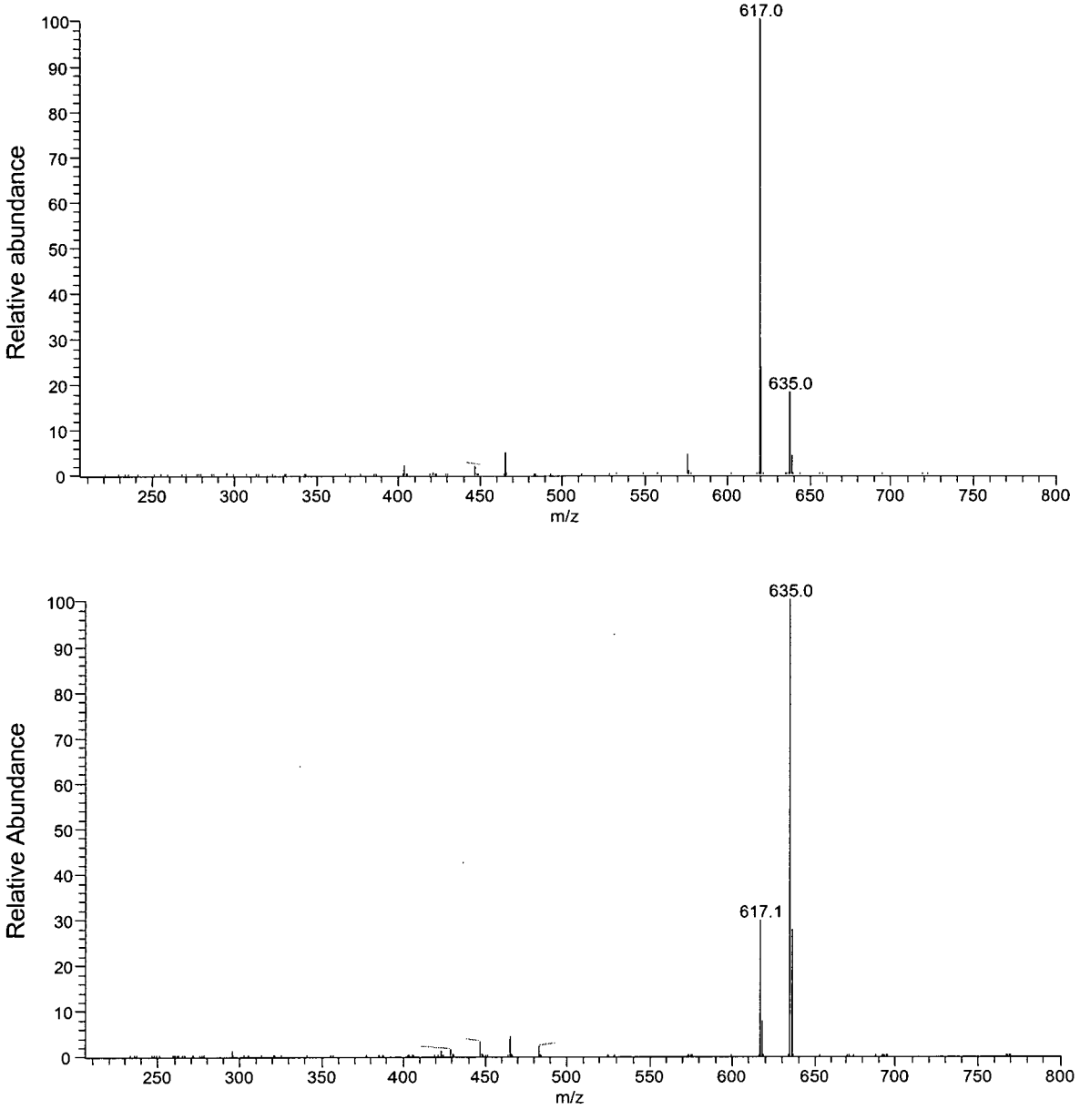
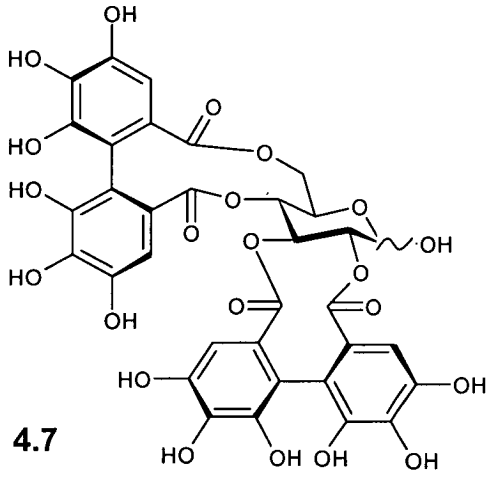


Figure 4.6. MS-MS daughters of the [M-H]- ion at m/z 787: a) from the 1,2,3,6-tetragalloyl-ß-D-glucose standard at 21.08 minutes; b) from the reaction zone extract peak at 21.08 minutes.

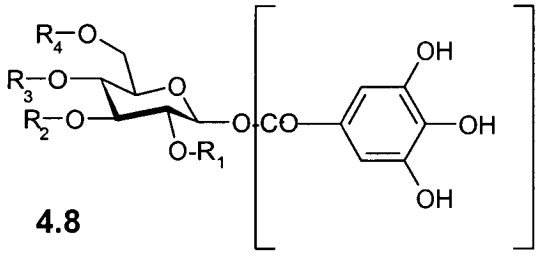
4.3.2 Compound identification

Table 1 outlines over thirty major and minor compounds discriminated from the *E. nitens* extracts, showing relative proportions (based on MS peak area) which have been classed arbitrarily as described. The sensitive MS system used in the present study also detected a multitude of smaller peaks which have not been analysed. Five ellagitannins (pedunculagin, tellimagrandin I and II, casuarinin and casuarictin), three

gallotannins (tri-galloylglucose, tetra-galloylglucose and penta-galloylglucose; abbreviated as Tr-GG, Te-GG and Pe-GG respectively) and three phenols (catechin, gallic acid and ellagic acid) were unequivocally identified. The structure of an abundant ellagitannin (pedunculagin) is shown in Fig. 4.7 and a variety of gallotannins are illustrated in Fig. 4.8. A number of assumed positional isomers and unidentified compounds are also presented in Table 4.1 and are discussed below.



4.7



4.8

Figure 4.7. Pedunculagin (2,3; 4,6-di-HHDP-glucose). **Figure 4.8.** Examples of the gallotannins with various numbers of galloyl groups (G, bracket) linked to the glucose core. Number of β positional isomers expected: GG = 1, Di-GG = 4, Tr-GG = 6, Te-GG = 4, Pe-GG = 1.β-Glucogallin: $R_1 = R_2 = R_3 = R_4 = H$; 1,6-Di-*O*-galloyl-β-D-glucose: $R_4 = G$, $R_1 = R_2 = R_3 = H$; 1,2,6-Tri-*O*-galloyl-β-D-glucose: $R_1 = R_4 = G$, $R_2 = R_3 = H$; 1,2,3,6-Tetra-*O*-galloyl-β-D-glucose: $R_1 = R_2 = R_4 = G$, R_3 =H; 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose: $R_1 = R_2 = R_3 = R_4 = G$.

Treatment of *E. nitens* reaction zone extracts (prepared as aqueous solutions) with sodium borohydride revealed that at least three prominent ellagitannins that form anomers are present, as three prominent "new" reduction product peaks were detected (Appendix 4.1). Two of these anomer-forming ellagitannins are pedunculagin and tellimagrandin I, and the third may be a compound with $[M-H]^-$ at *m/z* 951.

Table 4.1. Major and minor compounds present in the *E. nitens* wood extracts.

[M-H]⁻	RT (minutes)	Compound	Relative Abundance [a]	
			HS [b]	RZ
169	2.79	Gallic acid [c]	tr	+
289	10.03	Catechin [c]	+	tr
301	28.93	Ellagic acid [c]	+	++
481	1.55	HHDP-glucose	tr	+++
483	8.83	Di-GG [v]	tr	tr
	10.67	Di-GG [w]	tr	tr
	12.00	Di-GG [x]	tr	tr
	13.59	Di-GG [y]	tr	tr
	14.49	Di-GG [z]	tr	tr
633	9.61	HHDP-GG	tr	++
635	12.46	Tr-GG [w c]	+	+
	14.75	Tr-GG [x c]	++	++
	16.23	Tr-GG [y c]	++	++
	16.39	1,2,6-Tr-GG [c]	tr	tr
	16.79	Tr-GG [z c]	+	+
649	27.82	Me-(Tr-GG)	++	+++
783	2.40	di-HHDP-glucose	tr	++
	3.16/5.00	Pedunculagin [c]	+	++++
785	7.27/12.19	Tellimagrandin I [c]	++	+++
	13.52	HHDP-di-GG [x]	tr	++
	14.19	HHDP-di-GG [y]	+	++
	16.85	HHDP-di-GG [z]	+	++
787	16.29	Te-GG [w c]	+	+
	21.08	Te-GG [x c] + 1,2,3,6- Te-GG	++	+++
	22.86	Te-GG [y c]	tr	tr
	24.04	Te-GG [z c]	tr	tr
935	9.32	Casuarinin *	0	+
	15.02	Casuarictin *	0	+
	23.10	di-HHDP-GG	tr	+
937	16.91	HHDP-tri-GG [x]	tr	+
	18.02	Tellimagrandin II [c]	tr	+
	19.24	HHDP-tri-GG [y]	tr	+
	32.95	HHDP-tri-GG [z]	+	0
939	25.42	Pe-GG [c]	tr	+
951	5.64/10.84	(Trisgalloyl)-HHDP-glucose [y]	tr	+++
	6.88	(Trisgalloyl)-HHDP-glucose [z]	0	+++

[a] Relative scale; 0 = none detected, tr = 0-1 (trace amount); + = 1-10; ++ = 10-50; +++ = 50-100; ++++ = 100 +

[b] HS = healthy sapwood, RZ = reaction zone

[c] = unequivocal identification

[v, w, x, y, z] are to discriminate putative individual isomers

The general substitution pattern of HHDP and galloyl groups for a number of compounds in Table 1 has been determined from molecular weight information and characteristic MS-MS daughter ions. Where molecular weights of hydrolyzable tannins differ by two, it can be related to the difference between either an HHDP group or two galloyl groups. For example, by coupling two adjacent galloyl groups of the four from Te-GG (788) by intramolecular oxidation, tellimagrandin I (786) would be formed. Coupling of the two remaining galloyl groups from tellimagrandin

I would produce pedunculagin (784) (Fig. 4.7). The loss of two hydrogen atoms resulting from bonding of galloyl groups may occur similarly for other structures. The strong $[M-H]^-$ at *m/z* 481 in the reaction zone samples has been assigned as an HHDP-glucose, based on molecular weight and the presence of an intense daughter ion at *m/z* 301. The compound with $[M-H]^-$ ion at *m/z* 633, abundant in the reaction zone samples, has been assigned as an HHDP-galloylglucose (Table 4.1), from the MS-MS evidence of intense ions at *m/z* 301 and 463. The minor $[M-H]^-$ ions at *m/z* 483 have been assigned as di-galloylglucoses.

Casuarinin and casuarictin (both with an $[M-H]^-$ ion at *m/z* 935) could be distinguished by their retention time and also by the MS-MS spectra. The MS-MS daughters of the casuarinin $[M-H]^-$ ion showed a strong ion (30% relative abundance) resulting from a loss of water, due to its open glucose ring structure (*C*-glucoside rather than *O*-glucoside). For casuarictin this ion was only 0.2% relative to the strongest ion, the loss of 302 as discussed above. The peak eluting at 2.40 minutes prior to the two pedunculagin anomers at m/z 783 (Fig. 4.4) also showed a strong ion (20% relative abundance) resulting from a loss of water from the $[M-H]^-$ ion and therefore may be the *C*-glucoside version of pedunculagin.

Based on molecular weight and MS-MS data, the three abundant peaks with $[M-H]^-$ at *m/z* 951 in the reaction zone samples (Table 4.1) would appear to be tannins with one HHDP group and one trisgalloyl group (e.g. valoneoyl, tergalloyl, macaronyl). All three peaks lost 44 from the $[M-H]^-$ ion, consistent with a free carboxyl group as found on a compound such as praecoxin A. As mentioned, the two peaks at *m/z* 951 eluting at 5.64 and 10.84 minutes (Table 4.1) may be anomeric forms of one compound, therefore suggesting a free hydroxyl at the glucose C1. The single peak at 6.88 minutes may be a variation on this which involves bonding at the C1 position.

The main peak of Te-GG at 21.08 minutes in the *E. nitens* extracts (see Fig. 4.4) is likely to represent two isomers. The 1,2,3,6-tetra-*O*-galloyl-β-D-glucose standard eluted at this time and its MS-MS spectrum (Fig. 4.6a) showed two major daughter ions of which *m/z* 617 is 5-fold more abundant than *m/z* 635. However, the MS-MS spectrum of the *E. nitens* peak at 21.08 minutes (Fig.4.6b) revealed that the daughter ions are in reverse proportion, and therefore coming largely from a different isomer. This latter ratio was consistent in all reaction zone and healthy sapwood extracts

analysed. Four major Te-GG isomers were detected in the *E. nitens* extracts (Fig. 4.4 and Table 4.1). As the possible number of β isomers for Te-GG is four (see Fig. 4.8) it would be surprising if the 1,2,3,6- isomer was not present, and therefore it may be making a small contribution to the main peak in the *E. nitens* extracts. The number of possible positional isomers of the other gallotannins (Fig. 4.8) correlates likewise but not exactly to the number of peaks representing each gallotannin isomer in the wood extracts (Table 4.1).

4.3.3 Tannin levels and biological significance

For simplicity, compound abundance has been classified into groups based on MS peak areas (from a typical set of samples) as detailed in Table 4.1. The abundance of pedunculagin was found to be increased approximately 50-fold in the reaction zone compared to the healthy sapwood. Many other ellagitannin levels were also present at greatly increased levels in the reaction zone. There was at most only a slight increase in the levels of the gallotannins in the reaction zone samples. This suggests that the ellagitannins are more important in the antimicrobial effectiveness of the reaction zone. Gallotannins such as penta-*O*-galloyl-β-D-glucose may be important as ellagitannin precursors (Hatano *et al.*, 1986; Haslam, 1989). Interestingly, preliminary LC-MS studies of *E. nitens* leaves have shown that a range of gallotannins are present in light stressed leaves compared to normal leaves, but few/no ellagitannins (D.C. Close and N.W. Davies, unpublished).

Haslam (1989) has classified plants with penta-*O*-galloyl-β-D-glucose and one or more of tellimagrandin II, tellimagrandin I, casuarictin, potentillin and pedunculagin as having "group 2B" phenolic metabolites. *Eucalyptus nitens* wood metabolites therefore fall within this grouping, as has been found for the Myrtaceae family in general (Haslam, 1989). The range of closely related hydrolyzable tannins and isomers found in *E. nitens* wood is a common occurrence and may prove advantageous for defence (Zucker, 1983). For example, tannin-protein binding may be an important mechanism to halt fungal metabolism, and a range of tannin structures may promote a variety of specific tannin-protein interactions.

As casuarictin and casuarinin (and a compound at *m/z* 951 eluting at 6.88 minutes) were not detected in the *E. nitens* healthy sapwood these compounds may be

produced *de novo* in the reaction zone from pedunculagin (Okuda *et al.*, 1995). All other tannins are detectable in at least trace amounts in the healthy sapwood (Table 4.1). Concentration increases of the magnitude found in the *E. nitens* reaction zone would be expected to confer biological activity. For example, Hart and Hillis (1972) found that even two-fold increases of white oak heartwood extracts resulted in antifungal inhibition to *Poria monticola* in bioassay. The presence of increased levels of gallic acid and ellagic acid in the reaction zone may indicate hydrolysis of ellagitannins and gallotannins over time.

Catechin has not been previously detected in *Eucalyptus* sapwood (Y. Yazaki, pers. comm.), and this may be due to greater detection sensitivity in this study, or it may represent a novel finding for *E. nitens*. The decreased levels of catechin in the reaction zone may be due to the formation of condensed tannins and the purple/blue colour of the zone is suggestive of condensed tannins. However these compounds were not analysed in this study and there was no evidence of condensed tannins such as epicatechin gallate.

In summary, LC-MS provides a powerful and relatively quick technique to elucidate the complex nature of crude wood extracts. It provides a solid basis for identification with reference to retention times, unequivocal molecular weight assignment and characteristic daughter ions. At least thirty hydrolyzable tannins (including structural isomers) were detected in the *E. nitens* wood extracts. Eight of the hydrolyzable tannins and three phenols were unequivocally identified by comparison with standards. The ellagitannins were orders of magnitude more abundant in the reaction zone compared to healthy sapwood. The ellagitannin pedunculagin is particularly concentrated in the reaction zone and may play an important role in its antimicrobial function.

APPENDIX 4.1

Preliminary HPLC investigations

Initial studies of extracts from naturally-infected plantation-grown trees were completed with HPLC and UV detection. While the crude and complex nature of the extracts made resolution difficult, these studies provided a basis for subsequent LC-MS analysis.

High performance liquid chromatography (HPLC) was performed on a Waters HPLC consisting of two model 510 pumps, a 600E system controller and a 486 tunable UV absorbance detector. Samples were injected via a U6K sample injection unit onto a C18 Spherisorb ODS analytical column (4.6 x 250 mm) with a C18 Spherisorb guard column (4.6 x 10 mm). UV detection was at 275, 280, 260 or 245 nm.

A variety of solvent programs were used including most commonly:

P5: This was a two-step linear program from 1% acetic acid (solvent A) to 100% methanol (solvent B). The first step was a linear gradient from 100% A and 0% B to 40% A and 60% B over 40 minutes. The second linear gradient was to 0% A and 100% B over 10 minutes which was maintained for 2 minutes before returning to initial conditions.

NWP-2: This was an isocratic program (no gradient) consisting of a solution of 15% acetonitrile, 85% water and 0.2% phosphoric acid being constantly pumped through the column. Monitoring was typically over 20 minutes.

Wood extracts analysed included healthy sapwood, reaction zone and decayed wood. Decayed wood was typically very low in extractives compared to the other tissues (Fig. A.4.1.1). A range of standards were used, as already described in Chapter 4.

Some standards and samples were subjected to sodium borohydride ($NaBH_4$) treatment which reduces anomeric hydroxyl groups (Hatano *et al.*, 1988), resulting in a larger single chromatographic peak rather than two. This was illustrated with pedunculagin standards (Fig. A.4.1.2a-b). When reaction zone extracts were treated three new single peaks became prominent (Fig. A.4.1.3a-b). The earliest of these

peaks had the same retention time as the pedunculagin standard when treated. The other two peaks probably relate to single peaks of 2 other anomeric compounds. Decreased levels of tetragalloylglucose and increased levels of ellagic acid (the peak eluting at 43 minutes) indicate that the treatment may have caused some hydrolysis of the tannins.

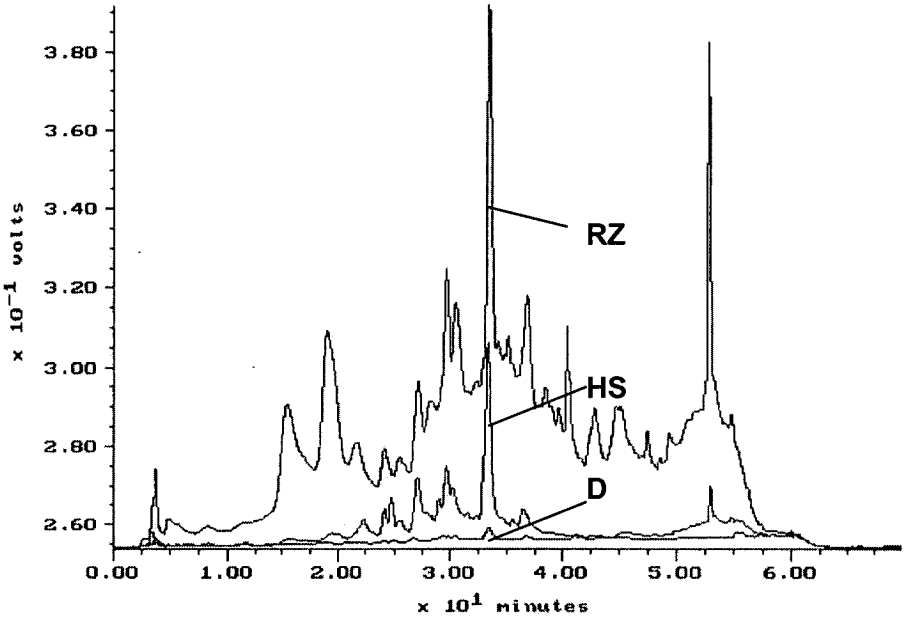


Figure A.4.1.1. Three HPLC chromatograms overlaid on one axis, including healthy sapwood (HS), reaction zone (RZ) and decayed wood (D). Program P5, detected at 275 nm.

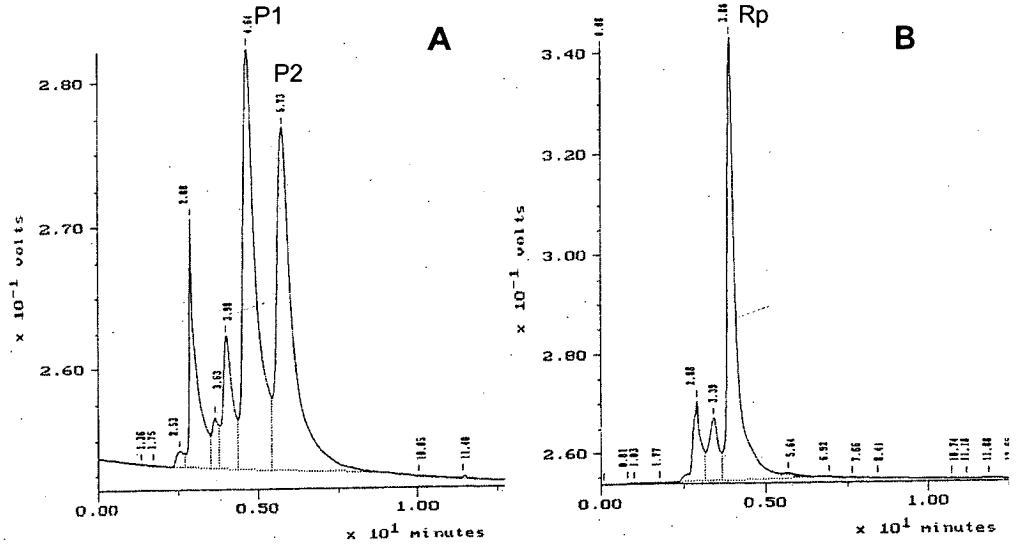


Figure A.4.1.2. HPLC chromatograms of pedunculagin (0.1 mg/ml); using program NWP-2, detected at 245 nm. A) normal standard, B) treated with $NaBH_4$. P1 and P2 refer to main anomeric peaks, Rp is reduced single peak.

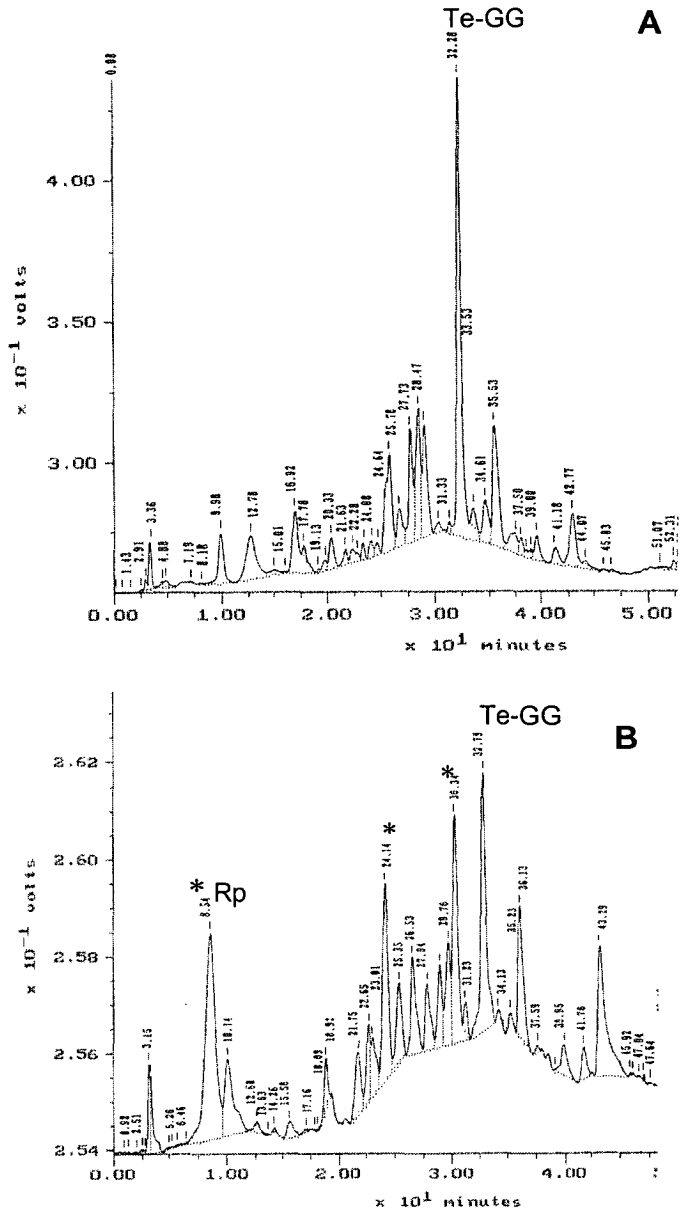


Figure A.4.1.3. HPLC chromatograms of reaction zone extract; using program P5, detected at 280 nm. A) normal extract, B) treated with $NaBH_4$. Asterix (*) indicate new peaks which resulted from the treatment, including reduced pedunculagin (Rp).

CHAPTER 5

COMPARATIVE LEVELS OF REACTION ZONE AND HEARTWOOD EXTRACTIVES IN *EUCALYPTUS NITENS* WOOD, INCLUDING ANTIFUNGAL ACTIVITY

5.1 INTRODUCTION

Previous studies of the *E. nitens* reaction zone have reported increased total phenol levels (Chapter 2) and found that hydrolyzable tannins account for the majority of the extractives (Chapter 4). Gallotannins dominate *E. nitens* sapwood and ellagitannins are found in trace amounts. In the reaction zone, some gallotannins (tetragalloylglucose and pentagalloylglucose) and ellagitannins (particularly pedunculagin) increase in concentration. Increased gallotannins have been found during the very early stages of wounding and infection, and may be precursors for ellagitannin production (Chapter 3).

The constituents of reaction zones are often similar to those in heartwood; including the mansonones E and F in elms and pinosylvin and pinosylvin monomethyl ether in pines (Kemp and Burden, 1986). Hillis and Inoue (1968) found that while pinosylvin and pinosylvin monomethyl ether were found in both "affected" sapwood and heartwood, some compounds were found in heartwood that were not found in the "affected" sapwood and vice versa. The trend found in a range of trees is that extractives increase at the heartwood transition zone and then decrease towards the pith. This may be accounted for by increased polymerization and insolubility (Hillis, 1987; Peng, 1991).

Accumulation of extractives affords an antimicrobial protection in tissues where living cells are not present. In non-living wood, heartwood is typically more decay resistant than sapwood due to impregnation with extractives such as tannins (Rayner and Boddy, 1988; Hillis, 1987). Reaction zones are often even more resistant than heartwood and this may be due to greater accumulation of extractives. *In-vitro* bioassays in a woody substrate (Hart and Hillis, 1972; Hart and Hillis, 1974) or artificial system such as on a thin-layer chromatogram (TLC) plate (Woodward and

Pearce, 1985) are useful to demonstrate the fungicidal or fungistatic potential of these compounds.

This paper provides quantitative results of total phenols, selected tannins and catechin in sapwood, reaction zone and heartwood samples from *E. nitens* plantation trees in Tasmania determined by high performance liquid chromatography - electrospray ionization mass spectrometry (LC-MS). An account of bioassay results using TLC plates is also presented for sapwood and reaction zone extracts.

5.2 MATERIALS AND METHODS

5.2.1 Plant material and sampling

Reaction zone material was obtained from pruned trees, sampled at four sites in Tasmania (Urana, Flowerdale, Evercreech and Hastings). Details of plant material used is provided in Table 5.1. A small number of trees (up to 9) were felled from each site and those with decay columns interfaced by a reaction zone were sampled (2-3 trees per site, often more than 1 decay column per tree). In most cases reaction zones were found to be in the sapwood (RZ or SRZ), however for the second sampling at Evercreech (Table 5.1) most reaction zones were present within the heartwood (HRZ).

Heartwood samples were also collected from one of these sites (Evercreech) and from a site of unpruned trees (Esperance). Material at four radial positions (sapwood, heartwood transition zone, outer heartwood and inner heartwood) was sampled from a billet at approximately 2.8 m height for Evercreech and 2.5 m for Esperance (in both cases well away from any decay columns).

5.2.2 Extraction of phenolics

Billets were transported to the laboratory in plastic bags and stored at 4 °C for 1-2 days prior to sampling. Disks (1 cm thick) adjacent to the material to be sampled were stained with dimethyl yellow (0.2% in 80% ethanol) to determine the heartwood/sapwood boundary (heartwood transition zone). Various wood tissues were sampled as chisel shavings (ca. 0.5 mm thick) and fresh weight was determined

immediately. In most cases fresh material was extracted (extraction method A) but for others the material was extracted after freeze-drying and milling (extraction method B). Aqueous acetone (70%) has been shown to be the most efficient for extraction of hydrolyzable tannins (Zhentian *et al.*, 1999).

The method used for each sample type is provided in Table 5.1 and the methods were:

Extraction method A: fresh wood shavings (typically 100-300 mg FW) were extracted in two successive volumes of 70% acetone, both for 24 hours at 4 °C in the dark (ca. 100 mg FW per ml solvent). After extraction, the liquid was removed with a pipette and placed at -20 °C in the dark. Before analysis, extracts were centrifuged to remove any remaining particulate matter.

Extraction method B: Larger samples were obtained (400-1800 mg FW) and the wood shavings were wrapped in miracloth before being dipped in liquid nitrogen. Samples were then freeze-dried and dry weight obtained. Samples were milled through a 0.1 mm sieve, weight of recovered sample was determined (between 200-800 mg) and samples were extracted immediately in 70% acetone (ca. 100 mg DW per ml solvent). Samples were extracted once for 24 hours at 4 °C and then filtered through Whatman No. 1 filter paper. Extracts were dried down (under a stream of nitrogen to remove the acetone followed by freeze-drying) and later re-dissolved to a known volume. Results were expressed on a DW basis.

Table 5.1. Details of plant material source, type, sampling and experimental procedures for *E. nitens* trees.

Site / altitude, coordinates	Tree age / yrs since pruning	Tissues sampled	Extraction procedure	Experiments
Urana 24B / 450 m, 41°23' S, 148°00' E	5 / 2	HS & RZ	A	Bioassays
Hastings 28B / 140 m, 43°24' S, 146°53' E	7 / 3	HS & RZ	A	TP, LCMS
Evercreech 29C / 670 m, 41°22'S, 147°56' E	7 / 4	HS & RZ	A	TP, LCMS
Flowerdale 37D / 260 m, 41°04' S, 145°29' E	7 / 3	HS & RZ	A	TP, LCMS
Esperance / 240 m, 43°17' S, 146°52' E	16 / not-pruned	HS, HTZ, OH & IH	A	TP, LCMS
Evercreech 29C / 670 m, 41°22'S, 147°56' E	8 / 4.5	HS, RZ, HRZ, HS, HTZ, OH & IH	B	Bioassays, TP, LCMS

HS= healthy sapwood, RZ= reaction zone (sapwood), HRZ = reaction zone (heartwood), HTZ = heartwood transition zone, OH = outer heartwood, IH = inner heartwood.

5.2.3 Total phenol analysis

Total phenols were estimated by the Folin-Ciocalteu method as previously described by Bonello and Pearce (1993) but with slight changes. Extracts were diluted appropriately to 3000 µl with water. To this, 1500 µl of 1:1 diluted Folin and Ciocalteu's reagent (Sigma Chemical Co., Sydney) was added and left for 3 minutes, followed by addition of 1500 µl of 1 M aqueous Na_2CO_3. The solution was shaken and placed at 50 °C for 5 minutes. The absorbance was measured at 725 nm with a spectrophotometer. Concentrations of total phenols were calculated with reference to a gallic acid standard curve (10-200 µg per ml dissolved in acetone) by applying regression analysis. Results were expressed as µg gallic acid equivalent per mg DW.

5.2.4 Analysis of phenolics by LC-MS

Internal standard. As an internal standard, rutin (Sigma Chemical Co.) was prepared in pyridine (5 mg/ml) and added to each extract prior to analysis.

LC-MS system. Samples were analysed by LC-MS (sample chamber maintained at 5 °C) by the following system. HPLC separations were carried out on a Waters Alliance 2690 using a Waters Nova Pak C18 column (150 mm x 3.9 mm). The mobile phase consisted of water/acetic acid 98:2 (solvent A) and methanol/acetic acid 98:2 (solvent B). Initial conditions were 5 % solvent B and 95 % solvent A. The program was a linear gradient to 54% solvent B and 46% solvent A over 40 minutes with a flow rate of 0.8 ml per min, before returning to initial conditions with 12 minutes re-equilibration between samples.

Mass spectrometry was carried out on a Finnigan LCQ (San Jose, USA) with an electrospray ion source, using LCQ Navigator Version 1.2 software. The instrument was operated in negative ion mode, scanning from m/z 125 to 1500 with an AGC target value of $2x10^7$ and maximum ion injection time of 100 ms. Operating conditions were as follows: sheath gas 90 psi, aux gas 50 psi, ESI needle voltage 4.5 kV, capillary temperature 270 °C and capillary voltage –30 V. Data-dependent MS-MS spectra were routinely acquired from the most intense ion in the spectrum, with a default collision energy of 30 % and a peak isolation width of 3 amu.

Monitoring and quantification. In all experiments, 2 ellagitannins, 2 galloylglucoses and 1 flavonoid were selected for monitoring by LC-MS. Previous identification of *E. nitens* reaction zone extractives (Chapter 4) revealed that of a number of ellagitannins, pedunculagin and tellimagrandin I were dominant and considerably increased compared to the sapwood. Both compounds consisted of two anomers and therefore required summation of two peaks. Of the gallotannins, tetragalloylglucose and pentagalloylglucose showed some increase and were also selected. There was a range of isomers of tetragalloylglucose but only the main peak showed an increase (Chapter 4). This peak was composed of two isomers including 1,2,3,6-tetragalloylglucose (Chapter 4). For pentagalloylglucose, a peak with equal retention time to the standard (one of several peaks in the sample with an $[M-H]^-$ at 939) was

chosen. Catechin levels (while generally low) showed an inverse trend which warranted further examination. The complexity of compounds in the reaction zone made some other compounds of interest difficult to quantify with good repeatability from sample to sample (due to slight changes in retention time confusing identification of a range of isomers).

The process of quantification by LC-MS is outlined in Appendix 5.1. To quantify peak areas of individual compounds, characteristic ions were selected. This required inclusion of not just the singly charged de-protonated molecular ions, but doubly charged molecular ions, daughter ions and adducts formed during ionization. This then simulated the total ion current for each compound while allowing the exclusion of co-eluting peaks.

Correction factors. Peak areas were converted from "rutin equivalents" to appropriate units by using response factors to hydrolyzable tannin standards (non-commercial, see Chapter 4) and a commercially supplied standard of catechin (Sigma Chemical Co., Sydney), as outline in Appendix 5.1. The response factor between rutin and 1,2,3,6-tetragalloylglucose was 1.45. Response factors were then used to relate amounts of 1,2,3,6-tetragalloylglucose to pedunculagin, tellimagrandin I, pentagalloylglucose and catechin were 3.83, 3.36, 1.92, and 4.15 respectively. These response factors were calculated from an average of 8 runs of standards (prepared in a mix). The purity of non-commercial standards was also calculated and incorporated into the results. This was complicated by impurities contributing to the total of other compounds (for example, tellimagrandin 1 had a ca. 13 % impurity of pentagalloylglucose) which was incorporated into the total amount calculated. Details of compound purity calculations are provided in Appendix 5.1. Concentration of compounds was expressed as μg per mg extracted DW.

5.2.5 Statistical analysis

Fishers t-test was applied to all results using SAS software version 6.11 (SAS Institute; Cary, NC, USA). Probability was assessed at the 5% level using type III data.

5.2.6 TLC and fungal bioassays

Separation of wood extracts on thin-layer chromatography plates was trialed by a number of methods. This included: (1) Silica gel 60 plates, 0.2 mm thick (Merck, Sigma-Aldrich, Sydney, Australia) developed in chloroform/methanol (1:9); (2) Cellulose plates, 0.1 or 1 mm, with fluorescent indicator (Merck, Alltech Chromatography, Melbourne, Australia) developed in iso-butanol/water (14:5). For two-dimensional separation on cellulose, plates were then developed in 100% water in the opposite direction.

Extracts were applied as a line (or as a spot for two-dimensional separation) and either crude or after partitioning: (i) Crude extracts (reaction zone extracts up to an equivalent of 60 mg fresh weight of wood tissue were used for bioassays as greater amounts overloaded the plates); (ii) Partitioned extracts were prepared by dissolving crude extracts in water and partitioning against two equal volumes of diethyl ether and retaining the water fraction, which was then further partitioned against two equal volumes of ethyl acetate; (iii) Alternatively a simplified form of separation was possible by adding water to a crude extract and allowing the water soluble compounds to re-dissolve. The remaining solid (a purple colour for reaction zone extracts) was re-dissolved in methanol.

Multiple TLC plates were prepared for both chemical detection of compounds and bioassays. Chemical detection of phenols was possible with chromogenic sprays including Folin-Ciocalteu reagent (Sigma Chemical Co., Sydney, Australia) which resulted in a blue-grey colouration. To distinguish different hydrolyzable tannins, "NSSC" was utilized (15 g Na_2SO_3: 3.5 g Na_2CO_3 : 350 ml water; Hart and Hillis, 1974). A range of hydrolyzable tannin standards were characterized by TLC but could not be used for bioassays as only very small amounts (< 1 mg) were available.

Rf values were calculated by the following equation: Rf = distance to centre of spot from starting point (/) distance to solvent front from starting point (x) 100.

Bioassay methods using TLC plates have been previously described (Woodward and Pearce, 1985). TLC plates for bioassay were dried and then fungal material was applied in a nutrient solution and the plates were incubated at 20 °C in the dark, in moist chambers. *Cladosporium cladosporioides* (Fresen.) was grown on 3% malt agar and a spore suspension was prepared by flooding 10-14-day-old plates with Czapek Dox medium and agitating the plate to dislodge conidia (a surfactant was not used). The spore suspension was filtered through two-layers of muslin, sprayed onto the plate and examined after 3 days incubation.

Mycelial suspensions were prepared of a wood-decaying basidiomycete isolated from naturally-infected pruning-wounds in Tasmania (from an Evercreech tree from series A above, see Appendix 2.5). This fungus remains unidentified and has been referred to as "isolate D", but has been described in Chapter 6. Cultures were maintained on 3% malt agar and sub-cultured to 2% malt extract (ca. 15 ml). Liquid cultures were incubated for 14 days in the dark at 20 °C on a shaker. Mycelial mats were then homogenized with a blender, followed by filtering with two-layers of muslin and subsequent application to the plate as a spray. Plates were examined after 5 days and to aid visualization of the hyaline mycelium an 0.05% solution of fluorescent brightener 28 (Sigma Chemical Co., Sydney, Australia) in 0.1 M phosphate buffer (pH 8.0) was applied. Plates were then viewed under UV light at 366 nm.

5.3 RESULTS

5.3.1 Reaction zones

Samples of reaction zones present within the sapwood were analysed from trees at three sites (Fig. 5.1) and although replicate numbers were relatively low, there was a significant interaction between site and tissue type differences for levels of pedunculagin (P= 0.0001), pentagalloylglucose (P= 0.0004) and catechin (P= 0.0001). Levels of pedunculagin were the greatest, being up to 35-fold greater on average in the reaction zone compared to healthy sapwood for the Flowerdale samples. Levels of all compounds were considerably greater in the sapwood of

Evercreech trees than at the two other sites. While levels of all tannins were increased in the reaction zone tissues, catechin was dramatically reduced compared to the sapwood and was not detected at all in the Hastings reaction zones.

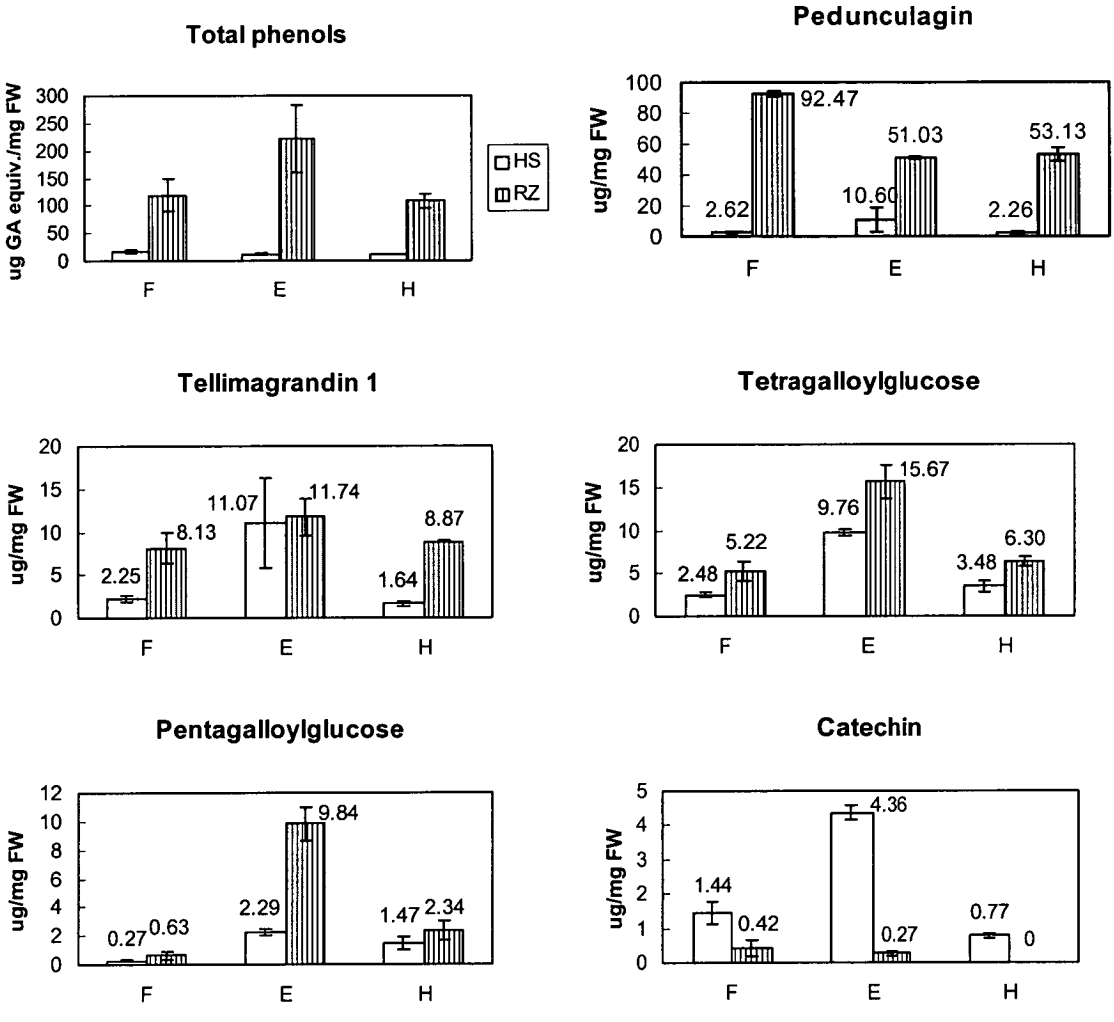


Figure 5.1. Analysis of healthy sapwood and reaction zone tissues of pruned *E. nitens*: Average concentration (µg/mg FW wood ± SE) of pedunculagin, tellimagrandin 1, tetragalloylglucose, pentagalloylglucose and catechin in trees presented by site (F = Flowerdale, H = Hastings and E = Evercreech).

In the second set of results comparing reaction zones to sapwood (Fig. 5.2), reaction zones within the heartwood were sampled. These zones included both dark purple (closest to the decay) and pink coloured zones (between the dark purple and sapwood). The purple zones were approximately 2-fold higher in total phenol levels than the pink tissue, and showed general increases for the tannins studied. Phenol levels of both zones of the heartwood reaction zone tissues were higher than the sapwood reaction zone. The three reaction zone tissues were greater in tannins than the sapwood, and there were significant differences for total phenol levels (P=0.0265; HS different from purple HRZ), pedunculagin (P= 0.0134; HS and SRZ different

from purple HRZ) and tellimagrandin 1 (P= 0.0435; HS different from purple HRZ). As above, catechin levels were dramatically reduced in the reaction zone compared to healthy sapwood, but this was not significantly different, probably due to the variation within the sapwood sample.

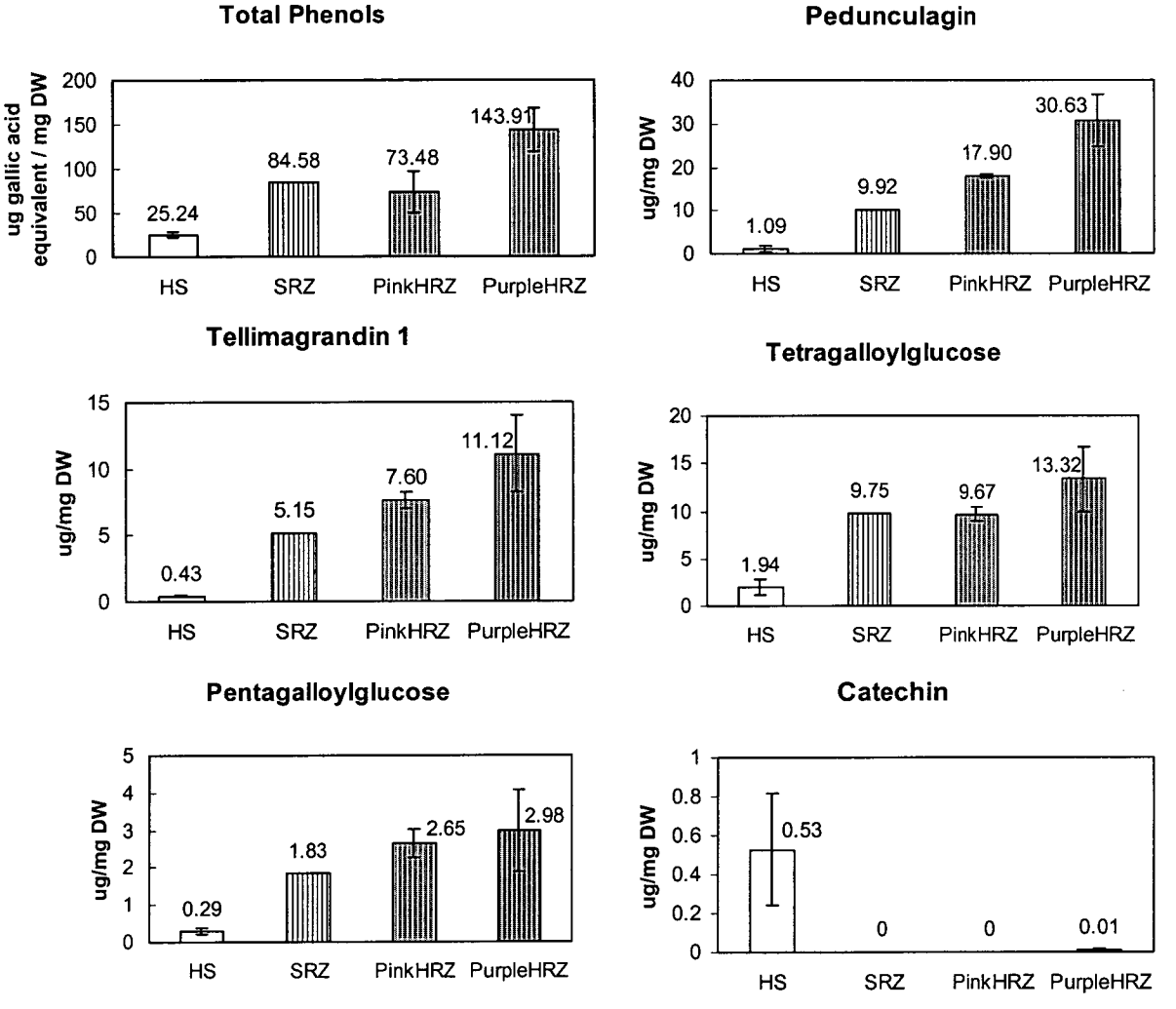


Figure 5.2. Analysis of extracts from two 8-year-old Evercreech *E. nitens* trees: Average concentration (μg/mg DW wood) of total phenols and individual compounds in the healthy sapwood and various reaction zone tissues (HS= healthy sapwood, SRZ= sapwood reaction zone, HRZ= heartwood reaction zone).

5.3.2 Heartwood

The constituents of the *E. nitens* heartwood were qualitatively similar to the reaction zone, as shown by the mass chromatograms of the two tissues from one tree (Fig. 5.3). Quantitative analysis of the selected compounds showed that while they were generally increased in the heartwood compared to the sapwood, this was not to the same extent as the reaction zone (Fig. 5.1 & 5.2 compared with Fig. 5.4).

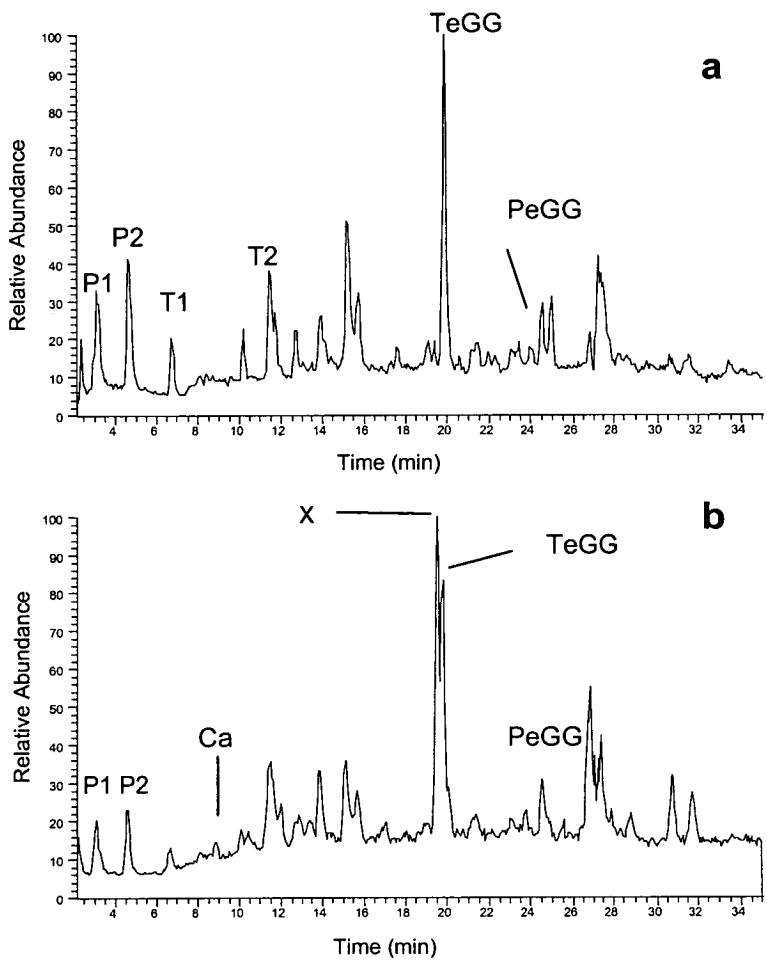


Figure 5.3a-b. Total Ion Chromatograms from LC-MS analysis of *E. nitens* extracts from an 8-year-old Evercreech tree. a) Reaction zone within heartwood tissue (HRZ purple); b) Outer heartwood. Peaks: P1and P2are anomers of pedunculagin, T1 and T2 are anomers of tellimagrandin 1, TeGG is tetragalloylglucose main peak, PeGG is pentagalloylglucose peak, Ca is catechin, x = non-tannin peak (possible contaminant).

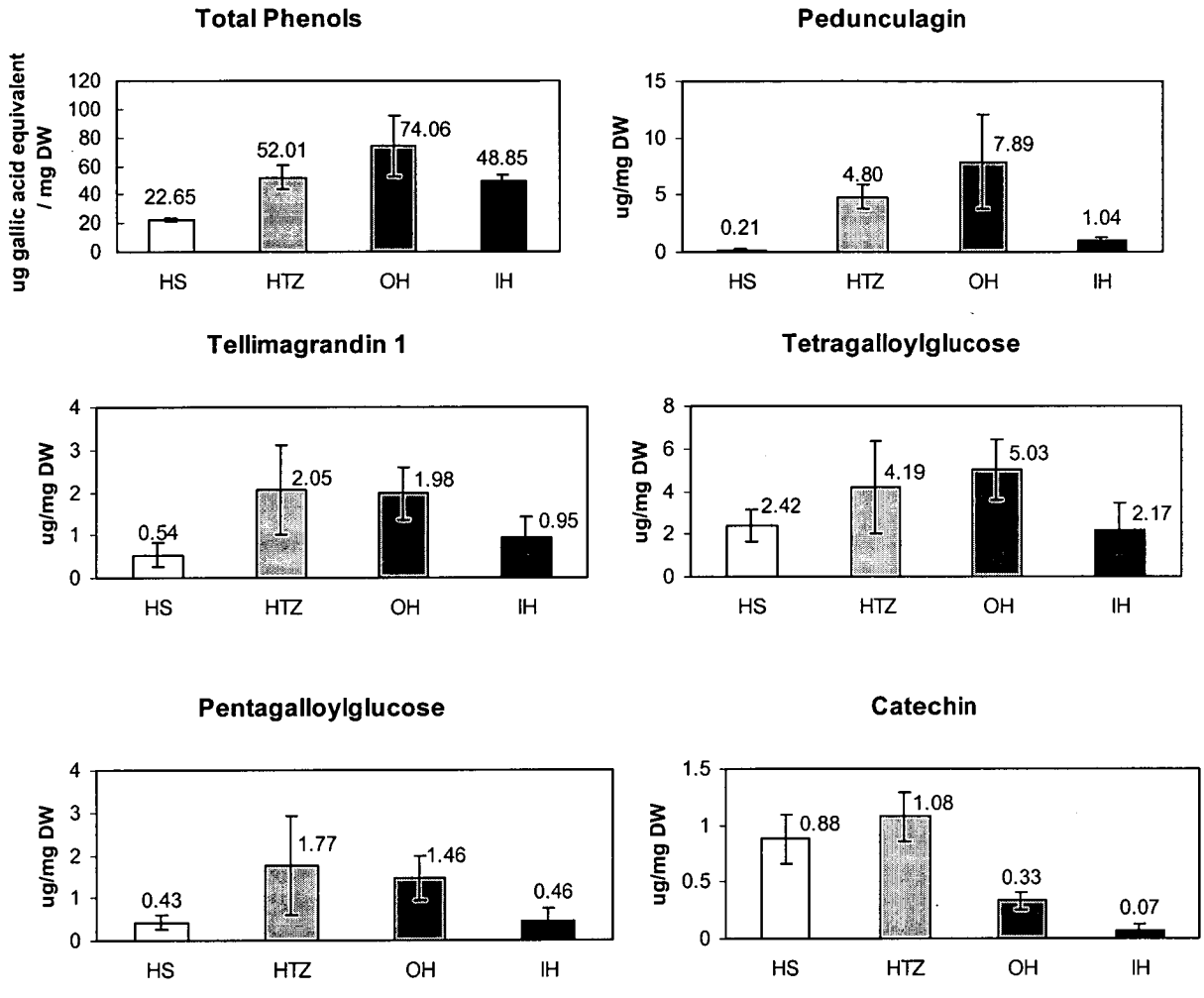


Figure 5.4. Analysis of sapwood and heartwood samples of three 8-year-old Evercreech trees: Average concentration (μg/mg DW wood ± SE) of total phenols and individual compounds. (HS= healthy sapwood, HTZ= heartwood transition zone, OH= outer heartwood, IH= inner heartwood).

Levels of phenols in the four radial zones of both pruned Evercreech trees (Fig. 5.4) and unpruned Esperance trees (Fig. 5.5) were generally not significantly different, but typically increased in the heartwood tissues compared to the sapwood. As the level of variation between trees was particularly high, increased replication may give the results more definition. Catechin levels were significantly different for both Evercreech (P= 0.0075) and Esperance (P= 0.0246) trees and while trends were slightly different, in both cases catechin was significantly lower in the outer and inner heartwood compared to the sapwood. Tetragalloylglucose levels were significantly different for the Evercreech trees (P= 0.0362). Levels of phenols were not directly comparable between the Evercreech and Esperance trees as the results

were expressed on a different basis (DW compared to FW). As moisture content tends to decrease towards the central tissues, results expressed on a DW basis will be most meaningful. As for reaction zone tissues, levels of pedunculagin were higher than the other compounds in the heartwood.

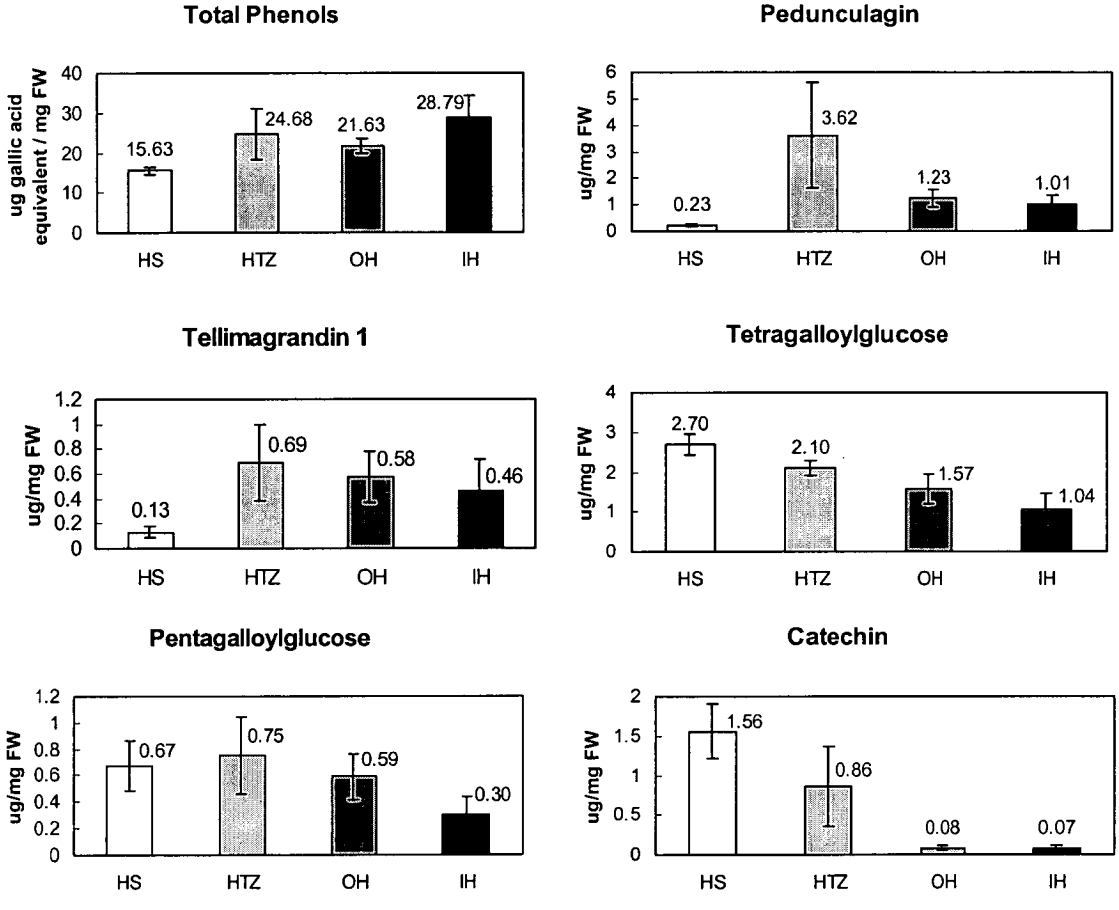


Figure 5.5. Analysis of extracts from three 16-year-old Esperance trees: Average concentration (µg/mg FW wood ± SE) of total phenols and individual compounds from various tissues (HS= healthy sapwood, HTZ= heartwood transition zone, OH= outer heartwood, IH= inner heartwood).

5.3.3 TLC and fungal bioassays

Analysis of a range of hydrolyzable tannin standards (Table 5.2) aided interpretation of TLC separations of complex *E. nitens* extracts. Using TLC method 2 (one-dimensional) Rf values were reasonably low (up to 33). Colour reactions agree with previous studies using "NSSC" (Seikel and Hillis, 1970; Hart and Hillis, 1972; Hillis and Yazaki, 1973). Eighteen-hours after spraying with NSSC all compounds faded to a light yellow/orange. All standards reacted with the Folin reagent to give a pale blue-grey colour.

Table 5.2. Characteristic Rf values and colour reactions with "NSSC" of hydrolyzable tannin standards on cellulose plates separated in 1 dimension (iso-butanol/water, 14:5).

Compound	Rf value	Colour (3 min.)	Colour (10 min.)
Pedunculagin	21	Pink/peach	Red/brown
Corilagin	18	Orange	No change
Tellimagrandin I	33	Orange	No change
Tellimagrandin II	28	Brown/yellow	Faint orange
1,2,6- trigalloylglucose	33	Brown/yellow	Faint orange
1,2,3,6- tetragalloylglucose	33	Brown/yellow	Faint orange
Pentagalloylglucose	33	Brown/yellow	Faint orange

E. nitens extracts were complex and of a polar nature and therefore difficult to separate into individual compounds by conventional TLC methods. The bulk of the crude extract (and water fraction) had low retention times similar to the standards (Table 5.2). The best separation method was two-dimensional on cellulose, as used by Hart and Hillis (1974) however this method required that extracts were applied as a spot which reduced the amount of extract that could be applied before the plate overloaded (which was not enough to obtain fungal inhibition). Therefore, extracts were typically separated using one-dimension, the extract was applied as a line and inhibition of bioassay test fungi was observable in broad regions.

Some inhibition of *C. cladosporioides* was detectable with the extracts from healthy sapwood while substantial inhibition resulted from the reaction zone extracts (Table

5.3). An example of a TLC bioassay plate is shown in Appendix 5.2. Partitioning of the reaction zone extract suggested that the purple fraction was not responsible for inhibition (Table 5.3). The water fraction contained most extractives by yield and was more inhibitory than the ethyl acetate fraction (Table 5.3) which had a negligible yield. The fact that both of these fractions showed some inhibition suggests that there is more than one compound inhibitory to the growth of *C. cladosporioides*. Bioassays completed with "isolate D" suggested that patterns of inhibition were similar to *C. cladosporioides*.

When regions of the extract corresponding to inhibition were removed from the preparative scale plates and analysed by HPLC, a range of compounds were often detected. While the nature of the antifungal compounds could not be discriminated, compounds that were not present could be excluded. Also, comparing patterns of extract development between plates used for bioassay and those assessed with chromogenic sprays (particularly NSSC) allowed some interpretation. Pedunculagin was most distinctive as it turned a bright pink upon staining with NSSC (as for the analysis of standards, Table 5.2) and was not associated with the antifungal region or detected on the HPLC analysis following preparative TLC. As the colours of other compounds were less distinctive, being brown or orange (Table 5.2) they were more difficult to distinguish in a poorly separated crude extract.

Table 5.3. Summary of TLC bioassay results for various *E. nitens* extracts (see Materials and Methods text for interpretation of TLC method and extract type).

Origin of material (tree number)	TLC method/ extract type	Extract and concentration	Intensity of fungal inhibition	
			C. cladosporioides	Isolate D
Urana (2A)	1/i.	HS (50 mg FW equiv.)	+	NT
		RZ (10 mg FW equiv.)	-	
		(30 mg FW equiv.)	-	
		(40 mg FW equiv.)	++	
		(75 mg FW equiv.)	++++	
Urana (2B)	1/i.	HS (60 mg FW equiv.)	-	NT
		RZ (60 mg FW equiv.)	+++	
Urana (5B)	1/iii.	RZ - Water (50 mg FW equiv.)	++	NT
		RZ - Methanol (purple, 100 mg FW equiv.)	-	
Evercreech 6 and 7 (Purple HRZ)	2/ii.	RZ – Water fraction (purple)		
		(20 mg DW equiv. ~ 1.0 mg yield)	++	++
		(50 mg DW equiv. ~ 2.5 mg yield)	+++	+++
		RZ – Ethyl acetate		
		(20 mg DW equiv. ~ 1.0 mg yield)	-	-
		(50 mg DW equiv. ~ 2.5 mg yield)	++	++

NT = not tested, - = no inhibition, + = inhibition.

5.4 DISCUSSION

All results were highly variable from tree-to-tree, although tissue differences were still apparent. Increasing replicate numbers substantially would be likely to improve results. Masson *et al.* (1995) found more tree-to-tree variation in levels of individual heartwood ellagitannins than species difference between pedunculate oaks (*Quercus robur* L.) and sessile oaks (*Quercus petraea* Liebl.). While differences in the age of the wood were important, the effects of height and orientation of sampling were not.

Analysis of reaction zone and heartwood extracts from *E. nitens* has revealed that they are qualitatively similar. That is, ellagitannins are prominent (pedunculagin is at the highest levels), gallotannins increase to some extent, but catechin levels diminish. This lends support to previous findings (Kemp and Burden, 1986) that both heartwood and reaction zone formations are similar processes.

In absolute terms, there are more tannins present in the reaction zone than heartwood. For example, pedunculagin in the heartwood reaction zone of Evercreech trees was present at an average of ca. 30 μg / mg DW (Fig. 5.2) compared to the normal heartwood samples up to ca. 8 μg / mg DW on average for outer heartwood (Fig. 5.4). In results from other trees (Fig. 5.1) averages for pedunculagin in reaction zones were up to ca. 92 μg / mg FW and would be expected to be even greater on a DW basis. Tree-to-tree differences probably cause this variation (as few trees were sampled from each site), but site differences may exist. Also, the ages of the reaction zones vary. These factors require further investigation. In the 16-year-old Esperance trees, levels of pedunculagin were found only up to ca. 4 μg / mg FW on average in the heartwood (Fig. 5.5).

In heartwood samples for both Evercreech (Fig. 5.3) and Esperance (Fig. 5.5) trees, levels of tannins decreased in the inner heartwood. It has been commonly found in other studies that the heartwood transition zone is richest in extractives and then levels decline towards the pith, including tannins from other eucalypts (Hillis, 1987) and oak tannins (Masson *et al.*, 1995). It has been proposed that due to polymerization and oxidation, extractives in the inner heartwood become progressively insoluble (Peng *et al.*, 1991). Alternatively, hydrolysis of ellagitannins may also occur as heartwood ages (Masson *et al.*, 1995).

In *E. nitens* there was little evidence of qualitative differences between reaction zone and heartwood extracts when examined by LC-MS (Fig. 5.3). In some other trees, qualitative differences have been found as heartwood ages. For example, vescalagin predominates in the heartwood transition zone in pedunculate and sessile oak but not in the inner heartwood, which may relate to its sensitivity to oxidation compared to other tannins (Masson *et al.*, 1995).

E. nitens reaction zone extracts have displayed antifungal behaviour to a sensitive test fungus (*Cladosporium cladosporioides*) and native Tasmanian wood-decay fungus (isolate D) in TLC bioassays. The compound/s responsible for this were not discernible with current methods. Given the amount of total phenols in the heartwood compared to the sapwood and reaction zone extracts (ie. between these tissue), heartwood extracts would be less likely to inhibit fungal growth per weight of wood extracted than the reaction zone, but more likely than sapwood. Further bioassay studies with *E. nitens* extracts would be advanced by obtaining large purified amounts of the tannins present (which are not obtainable commercially). These could be obtained from heartwood material, which is ubiquitous and much more abundant than reaction zone material.

Results with *E. nitens* extracts showed that pedunculagin was not antifungal in the bioassays, which agrees with previous bioassays using *Penicillium waksmanii* (Hillis and Yazaki, 1973). However, antifungal activity (to decay fungus *Poria monticola*) of some hydrolyzable tannins has been demonstrated in past studies, including ellagitannin D-6 and D-12 (Hart and Hillis, 1972). While some tannins may display fungitoxic properties in bioassay, tannins may operate as effective antimicrobial agents *in vivo* through different mechanisms, which do not operate in the artificial bioassay environment. For example, the ability of tannins to bind specific proteins may be inhibitory for fungal growth *in vivo*, where resources (and hence enzymes for metabolism) will be different from those provided in the bioassay.

As well as protein-binding inhibition mechanisms, tannins have been revealed as antioxidants with the capacity to halt radical reactions (Okamura *et al.*, 1993; Hagerman *et al.*, 1998) such as those involved in lignin degradation (Pearce *et al.*, 1997). Current theories of heartwood extractives propose that they can have two functions, as fungicidal and antioxidant agents (Schultz and Nicholas, 2000), and this would also apply to the reaction zone. Preliminary studies of *E. nitens* wood tissues by electron spin resonance (ESR) spectroscopy revealed little difference between free radical signals of healthy sapwood and reaction zone (Appendix 5.3). This may be because the antioxidant tannins are short-lived and readily consumed. This is in contrast to dramatic increases in "long-lived" free radicals associated with polymeric material in reaction zones of *Acer pseudoplatanus* and other hardwood trees (Pearce

et al., 1997). This may imply that the *Acer pseudoplatanus* reaction zone is also long-lived and more static in nature compared to the *E. nitens* reaction zone.

In summary, this study has quantified a range of compounds from *E. nitens* wood extracts with a precise method (LC-MS) allowing co-eluting compounds to be discerned on the basis of molecular weight. The *E. nitens* reaction zone extracts were similar in nature to the heartwood, but generally of greater quantity. The reaction zone extracts are inhibitory to fungi in *in-vitro* bioassay conditions, but the compound/s responsible for this have not been elucidated as yet. It is likely that this activity was due to a number of hydrolyzable tannins.

APPENDIX 5.1

Quantification of hydrolyzable tannins

The stages for quantification of selected compounds by LC-MS were as follows:

1. All ions produced by the MS process were determined for each compound of interest (to exclude ions from "overlapping" compounds). This included the molecular ions and isotopes (underlined), daughter ions and doubly charged molecular ions, and all ions are shown in Table A.5.1.1. As actual ion mass varied slightly across a peak (and from run to run), ions were monitored in a range. Retention times have already been reported (Chapter 4: Table 4.1).

Table A.5.1.1. Ions selected for quantification of compounds.

Compound	Ion ranges monitored
Pedunculagin	782.5-784.5, 431.6-433.0, 476.0-479.0, 803.5-806.0, 390.0-392.6, 863.7-866.0, 804.5-805.5
Tellimagrandin 1	784.5-785.5, 391.8-392.8, 882.1-883.1
Tetragalloylglucose	786.5-788.5, 392.0-394.5, 867.0-870.0
Pentagalloylglucose	938.5-939.5, 468.9-469.9, 517.6-518.6
Catechin	288.5-289.5, 348.5-349.5, 578.0-579.5
Rutin	608.0-613.0

2. The selected ions were monitored to determine peak area (PA) of the rutin internal standard (IS) and compounds of interest (C).
3. The amount of C per sample injection in units of "μg rutin equivalent" was calculated and multiplied to account for amount of internal standard per injection (e.g. 5 μg):

   C (μg rutin equivalent) = PA_C (/) PA_{IS} (X) 5
4. Using MS response factors between rutin and the compound of interest (determined from standards weighed on a six-point balance), units of rutin equivalents were converted to relevant units.
5. Figures were adjusted according to the purity of the standards. Purity was determined by MS runs of individual standards (Table A.5.1.2). Impurities in some standards contributed to the total amount of compound present, which was important where mixed standards were used (Table A.5.1.2).

Table A.5.1.2. Impurities contributing to amounts in a mixed sample.

	Proportion of main compound in standard (% of total ions detected)				
	Pedunculagin	Penta-GG	Tetra-GG	Tellimag. I	Tellimag. II
	79.17	81.86	86.01	67.51	67.23
Containing impurities					
Pedunculagin	NA	-	-	-	-
Penta-GG	-	NA	6.06	-	-
Tetra-GG	-	-	NA	-	-
Tellimag. I	-	13.06	-	NA	-
Tellimag. II	-	-	6.49	11.97	NA
Total in mix	79.17	81.86	98.57	79.48	67.23

6. Concentrations were then determined by considering amount of wood material extracted per sample injection.

APPENDIX 5.2

Thin layer chromatography bioassays

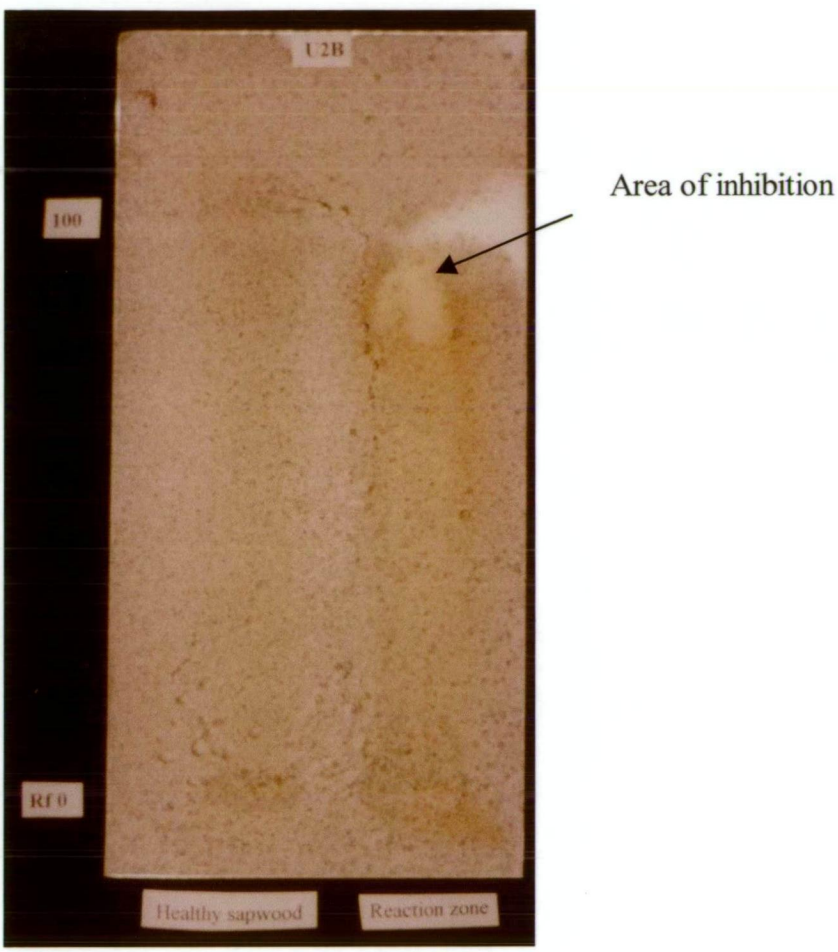


Figure A.5.2.1. TLC bioassay of 70% acetone wood extracts (U2B), healthy sapwood (left) and reaction zone (right).

APPENDIX 5.3

Detection of free radicals by electron spin resonance (ESR) spectroscopy

Small pieces (ca. $2mm^3$) of fresh wood tissue from naturally-infected, pruned, plantation-grown trees were excised from various tissues. These were transferred to 3 mm diameter ESR tubes. ESR spectra were acquired at 298 °K using a Bruker ESP 300 continuous wave X-band spectrometer equipped with an ER 4105 DR double rectangular X-band resonator, operating in the TE_{104} mode (Bruker Analytische Messtechnik GMBH, Karlruhe, Germany). Sweep width was 800 gauss, with a centre field of 3492 gauss. The modulation frequency was 100 KHz and modulation amplitude was 4 gauss. Free radical signals are detectable at ca. 3450 gauss. Manganese is also detectable on ESR spectra at this gauss range, as a series of 6 peaks.

The following figures (A.5.3.1a-e) provide preliminary data for various tissues, as recorded above the spectra. Generally, small free radical signals were detected from healthy sapwood and heartwood (Fig. A.5.3.1a&b) but there was no evidence of a signal from the decayed tissue (Fig. A.5.3.1c) or reaction zone samples (Fig. A.5.3.1d&e). Slightly greater levels of manganese were detected in the reaction zone spectra, which may suppresses detection of the free radical signal.

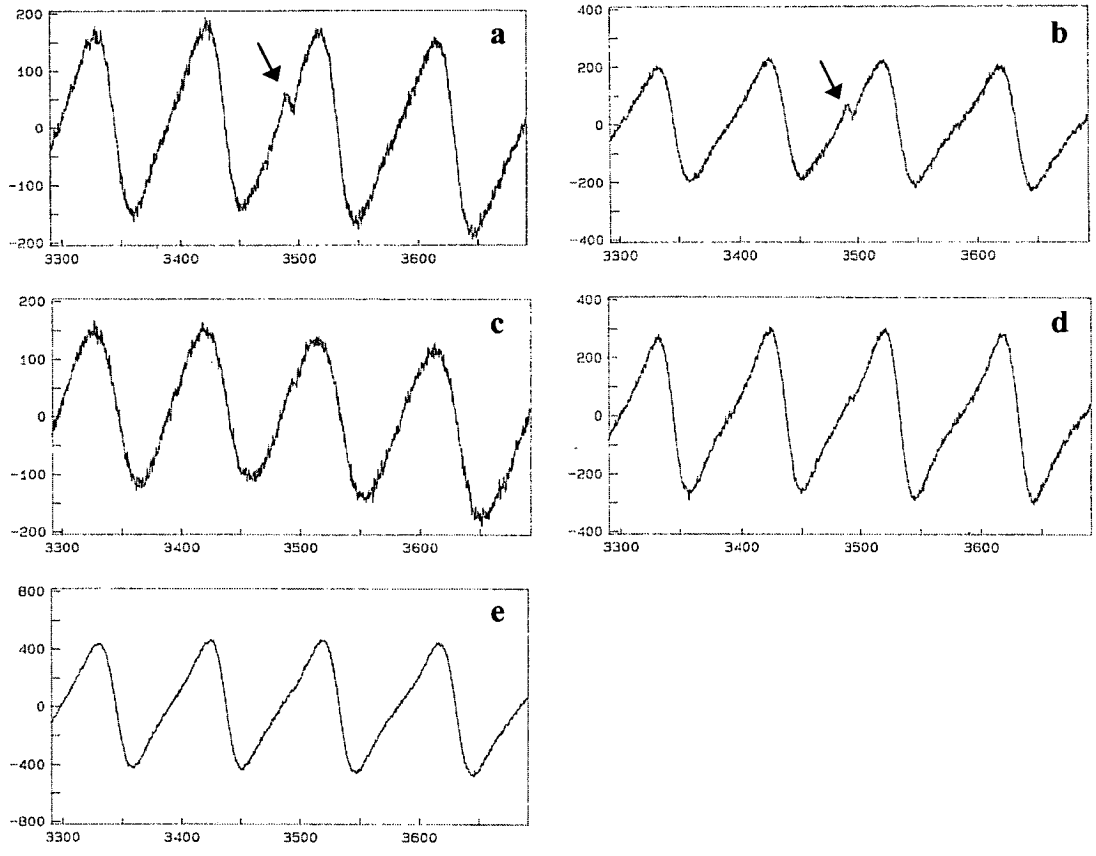


Figure A.5.3.1a-e. ESR spectra of wood tissues; a) healthy sapwood, b) healthy heartwood, c) decayed wood, d) reaction zone, replicate 1, e) reaction zone, replicate 2. Arrows indicate the free radical peak.

CHAPTER 6

EFFECT OF SEASON AND DIFFERENT FUNGI ON PHENOLICS IN RESPONSE TO XYLEM WOUNDING AND INOCULATION IN *EUCALYPTUS NITENS*

6.1 INTRODUCTION

Eucalyptus nitens (Maiden) is an important plantation-tree species being grown for solid wood products in Tasmania. As pruning is required, this is done early (beginning at age 3-4 years) to decrease the proportion of the knotty core. It is hoped that decay arising from the wounds will be restricted to the knotty core. Compartmentalization in woody xylem involves both reaction zones, and a barrier zone which is formed by the cambium at the time of wounding (Shigo and Marx, 1977; Pearce, 1996). Barrier zones are considered more resistant to fungal spread than reaction zones (Shigo and Marx, 1977; Bauch *et al.*, 1980) but are usually limited in extent above and below a wound. For decay columns spreading well beyond a wound, the reaction zone is likely to be more important long-term.

Wounds present the opportunity for a wide array of microorganisms to colonize compromised plant tissue, which has been termed "unspecialized opportunism" (Rayner and Boddy, 1988). The ability of a fungus to invade beyond this compromised tissue may depend upon its tolerance of the inherent nature of the wood environment (Shigo, 1974; Gramss, 1992) and whether a defence response is elicited. There is evidence that different degrees of reaction zone development occur when woody xylem is challenged with fungi of varying aggression (Pearce *et al.*, 1994; Pearce, 2000). In *Acer pseudoplatanus* challenge of xylem with weakly aggressive fungi such as *Ustulina deusta* or *Ganoderma adspersum* resulted in rapid expression of defence responses including phytoalexin-like coumarins, metal ions and water accumulation (Pearce, 2000). In contrast, there was no evidence of these defence responses occurring in response to the invasive fungus *Chondrostereum purpureum*.

The season in which wounds are created is an important variable influencing initial host-pathogen interactions. Differences in rainfall and temperature may influence the hydraulic status of xylem (rendering it more or less compromised by wounding) and other factors such as rate of phenol biosynthesis. Factors affecting fungal infection could include seasonal differences in sporulation and dissemination for various fungi (Gadgil and Bawden, 1981) as well as factors affecting subsequent growth rate.

In Tasmanian *E. nitens* plantations, pruned in different seasons and examined after 12 months, Mohammed *et al.* (2000) found a slightly increased number of decay outbreaks into sapwood in spring and summer. Decay columns were more extensive following pruning in summer and autumn. In wounded *Eucalyptus regnans* also in Tasmania, spring and summer wounds had significantly greater defect volume than autumn wounds during the first 6 months (White and Kile, 1993). This seasonal influence was no longer apparent after 12 and 24 months. Therefore it may be expected that differences in defence responses are also likely to be most distinguishable at the early stages following wounding. Mireku and Wilkes (1989) found that extent of infected tissue resulting from spring and summer stem wounds in *Eucalyptus maculata* was less than for autumn and winter wounds. In summer, higher phenol production was detected and wounds were sealed with kino, which is likely to have resulted in less infection.

In previous challenge experiments with wounded *E. nitens*, *Ganoderma adspersum* proved to be weakly aggressive and elicited increased levels of total phenols up to 6-fold (Chapter 3). In the present study we used a species of this genus (*Ganoderma applanatum*, which is present in Tasmania) for further inoculation studies to determine the effect of season on defence response. To determine how the defence response is affected by challenge of different fungi, a variety of fungi were used. These included some cosmopolitan decay species (*Ganoderma applanatum* and *Phellinus robustus*) and non-decay species (*Botrytis cinerea*). In addition, fungi associated with decay of pruned plantation-grown *E. nitens* were isolated, including an *Aleurodiscus* spp. and two species which remain unidentified. Decay-causing saprot fungi have been poorly studied in Tasmanian eucalypts (Kile and Johnson, 2000; T. Wardlaw, pers. comm.) and therefore selection of challenge fungi was restricted. Studies of fungi entering pruning wounds of *Eucalyptus delegatensis* in New Zealand have revealed that *Stereum purpureum* (= *Chondrostereum purpureum*)

was the most commonly isolated single species capable of causing decay (Gadgil and Bawden, 1981). This species has not been recorded in Tasmania (Anon., 2000) but may be common.

This paper presents results from three experiments. The hypothesis proposed in the first two experiments was that a more aggressive fungus may not elicit as much phenol production. Experiment number 1 involved pot-grown *E. nitens* which were wounded and inoculated with 4 different decay fungi (and an uninoculated treatment) in spring and analysed after 6 weeks. Experiment number 2 involved wounding young plantation-grown *E. nitens* in autumn and inoculating with 3 different fungi, with assessment after 6 months. In a third experiment (also with young plantation-grown *E. nitens*) the effect of season on defence response was evaluated. As well as total phenol levels, some key phenols (mainly hydrolyzable tannins) were quantified by liquid chromatography – mass spectrometry (LC-MS).

6.2 MATERIALS AND METHODS

6.2.1 Plant material

Experiment 1. Pot-grown 22-month-old *E. nitens* trees were used. Seedlings were raised in a glasshouse in seed raising mix and then transferred to tubes (4 cm diameter) containing commercial potting mix (80% composted bark, 20% coarse sand) supplemented with slow-release fertilizer (Osmocote). Seedlings were then transferred to 17 cm pots and placed outdoors under shade cloth. At approximately 16 months of age, they were transferred to 25 cm diameter pots, and placed in full sun. Pots were watered by a drip-irrigation system (up to 4 times a day in summer). Additional liquid fertilizer (Aquasol) was also added in the growing seasons. The 6-week experiment was conducted in spring (October - mid-November) 1999. During this experimental period, average daily minimum temperatures were 9.22 °C while maximums were 17.86 °C (based on Hobart data, Bureau of Meteorology, Hobart).

Experiment 2. Young *E. nitens* trees grown in a plantation in southern Tasmania were used. This site was a Forestry Tasmania coupe (Arve 26 E) at 43°08' S latitude, 146°49' E longitude and 230 m altitude, ca. 10 kilometers from Geeveston. Trees were of Toorongo provenance and were planted in December 1996. The experiments

were begun in April 1999 (autumn) when the trees were ca. 3 years old. Trees were selected for uniformity as a means of controlling micro-environmental and genetic differences. Trees of ca. 3 m height, without showing phase change to adult foliage, were selected. Treatments within the experiment were then distributed randomly. Trees were selected over an area of ca. 100 x 100 m. During the 6 month experimental period average daily minimum temperatures were 4.35 °C, maximums were 14.56 and average daily rainfall was 1.63 mm (based on Geeveston data, Bureau of Meteorology, Hobart).

Experiment 3. Plant material used was as for experiment 2. Meteorological data for Geeveston during each experimental season period is shown in Fig. 6.1.

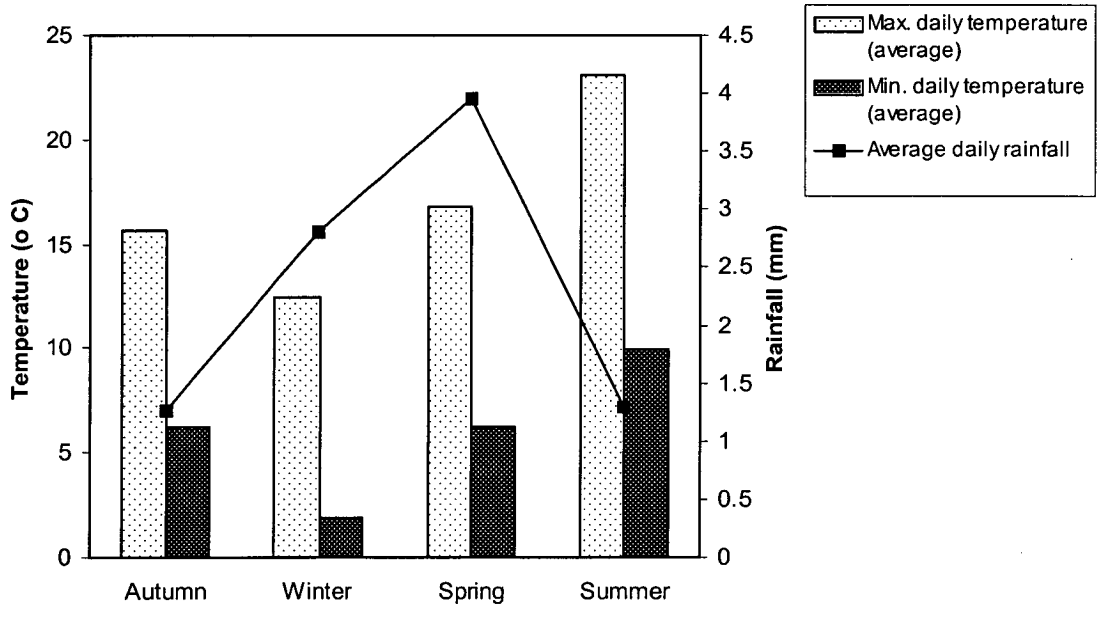


Figure 6.1. Meteorological data (temperature and rainfall) for Geeveston over the seasonal periods (1-month in the middle of each season) for experiment 3.

6.2.2 Fungal material

Ganoderma applanatum cultures were isolated from a fruit-body, growing on a *Nothofagus cunninghamii* in South-West Tasmania. A fruit-body of *Phellinus robustus* (growing on *Eucalyptus amygdalina*) was collected from Molesworth, southern Tasmania and cultures were isolated. A culture of *Botrytis cinerea* was obtained from a culture collection (University of Birmingham, UK). A species of

Aleurodiscus was isolated from sapwood of a dead 20-year-old *E. nitens* tree at a species-trial (Calder, North-West Tasmania). The white "tiled" hymenial layer was characteristic of *Aleurodiscus* and upon sectioning it was tentatively identified as *A. botryosus,* based on the presence of botryose acanthophyses (Cunningham, 1963). This has been illustrated in Appendix 6.1. This fungus has not been previously recorded in Tasmania (Anon., 2000).

Two fungi were isolated from white-rot decay columns of a naturally-infected pruned *E. nitens* tree (8-years-old) at Evercreech (site 29C, altitude 670 m, latitude 41°22'S, longitude 147°56' E). Isolations from the edge of a decay column (challenging the sapwood) resulted in one isolate type, labeled isolate D. Isolations from adjacent reaction zone tissue also resulted in only one isolate type which was labeled isolate R. The classification of both isolates as basidiomycetes was confirmed as follows. Isolate D had abundant clamp connections and while no clamp connections were observed for isolate R, both isolates grew on a selective media for basidiomycetes (described below) and produced peroxidase and laccase (enzymes frequently associated with white-rot fungi). Enzyme assays were performed by drop-tests on the margin of the fungal cultures (Stalpers, 1978). Isolate D has been found at a range of sites associated with large decay columns resulting from pruning wounds (K. Harrison, M.H. Hall and C.L. Mohammed, unpublished).

All fungi were maintained on 3% malt agar in the dark at 20°C. All fungi were characterized by growth rate, cultural morphology, hyphal structures (presence and type of clamps) and enzyme production. This information was required for accurate comparison of the fungi when re-isolated.

6.2.3 Wounding and inoculation

Experiment 1. For the pot-grown trees, single wounds were created at approximately 30 cm up the stem. Wounds were 18 mm long, 6 mm wide and 3-4 mm deep and were created with a sterile chisel. Rectangular pieces of fungal agar culture (or sterile agar) were adpressed to the wound and sealed with parafilm, followed by PVC tape. The five treatments included four different fungi on malt agar (*Ganoderma applanatum*, *Aleurodiscus* spp., isolate D and isolate R) and sterile malt agar. Five trees per treatment were wounded and inoculated.

Experiment 2. The fifteen plantation-grown trees were subjected to two inoculated wounds per tree. Wounds were larger than experiment 1, being 24 mm long, 12 mm wide and 10 mm deep. Both wounds were between 50-100 cm above ground level at least 10 cm apart and on opposite sides of the stem. There were three inoculation treatments included *G. applanatum*, *P. robusta* and *B. cinerea*. Five trees were wounded per treatment in mid-April 1999. Inoculation procedure was as for experiment 1. Six months after wounding and inoculation trees were harvested. All 30 wounds were assessed for lesion morphology and re-isolation of the challenge fungus, but only wound 1 was utilized for phenol extraction. Wound 2 was reserved for anatomical and histochemical studies.

Experiment 3. A total of twenty trees were wounded, divided between each of the four seasons. Wound size and inoculation procedure was as for experiment 2. The dates of inoculation were 12th April 1999 (autumn), 12th July 1999 (winter), 11th October 1999 (spring) and 10th January 2000 (summer). For each of five trees one inoculated wound per tree was made between 50-100 cm above ground level. All wounds were inoculated with *G. applanatum* and trees were harvested after one month.

6.2.4 Analysis of inoculated wounds

At the completion of each experimental period, the stem segment containing the wound was cut from the tree and analysed. Using a sterile chisel, stem segments were split axially through the centre of the wound. Extent of lesion development and discolouration was recorded by a tracing. The area of the lesion was later determined by making an enlarged copy of the tracing. The area of the lesion was carefully cut out and weighed to 1.0 mg. This was compared to a standard area of known size.

6.2.5 Re-isolation of fungi

For experiments 1 & 2, half of each stem segment was immediately transferred to a laminar flow cabinet and wood-chips were removed from areas of apparently infected tissue both above and below the wound. A small number of chips (3-4) were placed on both 1% malt agar selective for basidiomycetes and 3% malt agar. The

selective agar contained 50 ppm penicillin, 50 ppm streptomycin, 25 ppm polymixin and 230 ppm thiabendazole. Cultures were placed at 20°C in the dark. The number of wood-chips with typical decay fungi characteristics (as opposed to bacteria or other fungi) was recorded after 1 week. Mycelial growth which did not appear to be due to contamination (ie. non-sporulating white mycelium), was sub-cultured from wood-chips on both media onto 3% malt agar. Parent cultures were also sub-cultured for comparison. Growth of sub-cultures was observed and recorded after 1 week and then again after 6 weeks. The number of wood chips from which the challenge fungus was successfully isolated was determined and expressed as a percentage. In experiment 2, a range of isolates were obtained which were not identified as the inoculated fungi. These were semi-characterised by techniques described above including enzyme tests and microscopy. This provided preliminary information about common wound-infecting microorganisms, but was not a goal of this study.

6.2.6 Anatomy and histochemistry

For experiments 1 & 2, wood blocks were prepared from selected stems of each treatment and sectioned while fresh with a sliding microtome. Both transverse and radial longitudinal sections were prepared (10-50 μm thick). Sections were stained with 1% toluidine blue in 0.2 M phosphate buffer (pH 6.5) for general wood anatomy and to discriminate fungal hyphae. An aqueous solution of 0.5 % fast blue RR salt (FBRRS, Sigma Chemical Co.) was used to detect phenolics which stained a red colour.

6.2.7 Extraction of phenolics

For all experiments, wood shavings (ca. 0.5 mm thick) were prepared with a small chisel from an area of about 1 cm above and below the decay lesion, beyond the band of discolouration at the decay column "interface". Healthy sapwood was sampled on the opposite side of the stem to the wound, and approximately 5 cm below the wound. The fresh weight of the shavings (ca. 30 – 90 mg) was determined and then extracted in 750 μl of 70% acetone for 24 hours at 4 °C in the dark, followed by a second repeat extraction. Extracts were pooled (making 1.5 ml) and stored at –20 °C in the dark. Extracts were centrifuged before analysis. The extracted shavings were dried at 85 °C for 3 days and dry weight determined.

6.2.8 Determination of total phenols

Total phenols were estimated by the Folin-Ciocalteu method, adapted from Bonello and Pearce (1993). Either 50 or 20 µl of extract was diluted to 3000 µl water. To this, 1500 µl of 1:1 diluted Folin and Ciocalteu's reagent (Sigma Chemical Co., Sydney) was added and left for 3 minutes, followed by adding 1500 µl of 1 M aqueous Na_2CO_3. The solution was shaken and left to react for 1 hour. The absorbance was measured at 725 nm with a spectrophotometer. Concentrations of total phenols were calculated with reference to a gallic acid standard curve (10-200 µg/ml dissolved in acetone) by applying regression analysis and results were expressed as gallic acid equivalent (µg) per mg extracted dry weight (DW) of wood.

6.2.9 Analysis of phenols by LC-MS

Internal standard. As an internal standard, rutin (Sigma Chemical Co.) was prepared in pyridine (5 mg/ml) and added to each extract prior to analysis.

LC-MS system. Samples were analysed by LC-MS (sample chamber maintained at 5 °C) by the following system. HPLC separations were carried out on a Waters Alliance 2690 using a Waters Nova Pak C18 column (150 mm x 3.9 mm). The mobile phase consisted of water/acetic acid 98:2 (solvent A) and methanol/acetic acid 98:2 (solvent B). Initial conditions were 5 % solvent B and 95 % solvent A. The program was a linear gradient to 54% solvent B and 46% solvent A over 40 minutes with a flow rate of 0.8 ml per min, before returning to initial conditions with 12 minutes re-equilibration between samples.

Mass spectrometry was carried out on a Finnigan LCQ (San Jose, USA) with an electrospray ion source, using LCQ Navigator Version 1.2 software. The instrument was operated in negative ion mode, scanning from m/z 125 to 1500 with an AGC target value of $2x10^7$ and maximum ion injection time of 100 ms. Operating conditions were as follows: sheath gas 90 psi, aux gas 50 psi, ESI needle voltage 4.5 kV, capillary temperature 270 °C and capillary voltage –30 V. Data-dependent MS-MS spectra were routinely acquired from the most intense ion in the spectrum, with a default collision energy of 30 % and a peak isolation width of 3 amu.

Monitoring and quantification. Methods for quantifying five compounds (pedunculagin, tellimagrandin 1, tetragalloylglucose, pentagalloylglucose and catechin) were as for Chapter 5.

6.2.10 Statistical analysis

For all data sets, a Fishers t-test was applied using SAS software version 6.11 (SAS Institute; Cary, NC, USA). All statistics were assessed at the 5% probability level. Where 2-factor tests were used, significant P values for factor interactions were analysed.

6.3 RESULTS

6.3.1 Re-isolation of fungi

Re-isolation of challenge fungi from experiment 1 (the pot-grown trees) was relatively successful. From the more extensive decay lesions (isolate D and *Aleurodiscus* spp.), challenge fungi were successfully re-isolated from each wound. Overall isolation success for these fungi was 71% and 44% of wood-chips respectively. For all unsuccessful isolations from wounds inoculated with isolate D, no microorganism being isolated. In contrast, "contaminant" microorganisms were isolated frequently from wood-chips of wounds inoculated with *Aleurodiscus* spp. For wounds inoculated with isolate R and *G. applanatum*, the challenge fungi were re-isolated from four and two of each five wounds respectively. The proportion of "contaminant" microorganisms was high from all of these wounds. From wounds inoculated with sterile agar, "contaminant" microorganisms were isolated from each lesion and were dominated by *Penicillium* spp.

For experiment 2 (plantation-grown trees) the challenge fungi were not re-isolated from decay columns (except for two isolates of *B. cinerea*). Of twenty-five sub-cultured isolates, twelve had white cord-forming mycelium similar morphologically to xylariaceous fungi (ascomycetes). None of these isolates grew on the agar selective for basidiomycetes or had clamps connections. For three of these isolates, peroxidase was detected and in two of these laccase was also detected. Three isolates

(one of which produced peroxidase and laccase) produced a patchy black colouration on the reverse. Two of the twenty-five isolates were basidiomycetes as clamp connections were observed, they grew on the selective agar, and produced peroxidase, laccase and tyrosinase. The other eleven isolates were varied in character and further identification was not attempted.

6.3.2 Morphology of lesions and response

For experiment 1 (pot-grown trees analysed after 6 weeks) lesion area was significantly greater for wounds challenged with isolate D (P= 0.0026) (Fig 6.2). There were no significant differences between lesion area for the un-inoculated wound and wounds inoculated with *G. applanatum*, *Aleurodiscus* spp. and isolate R. Wounds inoculated with sterile agar did not appear to remain sterile as some fungi were isolated, however this treatment was associated with the lowest average lesion area.

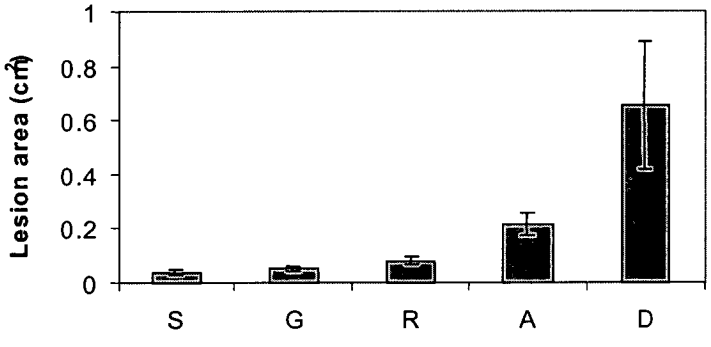


Figure 6.2. Lesion area (cm^2 ± SE) 6 weeks after wounding and inoculation with a variety of treatments (S = sterile, G = *G. applanatum*, R = isolate R, A = *Aleurodiscus* spp., D = isolate D).

Regions of defence beyond the decay columns varied substantially in colour and intensity. Discolouration at the interface of the decay lesion was previously found associated with intense phenolic accumulation (Chapter 3) but phenols were also induced beyond this zone. Discolouration was most prominent at the boundary of decay lesions of isolate D and *Aleurodiscus* spp., being slightly purple in colour for more extensive decay columns (Fig. 6.3). Discolouration was brown/orange for wounds inoculated with *G. applanatum* (Fig. 6.4) and typically had a water-soaked appearance. There was little discolouration associated with wounds inoculated with sterile agar and isolate R. Induction of phenolics both at the interface of the decay

column and beyond are considered part of the defence process. Formation of callus was substantially greater in wounds inoculated with sterile agar, isolate R and *G. applanatum*, while in most cases it did not form around wounds inoculated with isolate D and *Aleurodiscus* spp.

For experiment 2 (after 6 months) decay columns were extensive (Table 6.1). As treatment affects were not realized (that is challenge fungi were not re-isolated) examination of results on this basis would be superficial. However, results could be interpreted on the basis of observed categorical differences in response between wounds. That is, in some cases distinct purple zones were observed beyond the brown interface (13 of 30 wounds). It was apparent that purple zones were associated with larger lesions (Fig. 6.5). When results were analysed on this basis, average lesion area was 6-fold greater (Table 6.1) which was statistically significant (P = 0.0004). Purple colouration was commonly found in the position of the barrier zone (Fig. 6.5) and beyond this to wood formed after wounding.

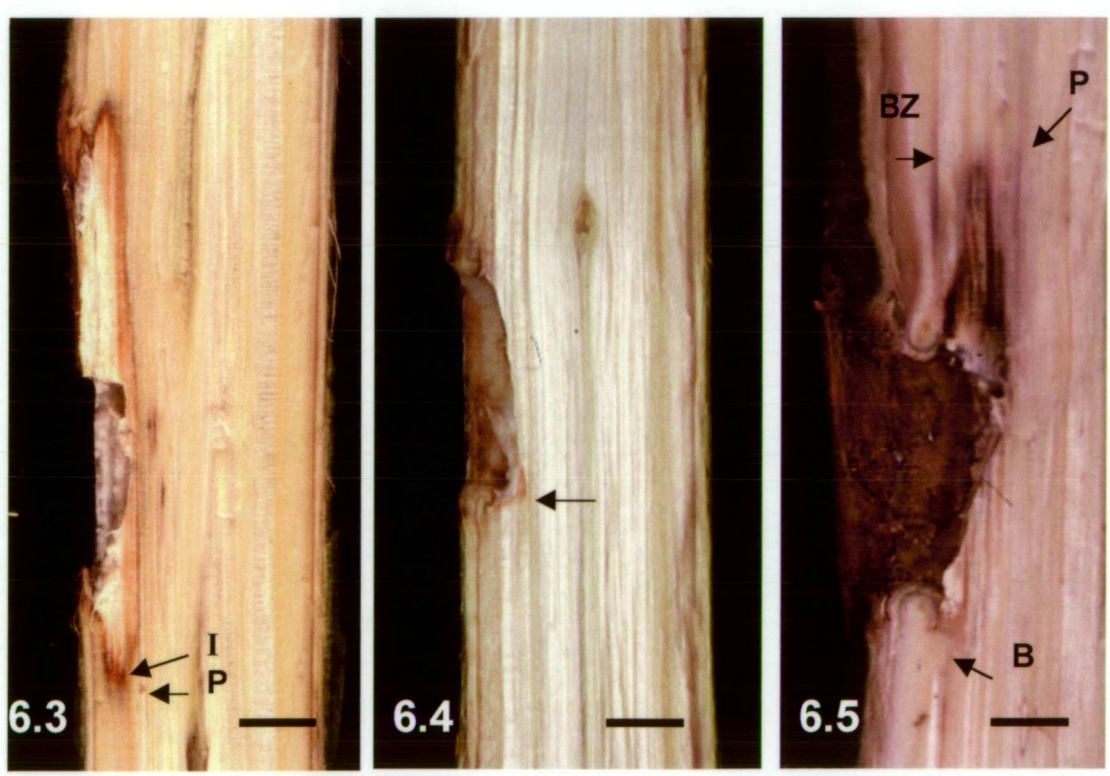


Figure 6.3-6.5.
6. 3. Decay lesion caused by isolate D, with brown discoloured interface (I) and purple zone (P) beyond, both above and below the infection. Scale bar = 10 mm. **6.4.** Area of orange/brown discolouration below a wound inoculated with *G. applanatum*. Scale bar = 8 mm. **6.5.** Decay lesions from a wound of experiment 2. Above the wound the lesion is associated with distinct purple reaction zones (P), but below the tissue is a pale brown (B). The barrier zone (BZ) is also associated with purple discolouration. Scale bar = 10 mm.

Table 1. Average lesion area (cm^2 ± SE) for experiment 2 classed by type of reaction zone formed.

Decay column	Average lesion area (cm^2 ± SE)	
Total	1.24	(0.29)
Associated with purple zone	2.34	(0.62)
Associated with brown/no RZ	0.39	(0.07)

For experiment 3, the lesion area one month after wounding and inoculation was small and did not alter significantly (P = 0.0871) between season (Fig 6.6). However, an increase in lesion size for summer was apparent, with lesion size being the least in winter.

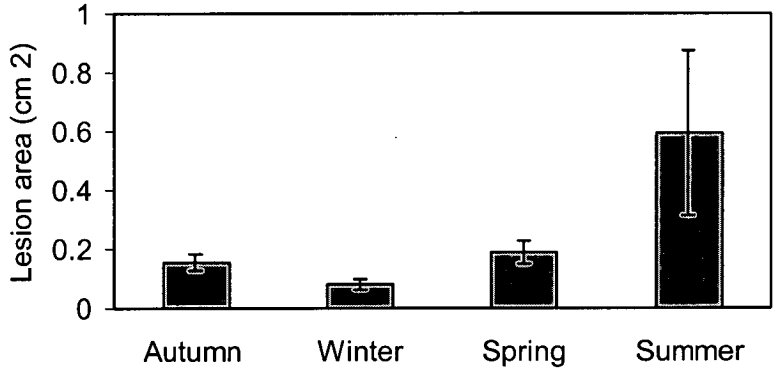


Figure 6.6. Lesion area (cm^2 ± SE) one month after wounding and inoculation with *G. applanatum* in each season for experiment 3.

6.3.3 Anatomy and histochemistry of xylem

Sections of xylem from a selection of the pot-grown trees (experiment 1) were examined for hyphal presence. For those fungi with clamp connections, these structures were regularly observed on hyphae within the various xylem cells. Hyphae invaded both vessels, fibres and parenchyma to the margins of the decay column but were not seen beyond.

Sections were also prepared from the material of experiment 2. The boundary of infection (interface) was associated with heavy brown deposits staining slightly red

with FBRRS (Fig 6.7, left). Beyond this interface, ray parenchyma and vessel tyloses of the purple and brown zones stained light red with FBRRS (Fig. 6.7, right).

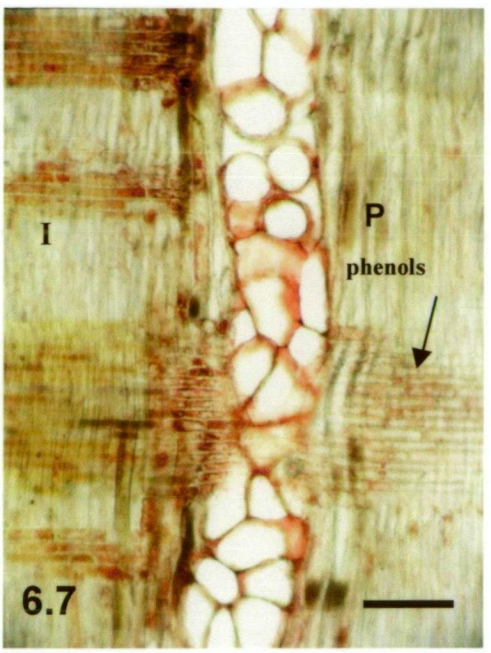


Figure 6.7. Radial longitudinal section (30μm) stained with FBRRS for phenol detection. On the left of the vessel is discoloured interface (I) and on the right is the purple region (P). Scale bar = 100 μm.

6.3.4 Phenolics

For experiment 1, there was a marked affect of different challenge fungi on the accumulation of phenolics adjacent to the lesion (Fig. 6.8). A trend is apparent from both the total phenol analysis and individual compounds. Inoculation with isolate D and *Aleurodiscus* spp. resulted in greater levels of phenols being induced than the other treatments. This trend is masked for pedunculagin by extreme variation within the *G. applanatum* treatment, and is not apparent for catechin. In this latter case catechin is the compound least increased in the reaction zone for the *Aleurodiscus* spp. inoculations, which is essentially an inverse trend to the tannins. For all compounds (except pedunculagin) there is significant interaction ($P = 0.0001$ in all cases except 0.0002 for pentagalloylglucose) between treatment and tissue type differences.

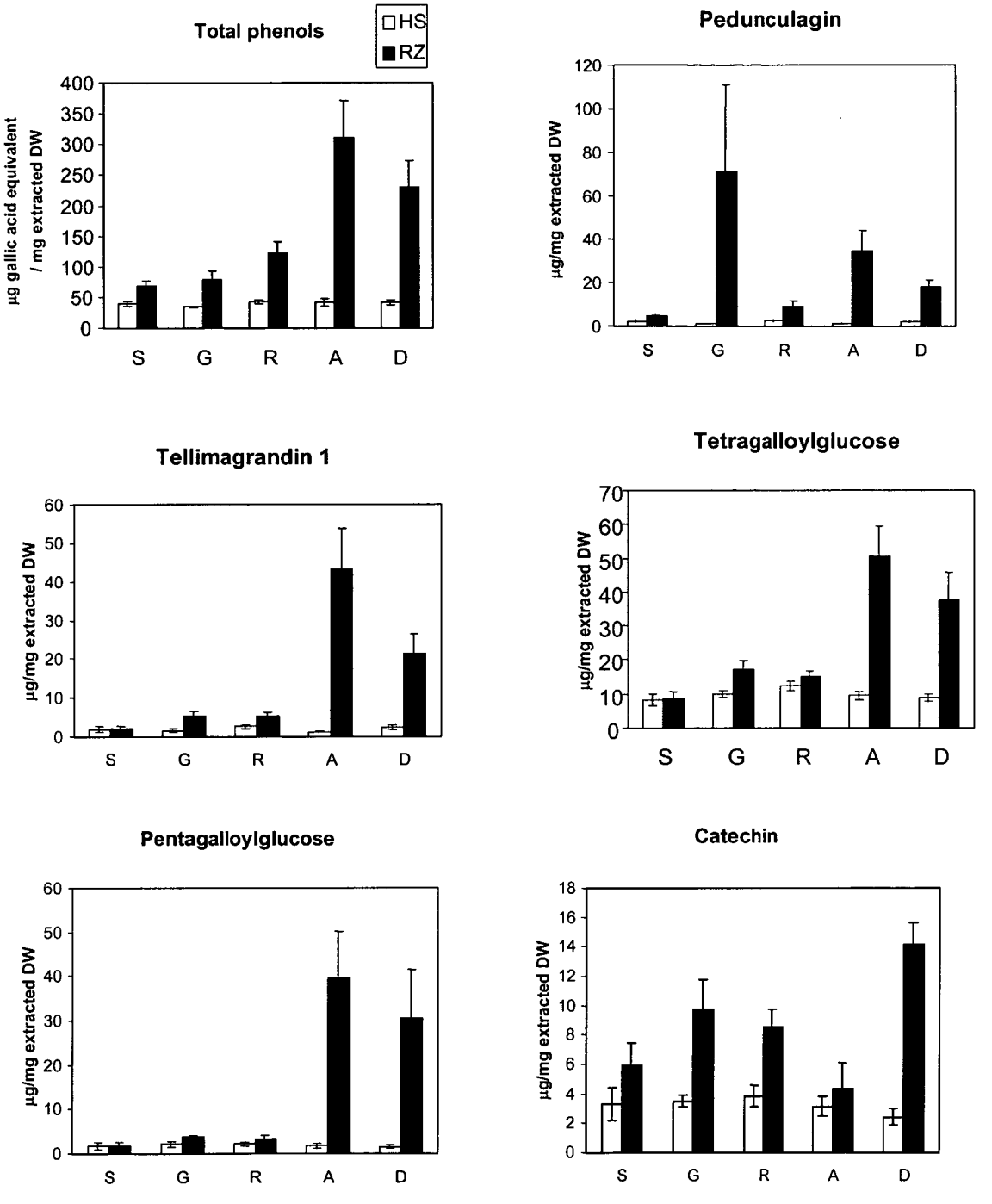


Figure 6.8. Total phenol levels (µg gallic acid equivalent / mg extracted DW) and concentration of individual tannins and catechin (µg / mg extracted DW) between healthy sapwood (HS) and reaction zone (RZ) for the pot-grown trees challenged with different fungal treatments (S = sterile, G = *G. applanatum*, R = isolate R, A = *Aleurodiscus* spp., D = isolate D).

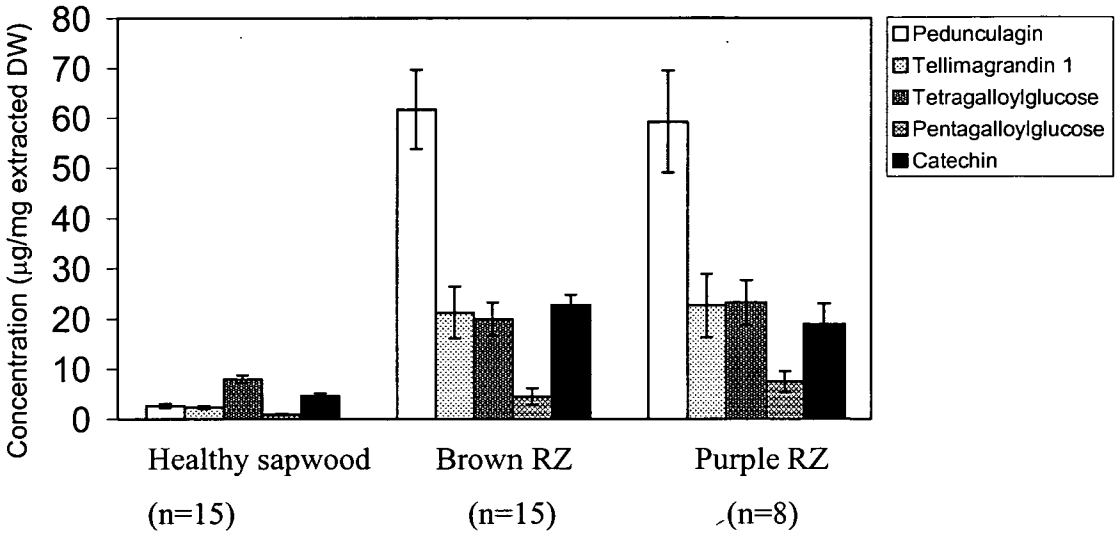


Figure 6.9. Concentration of hydrolyzable tannins and catechin (µg / mg extracted DW) for three different tissues of fifteen wounded and inoculated plantation-grown trees of experiment 2.

As treatment effects could not be concluded in experiment 2 (challenge fungi were not re-isolated) results were analysed as before on the basis of which type of reaction zone was elicited (that is whether a purple zone was present). In all 15 cases (NB. wound 1 only) the brown zone was sampled and in 8 cases a purple zone was also present. Analysis of individual compounds revealed that the concentration was extremely similar in both these tissues (Fig 6.9). Increases in pedunculagin in both tissues were highest, being 23- and 22-fold greater than healthy sapwood in the brown RZ and purple RZ respectively. Averaged mass spectra were prepared for a number of extracts to compare all compounds between the brown and purple zones (Fig. 6.10). Major qualitative differences were not apparent.

For experiment 3, total phenol levels produced in response to wounding and inoculation with *G. applanatum* were varied throughout different seasons (Fig. 6.11). Levels of tetragalloylglucose, pentagalloylglucose and tellimagrandin 1 were significantly different throughout the year in both the healthy sapwood and reaction zone ($P < 0.0001$, $P = 0.005$ and $P = 0.002$ respectively). Tetragalloylglucose was highest in the summer, pentagalloylglucose highest in winter and tellimagrandin 1 lowest in the summer.

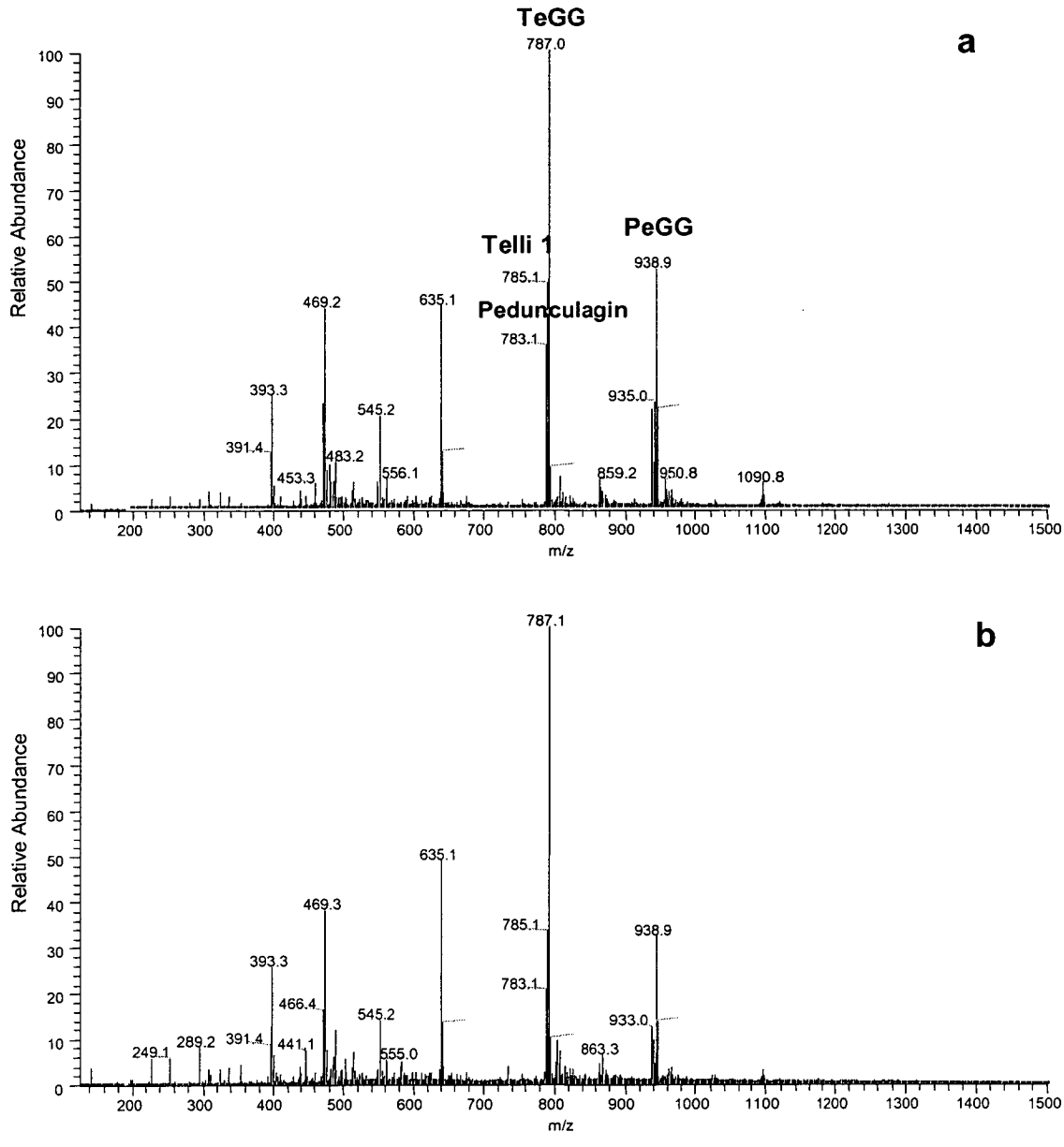


Figure 6.10. Averaged negative ion electrospray mass spectra of reaction zone extracts from experiment 2: a) Brown RZ; b) purple RZ.

The greatest increase throughout all seasons for reaction zone extracts compared to healthy sapwood was found for catechin (Fig. 6.11). This was particularly increased in response to autumn (4-fold) and summer wounding (3-fold). There was a significant interaction effect ($P = 0.004$) as well as highly significant differences between tissue types ($P<0.0001$) and season ($P = 0.0006$). There was a significant difference in pedunculagin levels between tissue types ($P = 0.02$) but no significant seasonal effect.

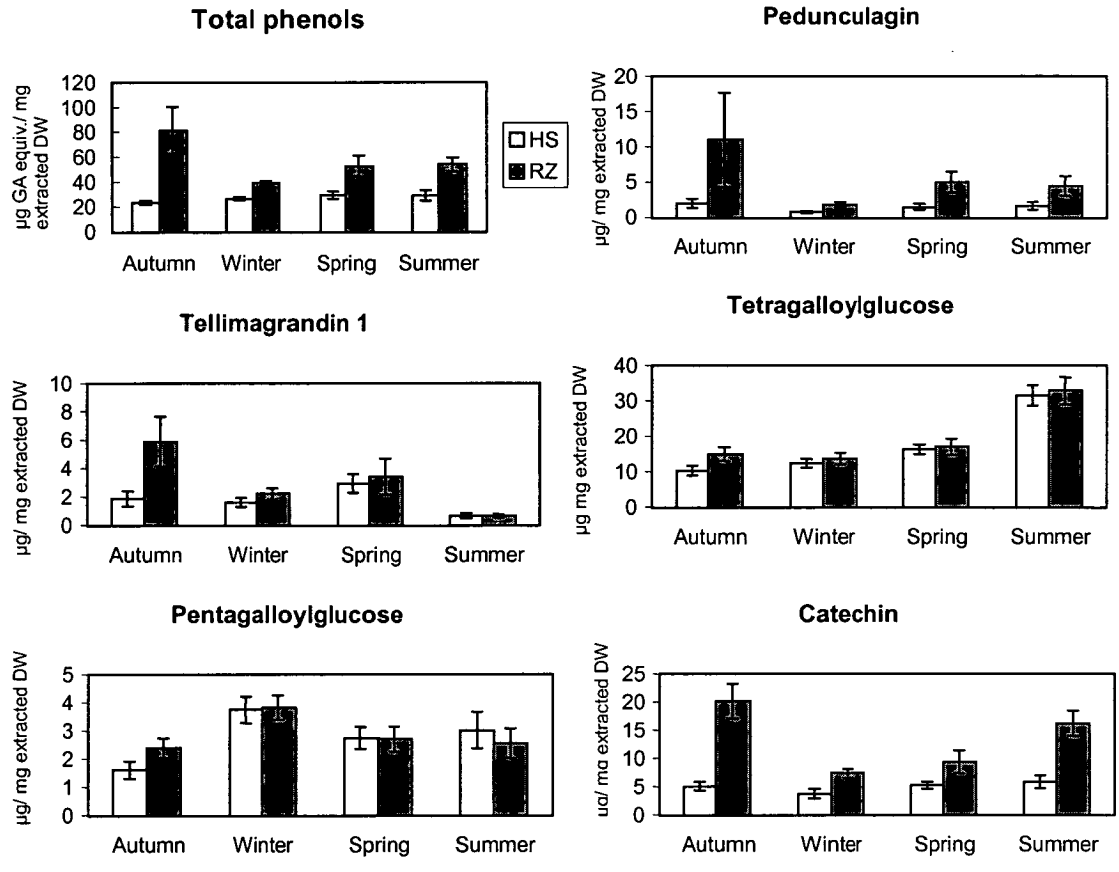


Figure 6.11. Concentration of total phenols (µg gallic acid equivalent / mg extracted DW) individual compounds (µg / mg extracted DW) in response to wounding and inoculation with *G. applanatum* in different seasons.

6.4 DISCUSSION

6.4.1 General trends in phenol accumulation

Previous studies of purple reaction zones in plantation-grown *E. nitens* (Chapter 4 and 5) have reported increased amounts of both ellagitannins and gallotannins to varying degrees, and consistent decreases in catechin levels. In the current studies of younger trees (both pot-grown and field grown) where reaction zone formation is at an earlier stage, the most noticable difference to the previous studies is an increase in catechin levels. This occurs across a range of defence zone types, including purple zones (Fig. 6.9). Increased catechin levels were also found at the heartwood transition zone of eight-year-old trees (Chapter 5) prior to decreases in older heartwood. Therefore, increased catechin levels are associated with the earlier stages of defence, and may be reduced by conversion to a variety of condensed tannins over time.

6.4.2 Effect of different fungi (experiment 1 & 2)

Studies of differential defence responses in *E. nitens* according to the challenge fungi tested showed that the least infection (Fig. 6.2) was associated with the least production of phenolics and *vice versa* (Fig. 6.8). Less phenolic production and discolouration was observed for wounds inoculated with sterile agar than other treatments. Increases in reaction zone total phenols for *E. nitens* wounds inoculated with *G. applanatum* (ca. 2-fold) were not as great as those found previously with *G. adspersum,* which were up to 6-fold (Chapter 3). Increases in total phenols for decay columns caused by isolate D and *Aleurodiscus* spp. were 8-fold and 6-fold respectively (Fig. 6.8). These two fungi appeared to be comparatively pathogenic as the cambium was killed around the wound and no callus developed. It is interesting that catechin levels are greatly increased (ca. 7-fold) in responses to isolate D, while marginally increased for *Aleurodiscus* spp. (Fig. 6.8). Catechin levels appear to behave inversely to the tannins studied.

Studies of *Acer pseudoplatanus* revealed that green reaction zone deposits were formed when wounds were inoculated with sterile agar and a range of non-aggressive fungi (Pearce *et al.*, 1994; Pearce, 2000). Lesions caused by aggressive, pathogenic fungi (*Chondrostereum purpureum*) were not associated with green deposits or other features of the reaction zone (Pearce *et al.*, 1994). The reverse trend was found in this study of *E. nitens*. If fungi of greater aggression (than those used in this study) were used to challenge *E. nitens*, a similar lack of defence responses may result. However, as both the *Aleurodiscus* spp. and isolate D appeared pathogenic, results suggest that a different system of defence operates in *E. nitens* compared to *A. pseudoplatanus*.

Results from the experiment with plantation-grown trees revealed that purple reaction zones (as observed previously in pruned plantation-grown *E. nitens*; Chapter 2 and Chapter 4) were formed in association only with extensive decay columns. The compound responsible for the purple colouration could not be determined in this study. As the purple colour is extractable with the solvents used, it may be in very low concentrations but producing a significant effect on the wood colouration.

In summary, results from this study suggest that more aggressive fungi (causing larger lesions) are able to elicit greater defence responses in *E. nitens*, represented by a range of phenolics. It is envisaged that greater phenol production is associated with more aggressive fungi because xylem cells are being continually challenged and consequently defence mechanisms are constantly elicited. This fits the model of reaction zone formation as a dynamic process (Shain, 1979). In contrast, weakly aggressive fungi are associated with poorly formed, stationary zones in *E. nitens*, as seen in other hardwoods (Pearce, 1996). There probably exists a continuum between these processes. Further studies of the major decay-causing fungi in Tasmanian plantation forests are required and this is currently being attempted.

6.4.3 Effect of season (experiment 3)

This study revealed little seasonal difference between lesion size produced by *G. applanatum* after 1 month (Fig. 6.6). Decay area was greater for summer wounds and least from winter wounds which concurs with previous studies of *E. nitens* in Tasmania (Mohammed *et al.*, 2000) and is correlated with general temperature trends (Fig. 6.1). Increased temperature may facilitate fungal growth as well as leading to greater xylem drying following wounding which would make infection more rapid. Alternatively drying of the wound may limit moisture for fungal growth.

Analysis of extractives from healthy sapwood and reaction zone tissue showed some interesting seasonal changes. For example, the greatest increase in pedunculagin, tellimagrandin I and catechin was in autumn (Fig. 6.11) when the temperature was similar to spring but having ca. 3-fold less rainfall (Fig. 6.1). Rainfall may influence a range of processes including fungal sporulation and dissemination, as well as tree physiology and biochemistry. Other seasonal factors may influence the plant-pathogen interaction. A speculative example is that in spring and summer there may have been more new xylem growth and therefore a greater proportion of young cells wounded (as in all seasons wounds were made to approximately the same depth). Masson *et al.* (1995) found the age of wood important in oak ellagitannin content (but not height or orientation). Alternatively, distribution of resources may change with varied environmental factors (Wainhouse *et al.*, 1998). As wounds would have been variably shaded from direct sun, temperature data would not relate directly to the temperature conditions experienced at each wound site.

The biosynthetic pathway between ellagitannins was first indicated by seasonal studies of deciduous leaves in *Liquidambar formosana* (Hatano *et al.*, 1986). While woody xylem is a perennial tissue, seasonal balances between compounds may also reflect biosynthetic relationships. For example, levels of pentagalloylglucose (the immediate precursor to ellagitannins) is highest in winter while the ellagitannins are reasonably low. Therefore conversion may not be occurring in winter due to low temperatures, allowing the precursor levels to increase. Conversely, tetragalloylglucose is highest in summer when tellimagrandin 1 levels are lowest.

This study has shown some difference between decay lesion development which was greater in summer (but statistically insignificant). As phenol content was not generally decreased in this time (except for tellimagrandin 1) other factors must be important for decay development, including seasonal differences in fungal sporulation. These results contrast seasonal studies of *E. maculata* (Mireku and Wilkes, 1989) who found decreased decay correlated with increased phenol production. As their study was completed in mainland Australia, seasonal changes may be different to Tasmania and it would be expected that different decay fungi were present. Defence responses and capacity probably differ within the *Eucalyptus* genus.

6.5 CONCLUSION

It is of practical relevance that phenol levels were altered by different treatments (fungal challengers or season) in *E. nitens* and is suggestive that with different sites or conditions (where fungal flora may differ) responses may also vary. Where many wounds are created during pruning, the reduced requirement for phenol production will afford greater resources for growth (Wainhouse *et al.* 1998).

APPENDIX 6.1

Characterization of *Aleurodiscus* spp.

Dead branch material of *E. nitens* was collected from a species trial site (Calder, NW Tasmania). By the morphology of the hymenial layer (Fig. A.6.1.1), the fungus was placed in the *Acanthophysium* subgenus of *Aleurodiscus* (Cunningham, 1963).

Sections of the hymenial layer were prepared by Mr. Ron Rainbow at the Royal Hobart Hospital. Bark material was fixed in a neutral buffered formalin fixative and then embedded in paraffin. Sections of ca. 10 μm were prepared. Sections were stained with toluidine blue (1%).

From anatomy of the hymenial layer, a number of features including the botryose acanthophyses (Fig. A.6.1.2) and smooth obovate spores (Fig A.6.1.3) indicate that it is *Aleurodiscus botryosus (Acanthophysium botryosus)*.

Figure A.6.1.1. White hymenial layer on an *E. nitens* branch.

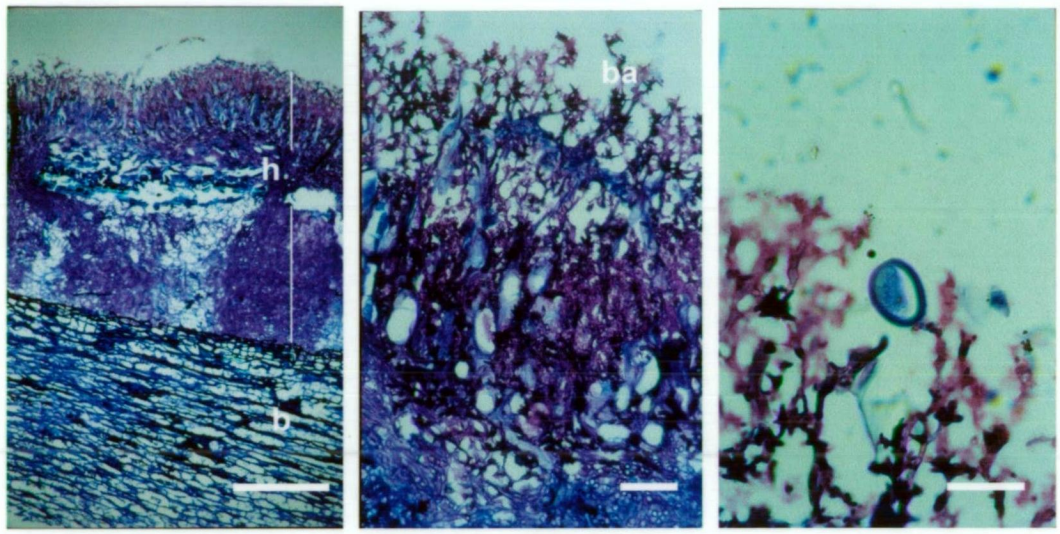


Figure A.6.1.2-4. Sections of hymenial layer stained with toluidine blue. **A.6.1.2.** Hymenial layer (h) and bark tissue (b). Scale bar = 100 μm. **A.6.1.3.** Botryose acanthophyses (ba) of the hymenial layer. Scale bar = 50 μm. **A.6.1.4.** Smooth spore arising from acanthophyses. Scale bar = 20 μm.

CHAPTER 7

GENERAL DISCUSSION

7.1 Experimental approaches

During this study of *E. nitens*, a number of aspects of xylem-fungal interactions (especially defence-related) have been investigated. In order to assess which factors were the most important in defence, a range of detailed investigations (e.g. phenolics and moisture content) were completed, as well as more preliminary studies (e.g. histochemical studies, free radicals). The formation of phenolics is a response which varies quantitatively, therefore may be a useful indicator of defensive capacity.

7.2 Characteristics of defence response

7.2.1 Water content

The moisture content of purple reaction zones in plantation-grown *E. nitens* was decreased on average compared to the healthy sapwood. There was some evidence of an increased moisture content in the reaction zone of wounded saplings by NMR imaging, however this was not substantial and did not occur as an early event. Therefore, accumulation of water does not appear to be an active defence in *E. nitens* (unlike for deciduous angiosperms such as *Acer pseudoplatanus*). Levels of moisture in the healthy sapwood were high (average of 140%) and comparable to typical levels in gymnosperms, which also have reduced reaction zone moisture levels. It may be that the inherent moisture content of the sapwood in these cases is sufficient to be inhibitory. This may also explain the lack of suberin in the reaction zone, which may have a "water-proofing" function.

In excised stems, a challenge experiment revealed that "dead" stems of similar moisture content to "living" stems were unable to inhibit fungal colonization. This experiment indicated that microenvironment alone was not effective as a defence, however the autoclaving treatment may cause ultrastructural changes (thereby actually disrupting the microenvironment).

7.2.2 Mineral content

The lack of alteration in levels of inorganic elements in the *E. nitens* reaction zone from the healthy sapwood, is in contrast to most other studies of both hardwoods and conifers where they are consistently increased. This result was also indicated by studies of discoloured wood in other *Eucalyptus* species (Wilkes, 1985). Infected, discoloured wood and reaction zones of many trees have been associated with increased mineral levels, particularly cations (Shigo and Hillis, 1973; Pearce, 1996). For example, many studies of trees in the *Acer* genus have revealed massive increases in potassium, manganese, calcium and zinc (Shortle and Shigo, 1973; Grime and Pearce, 1995). On this basis the "Shigometer" was developed as a means of detecting decay non-invasively as resistance to a pulsed electric current (Shigo and Hills, 1973). Grime and Pearce (1995) state that high manganese and zinc contents of reaction zones may be a relic of the enzymes involved in phenol polymerization, and that calcium accumulation in the decayed tissue may reflect xylem cell wall degradation.

Manganese levels are essentially unchanged in the reaction zone compared to healthy sapwood of *E. nitens* and this may afford a better resistance to phenolic de-toxification than if levels were increased. That is, Shortle *et al.* (1971) studied the effect of adding manganese to fungal bioassays, and suggested that changes in manganese concentrations in living trees could help fungi overcome the toxic effects of phenolics observed in bioassay. Levels of other elements may also effect the biochemistry of the host-pathogen interaction. For example, supplemental copper can increase the production of *C*-glycosidic ellagitannins in *Quercus alba* callus cultures (Zhentian *et al.*, 1999). In a forestry context, it is possible that sites deficient in copper may result in less efficient tannin biosynthesis.

Inorganic elements may be withdrawn from the dying reaction zone tissues in a similar manner to heartwood formation. Study of a sample of *E. nitens* reaction zone by proton induced X-ray emission analysis revealed that levels of a range of cations were extremely reduced in the reaction zone (more so than determined by acid digestion and flame photometry). However, this was not supported by PIXE mapping analysis, which showed little change from tissue to tissue. The fact that mapping

analysis results are different to those results reported on a dry-weight basis, suggests that results may be distorted when presented on a dry-weight basis rather than volume. That is, a decayed tissue is less dense than the sapwood while the reaction zone may be slightly more dense due to extractives. However, further replication is required with PIXE methodology.

7.2.3 Phenolics

Prior to this study of *E. nitens,* the nature of phenolics had not been examined in the reaction zone of any eucalypt. Initial HPLC investigations of a range of samples revealed a number of peaks particular to the reaction zone. These were the first focus of study. Potential candidates for these included stilbenes such as resveratrol, which is found in a range of *Eucalypts* and has antifungal properties (Hart and Hillis, 1974). Stilbenes produce a strong fluorescence under long-wave ultraviolet light (Pearce, 1996). Fluorescent stilbenes were detected in leaf extracts of various *E. nitens* provenances (Pederick and Lennox, 1979) and those that did not contain them (and had other morphological differences) were later separated into a new species - *Eucalyptus denticulata* (Cook and Ladiges, 1991). Examination of sections of decayed *E. nitens* material showed that the reaction zone did not fluoresce, but the bark did (results not presented). Therefore it was unlikely that stilbenes are found in the wood, but may be present in the bark.

The polar nature of the *E. nitens* wood extracts suggested that many of the compounds in the extract could have a number of hydroxyl groups or sugars. Hydrolyzable tannins have a sugar core and many hydroxyl groups. By obtaining standards of hydrolyzable tannins typically found in eucalypts and analyzing them by HPLC, it was found that the peaks induced in the reaction zone were hydrolyzable tannins. This was confirmed for two anomeric tannins (pedunculagin and tellimagrandin 1) by using a treatment of sodium borohydride which reduces the two anomeric peaks to one peak. This information allowed interpretation of a more detailed analysis of other compounds in the extracts by LC-MS.

Particularly in younger, stem-wounded material, phenols were observed accumulating in the parenchyma and vessel tyloses of the *E. nitens* reaction zone. These compounds may not be widely distributed throughout the other cell types. In

older reaction zone material, detection of phenolics in the reaction zone was not as strong. Difficulties associated with visualizing hydrolyzable tannins are common in eucalypt wood (L. Wilson, pers. comm.; J. Ilic, pers. comm.). It may be that the "discolouration" process (for which observation of deposits is much more apparent) is less distinct from the reaction zone in the early stages of defence, and therefore deposits are widely observable. The localization of the phenols may change over time. For example, tannins may infiltrate cell walls over time (Watanabe *et al.* 1997, Streit and Fengel, 1994) and this may be due to lignin-tannin interactions (Helm *et al.*, 1997). Further studies with ultra-thin sections and scanning electron microscopy may elucidate this further.

If phenols become widely distributed and infiltrate the walls of all cell types in the reaction zone, it may provide a strong defence. However, recent studies have revealed the ability of some white-rot fungi (*Inonotus hispidus*) to penetrate reaction zones of some European hardwoods (London plane) by a soft-rot mechanism, involving tunnelling through the cell wall to avoid the cellular contents (Schwarze and Fink, 1997). Other mechanisms of reaction zone penetration include degradation of beech (*Fagus sylvatica*) polyphenolics by *Ganoderma adspersum* (Schwarze and Baum, 2000). As *G. adspersum* did not appear to be an aggressive invader of *E. nitens* wounds, it is probably not able to degrade polyphenols such as hydrolyzable tannins.

In an experiment with *E. nitens* stem segments, the effect of oxygen access upon phenol accumulation was tested. There was more phenol accumulation in the stems for which the uninoculated end was left open (however this was not statistically significant). Nitrogen may also effect phenol accumulation, with increased levels leading to greater production of *C*-glucosidic ellagitannins in oak callus cultures (Zhentian *et al.*, 1999). There is some other evidence that oxygen access is important for phenol production in *Eucalyptus* species. In studies of *Eucalyptus marginata*, stems colonized with *Phytophthora cinnamomi* produced more soluble phenolics when roots were under normal oxygen conditions than roots under hypoxic conditions (Burgess *et al.*, 1999). This was generally associated with an increased percentage of activity of some enzymes in the phenylpropanoid pathway, however these were probably not all related to defence. In *Eucalyptus maculata*, a correlation was found between phenol oxidizing enzymes and concentration of phenols forming

in the marginal zone (reaction zone) after wounding (Mireku and Wilkes, 1988). In histochemical studies of wounded *E. nitens,* detection of peroxidases by common staining techniques did not indicate any particularly localized increases associated with wounding or infection (results not presented). The involvement of these enzymes in the defence system remains poorly understood, but their role is probably most important in this case by facilitating formation of phenolics. The biosynthetic formation of hydrolyzable tannins (especially ellagitannins) is still being elucidated (Helm *et al.*, 2000).

As mentioned, phenolic compounds may be the most useful indicator of defence capacity. Tree-to-tree variations in phenol levels are relatively high, which implies that future investigations of this nature must involve large replicate numbers. In order to study the effect of various treatments on elicitation of phenolics, it would be desirable to use clonal plant material. This is not easily available for most eucalypts, due to the poor rooting ability of cuttings.

7.2.4 Purple colouration

Blue and purple colours are not commonly seen in healthy wood. An example is the heartwood of *Millettia* spp. which changes to purple rapidly after sawing (Kondo *et al.*, 1986) and is accounted for by a methoxyisoflav-3-ene (Mitsunaga *et al.*, 1987). The purple colour associated with decay in *E. nitens* wood is apparent directly after cutting, and does not appear to occur as a change associated with cutting or exposure to air.

Blue and purple stains of standing trees and wood products are commonly associated with microbial infection. Blue-stain fungi such as *Ceratocystis* spp and *Alternaria* spp. have melanized hyphal walls which causes the infected wood to be coloured (Zink and Fengel, 1989). Many of these blue-stain fungi are associated with beetles (Paine and Stephen, 1987; Krokene and Solheim, 1997). There is no evidence for these microorganisms in the *E. nitens* purple zone; in fact, no particular type of microorganism was routinely isolated. Purple-brown staining has been associated with wood decay column boundaries in some trees infected by *Heterobasidion annosum*, such as in white fir roots (Garbelotto *et al.*, 1997) and Norway spruce stems (Shain, 1971).

Qualitative mass spectrometry comparisons between different zones has not indicated which compound could be responsible for the purple colouration of the reaction zone. While the purple colour can be extracted from the wood, the compound responsible may not be detectable be chromatography. One possibility is that an interaction between tannins and iron may produce this colour which is disrupted upon analysis. This suggestion is made on the basis that when an iron tool is used to cut *E. nitens* wood, a purple colour results. Preliminary studies of iron levels by proton-induced X-ray emission (PIXE) analysis show that there is more iron in the reaction zone, but this was near the minimum instrument detection limit therefore results may be unreliable. This theory requires much more exploration.

7.3 Models of a dynamic host-pathogen interaction

The concept of how living xylem defence operates has often been based on characterizing zones found associated with infection. The earlier models (CODIT and "dynamic" reaction zones) were particularly based on the presence of distinct zones and the function of these zones. In some xylem-microbe interactions, defence zones may not be present (e.g wounds infected with *Chondrostereum purpureum* in *Acer pseudoplatanus*; Pearce et al., 1997, Pearce, 2000) indicating that defence is prevented or quickly surpassed. In *E. nitens*, a range of zones have been observed. These were not constant from interaction to interaction and also appeared to change over time.

Wardlaw and Neilsen (1999) briefly described the purple wood associated with decay in *E. nitens* as similar to the "aniline" wood found in Norway spruce (Shain, 1971). The Norway spruce model indicates that the aniline wood is part of the incipient decay process, occurring before the much wider and diffuse reaction zone. While there are similarities (particularly in colour), it is felt that the *E. nitens* purple zone is in fact a reaction zone (and not aniline wood) due to a number of reasons:

- It is less commonly associated with microorganisms than the surrounding tissues (such as the brown discoloured tissue) as has been shown by isolations.
- It only appears to be formed in sapwood (although it can become "included" in heartwood) and may therefore be associated with processes of living cells (an

active response). Decay columns formed within the heartwood are preceeded by brown discolouration (incipient decay) only.

- It is diffuse and reasonably wide (up to 1 cm) unlike aniline wood.

While this purple zone has been a focus of this thesis, the defence process probably encompasses more than just the purple zone. Studies presented within this thesis have allowed the development of decay and defence models for *E. nitens*. In the early stages of wound response, decay was originally associated with a brown discolouration at the interface of the infection, where phenols were deposited intensely. While this reflects a reaction zone, in the later stages of decay the discoloured wood is associated with decay fungi and therefore whether it is incipient decay or a "defeated" reaction zone is difficult to ascertain.

The brown discoloured wood is similar to the marginal zone described by Wilkes (1985) for other eucalpyt species. In advance of the zone of discolouration in the early stages of decay, the xylem appeared unchanged but phenolics were detected in increased amounts (compared to the normal sapwood on the un-wounded side of the stem). This region is part of the reaction zone and becomes more distinct over time. The term "column boundary layer" is probably a suitable terminology for *E. nitens* to describe a variety of responses occurring at lesion margins. The distribution of zones observed in the early stages of *E. nitens* xylem-fungal interactions has been summarized in Figure 7.1a.

At later stages (e.g. revealed with young plantation trees 6-months after wounding) purple zones were formed around the decay lesion. These zones did not always completely surround the decay column, but pale brown zones were present which were of the same phenolic content. This is summarized in Figure 7.1b. Smith and Shortle (1993a) noted that distinct orange column boundary layers (reaction zones) did not consistently form between normal sapwood and wound-initiated discolouration in living hybrid-poplars. They concluded that the extent of compartmentalization was not only determined by column boundary layers. Our concept of defence in terms of visible boundaries may be exaggerated, as colour reflects only a small aspect of the wood's properties, possibly only accounted for by one compound.

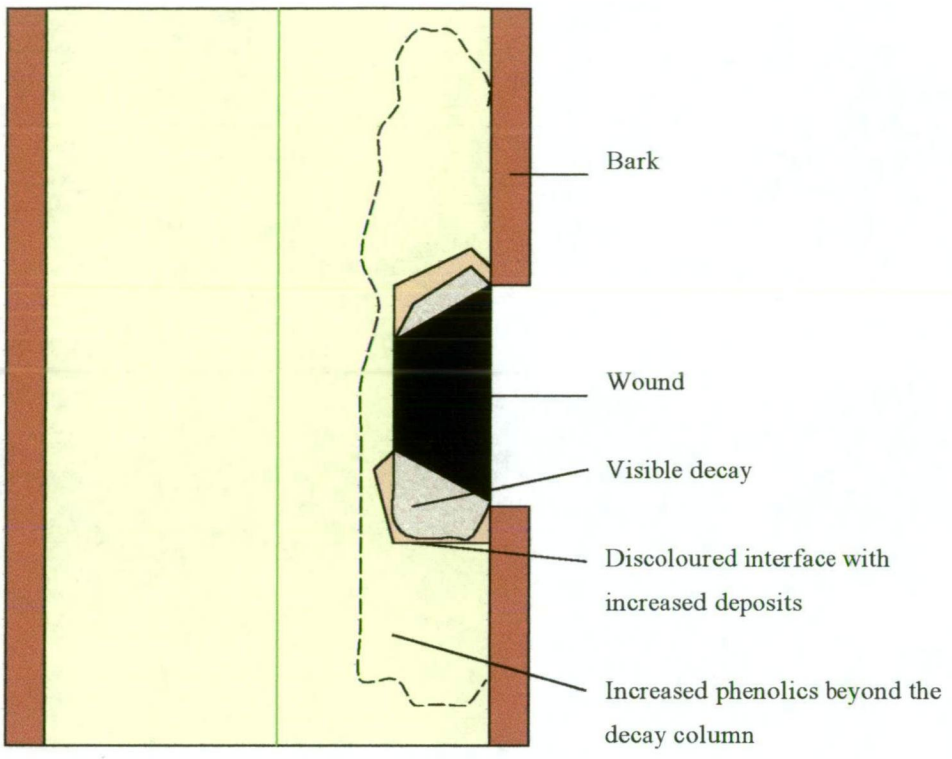


Figure 7.1a. Early stages of reaction zone formation.

Figure 7.1a and 7.1b suggest that reaction zone formation in *E. nitens* could often be a dynamic process, involving a continuum of zones. Of interest, these studies also revealed that the purple reaction zone was sometimes found associated with the barrier zone and in the new wood. This has been rarely described in woody xylem defence, and suggests that the decay is challenging the new wood. However, decay itself was not found beyond the barrier zone. It is suspected that the barrier zone in conjunction with the reaction zone would present a very strong barrier and this may be required to contain particularly aggressive fungi.

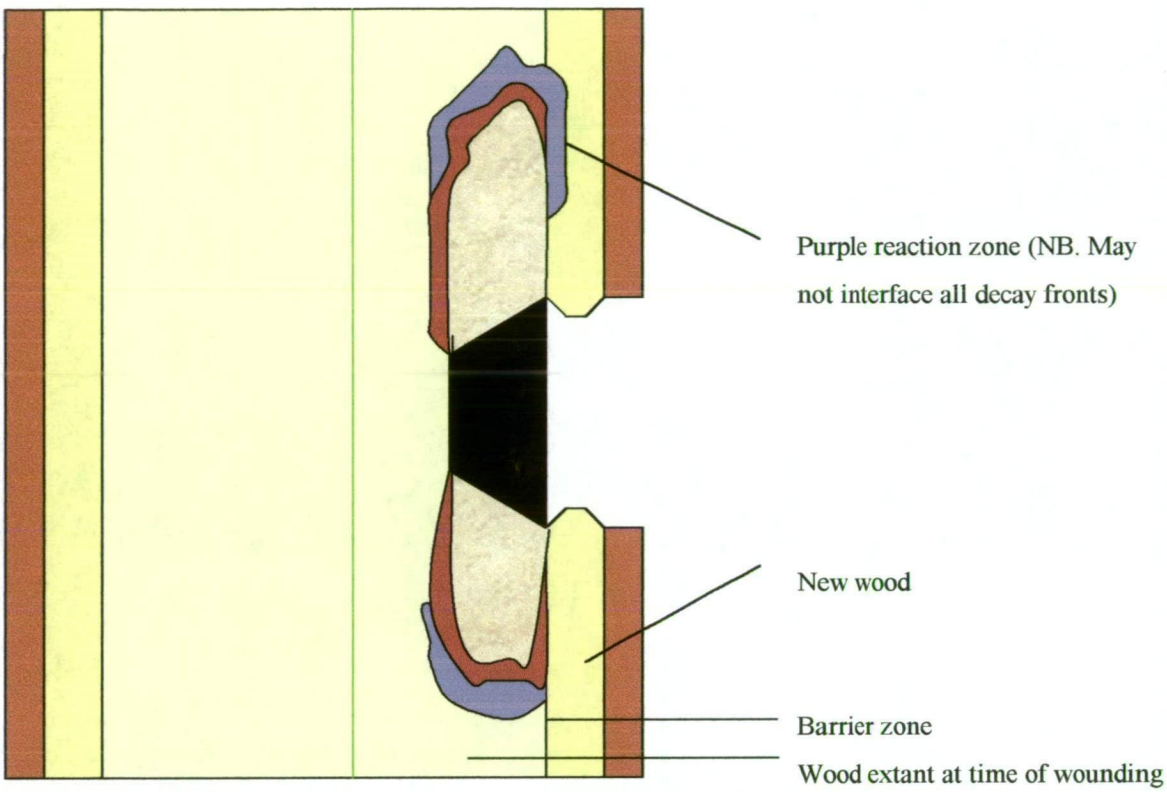


Figure 7.1b. Progression of decay and defence following wounding.

An advanced stage of decay and reaction zone development was observed and analysed in the first chapter of this thesis. A model of this is represented by Figure 7.2. As mentioned, the brown discoloured wood has been classed as incipient decay, but requires further examination. It may infact be similar in nature to the brown interface described above and therefore involved in the defence process. A microscopic comparison of this discoluration at the boundary of decay in heartwood compared to sapwood may elucidate this further.

The purple reaction zone is formed in the sapwood and is only present within the heartwood if it remains stable and becomes incorporated in the heartwood. How long it remains stable is unknown at present, but an *in vitro* storage experiment suggested that it is durable throughout conditions optimal for decay fungi (moist and at room temperature) for up to 9 months. It is expected that it will remain stable for much longer than this *in vivo*. The occasional presence of a dry white zone preceeding the reaction zone is illustrated in Figure 7.2. While this zone has not been fully

investigated, it may be similar in role to the transition zone described in conifers. That is, it may play a role in renewed phenolic production (histochemically, phenols were stronger here than the reaction zone) suggesting that the purple reaction zone is due to be defeated.

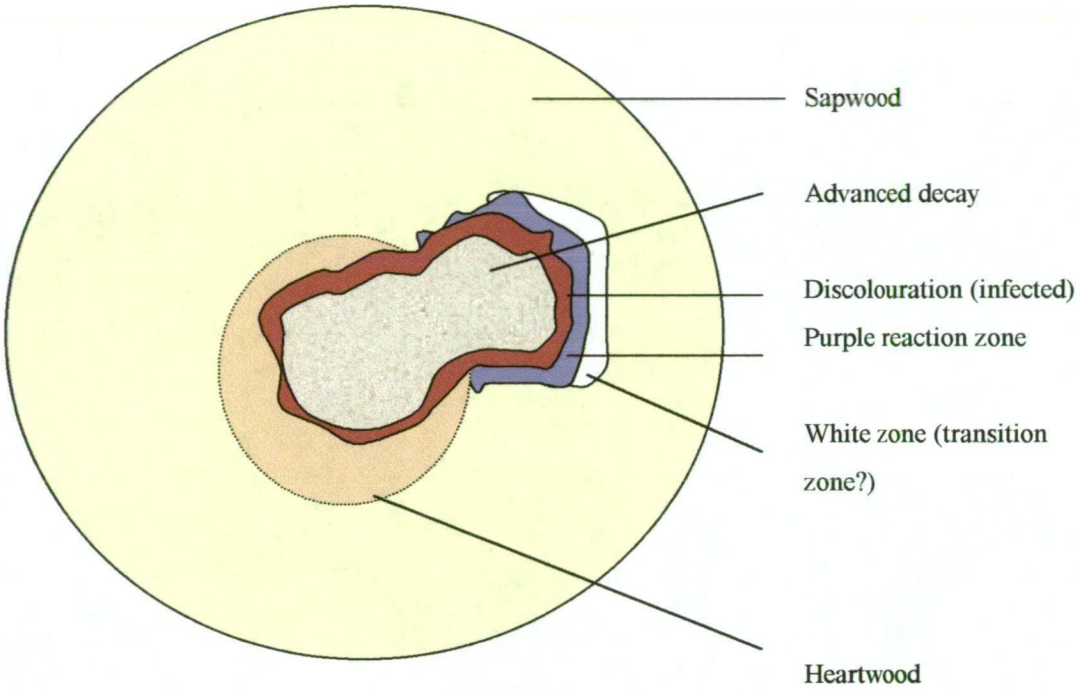


Figure 7.2. Stylized model of zones associated with advanced decay in *E. nitens*.

In accordance with the diagrams above, it is proposed that *E. nitens* reaction zone development is a dynamic process. This is supported by the fact that "relic" reaction zones were never observed during sampling. The rate at which the reaction zone is dynamic may differ according to which microorganisms are responsible for advancing the decay front. For example, where the fungal agents are weakly aggressive (e.g. *Ganoderma adspersum*, *Ganoderma applanatum* or "isolate R") the reaction zone may become stationary very quickly (this is not defined as static). It also need not be strongly developed to withstand penetration by these fungi, indicated by considerably less phenolic accumulation than that associated with more aggressive fungi. It is of interest that although isolate R was isolated from an *E. nitens* reaction zone, it did not appear to be aggressive in the wounding experiments.

It is likely that an interaction of xylem microenvironment and induced defences contribute to patterns of host-fungal interaction. For example, fungi which are able to quickly colonize sapwood which is compromised by wounding (due to easy access into severed vessels and a subsequently dried tissue) may grow through the wood until they reach a region of both living cells and a higher moisture content (conditions normally restrictive). The fungus (or a combination of microorganisms) may then colonize the living tissue to a greater or lesser extent depending on its tolerance of this environment. This would involve a dynamic stage where defence responses may be in the process of forming. When the active xylem responses become increased to such an extent that they inhibit the fungus, the process would become stationary. For example, the aggressive *Aleurodiscus* spp. and "isolate D" used to challenge *E. nitens* wounds were associated with greatly increased phenol accumulation. To determine whether this is ultimately able to restrict their spread, experiments would need to be analyzed at various time stages. The fact that "isolate D" was inhibited by reaction zone extracts in TLC bioassays suggests that levels can become inhibitory.

7.4 Perspectives for eucalypt forestry

The long-term stability of reaction zones may be more important in determining the potential spread of decay from wounds than the barrier zone. While barrier zones may be stronger (ie. "wall 4") they are usually limited in extension from the wound. For example, in some eucalypts, barrier zones only extend up to 10 cm axially above and below wounds (Wilkes, 1986). However, reaction zones can form where required. Decay lesions can extend axially well beyond the wound position (and barrier zone) and may then challenge the xylem in an outward fashion. For a series of wounds (such as for pruning) the situation may be different. In the expanded concept of the CODIT model, Shigo (1979) suggested that barrier zones form continually between pruning wounds (Refer Fig. 1.1). It is currently unknown whether this can occur in *E. nitens* and if so would present a considerable barrier resisting spread of decay from the knotty core.

Purple reaction zones in wood formed after wounding (ie. beyond the barrier zone) were observed occasionally in *E. nitens.* As the common definition of the reaction zone is that it is formed in pre-existing wood (Shain, 1967; Smith and Shortle,

1993b; Pearce, 1996), this appears unusual. This suggests that the young xylem and barrier zone cells can produce phenolics. It also suggests that the barrier zone is being penetrated, or at least elicitation of defence responses through the barrier zone to the new wood has occurred. The evidence that reaction zones can be evidenced as stationary boundaries within the heartwood may be very important for the long-term outcome of decay spread. If this were not the case and reaction zones were defeated once within the heartwood, decay would keep advancing with the heartwood transition zone (ie. continually challenging the sapwood and being met by a reaction zone in either a dynamic or static manner). In this case, the decay core would have the potential to become extremely large.

Based on a small number of previous field studies, it is suspected that *E. nitens* displays relatively successful xylem defence mechanisms. Defence that is "successful" may be a combination of inherent wood qualities (including heartwood resistance), reaction zone success and other factors, and will differ depending on what type of situation is described (for example, naturally occurring decay in unwounded trees compared to decay arising from wounds). For example, in unpruned plantation-grown *E. nitens* trees decay was detected rarely, while in *E. regnans* an "alarming incidence of decay" was detected (Yang and Waugh, 1996). Low levels of decay in *E. nitens* was suggested to be associated with kino barriers forming around self-pruned branches. In drill-wounded *E. nitens* and *E. regnans* of different provenances (White *et al.*, 1999), an average comparison can be made between species. This indicated that longitudinal spread of decay was less in *E. nitens* even though wounds were greater in radial length, however, as trees were at different sites it is probably not a valid comparison.

Although the requirements for successful defence have not been fully clarified (as they differ from tree to tree) it is possible to compare the effectiveness of defence responses between different trees. In South-East Asia, *Acacia mangium* has been widely planted for its fast growth, good wood qualities and adaptability to poor soils, however, decay incidence is high. Schmitt *et al.* (1995) found that the initial intensity of wound reaction was low in *A. mangium* (when compared to some European hardwoods), there was a lack of complete vessel occlusions (resulting in increased xylem drying) and secreted substances were degraded by fungal hyphae and therefore hardly toxic. It is therefore likely that both prevention of continued air access, and

formation of toxic phenolics is important for wound defence. *E. nitens* is efficient in producing tyloses, produces phenols which are of a toxic nature and the reaction zone can become a stationary, durable barrier. This probably indicates that it is essentially a successful defence. Surveys suggest that decay is restricted to the knotty core in *E. nitens* at least nine years after pruning (Wardlaw and Neilsen, 1999). Assessment of decay spread over time is a useful indicator of successful defence. Mireku and Wilkes (1988) found that microbial deterioration did not extend significantly between 90 and 240 days after wounding of *E. maculata*. Similar studies for *E. nitens* (and other eucalypts) would be valuable.

Within a species, decay spread and compartmentalization capacity may vary. Wounding studies of different provenances of *E. nitens* have not been able to distinguish differences in decay spread (White *et al.*, 1999). However, differences in decay incidence between sites has been found for pruned *E. nitens* in Tasmania (Mohammed *et al.*, 2000). A number of features will determine the decay incidence associated with pruning wounds; for example, the branch size, fungal species and general host condition (Mohammed *et al.*, 2000). On sites where decay incidence is high, the future extent of the decay spread will be determined by the interaction of the causal fungi and the xylem defences (the reaction zone and barrier zone). Further information of casual fungi is required, including site and seasonal differences in occurrence.

Characterization of reaction zones in *E. nitens* provides a base understanding of plant-fungal interaction which will aid future studies of decay spread potential in a plantation-forestry context. Future studies will be able to compare properties of reaction zones (particularly phenolic accumulation) to other major plantation species. For example, studies are currently being established to determine if *Eucalyptus globulus* holds a similar defence capacity. Preliminary pruning experiments have revealed that a purple discolouration is also frequently observed (M.H. Hall, A.D. Mollon, K.M. Barry and C.L. Mohammed, unpublished). The effectiveness of this defence compared to *E. nitens* could be quickly determined by controlled, quantitative studies. These would include assessments of decay spread for a range of chosen fungi, phenols induced and a comparative study of xylem anatomy. This may help predict which species are more likely to successfully contain decay to the knotty core, and may be a consideration in planting schedules.

Further surveys to assess decay spread towards harvest age of pruned trees will be required to determine whether the reaction zone remains effective. This may vary from site to site, depending on which fungi are present and their ability to penetrate the reaction zone over time. Environmental stresses and mechanical disruptions (such as insect borers) to the reaction zone may also compromise the reaction zone.

7.5 Conclusion

Xylem defence mechanisms in *E. nitens* appear to be well developed. Whether *E. nitens* is representative of other eucalypts is yet to be addressed. Responses observable after wounding and inoculation and in "fully-formed" reaction zones include:

- phenolic accumulation
- tylose formation
- decrease in potassium levels
- decreased pH.

All of these responses appear to be an accentuation of processes occurring in heartwood. Typical reaction zone trends shown by other hardwoods, which were not present included:

- mineral accumulation
- suberization
- long-lived free radicals
- water accumulation (some evidence following wounding).

E. nitens reaction zones are an apparently successful antimicrobial defence, and therefore the factors not observed are unlikely to be required for *E. nitens* xylem defence. As *E. nitens* is an evergreen hardwood, it may be expected that different processes (especially concerning water relations) may occur compared to deciduous hardwoods.

7.6 Recommendations for future research

To predict the long-term impact of decay spread in *E. nitens*, a greater understanding of reaction zone defeat will be required. This involves identifying the most common decay-causing fungi (a project currently in progress) and assessing their ability to defeat a reaction zone once it has become incorporated in the heartwood. Histochemical studies with ultra-thin microtome sections will allow visualization of fungal hyphae within the discoloured interface of the decay column. Studies with transmission electron microscopy (TEM) would be advisable for further studies of the distribution of tannins in the purple zone. This would provide an insight into how tannins provide a barrier to fungal spread if they are not present as deposits in the lumen.

Environmental factors that may influence defence responses (such as phenol accumulation) should be investigated, including differences due to fertilization and site. In order to assess these differences, a routine inoculation fungus would be used in preference to allowing the wounds to become naturally-infected. This would control variation due to different fungi and hopefully treatment effects could be seen beyond tree-to-tree variation.

Assessment of other zones with an antimicrobial function would provide a greater understanding of defence in Eucalypts. This includes barrier zones (which do not form as kino veins in *E. nitens*, unlike many other Eucalypts) and the protective zone formed in senescent branches.

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