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Heartwood formation in young *Eucalyptus bosistoana*

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TABLE OF CONTENTS

Heartwood formation in young *Eucalyptus bosistoana* 1
EXECUTIVE SUMMARY 1
INTRODUCTION 2
METHODS 3
Wood material and fixation 3
Microscopy 3
Conventional histochemical staining 3
Confocal microscopy 3
Sample preparation for immunolabelling 4
Image modifications 4
RESULTS 5
Starch 6
Nuclei 6
Tyloses 6
Wood types 6
Extractives 7
CONCLUSION 8
REFERENCES 9

EXECUTIVE SUMMARY

Little is known about heartwood formation in young trees and published literature reports are based on heartwood of old trees. However, in the context of fast-growing short-rotation durable eucalyptus plantations, heartwood formation in young trees is important. There are indications that heartwood formation in young trees differs from that in old trees in the sense that the parenchyma cells in the transition zone, which synthesise the heartwood extractives, remain active for a longer period. This would result in a wider transition zone which is not 'true heartwood' – the part of the stem in which all parenchyma cells have died. The implication would be that the extractive content (and consequently wood quality) at centre of the stem could improve over time in young trees.

The hypothesis that there is prolonged heartwood formation in young trees was tested by looking for living parenchyma cells in radial profiles of 6 year-old *E. bosistoana* trees. This was done by microscopy and different staining techniques. The observations revealed that 6 year-old *E. bosistoana* trees contained 'true' heartwood. In this tissue cell organelles (nuclie and vacuoles) and reserve material (starch) were absent; autofluroscence spectra indicated the presence of extractives; and vessels were blocked by tyloses.

As a consequence extractive content does not increase over time at the centre of 6 year-old *E. bosistoana* trees due to local synthesis and deposition of these compounds.

INTRODUCTION

Wood (xylem) cells undergo a complex metabolic and physiological process to form heartwood. The heartwood, which is rich in bioactive compounds termed 'extractives' is a defence system developed by trees. The economic and aesthetic value of wood is often determined by the heartwood. The key features of heartwood are the formation of extractives that impart natural durability and colour, sought after wood properties. Heartwood formation is a form of senescence or programmed cell death (PCD) of parenchyma cells (radial and axial) in the transition zone, the boundary between sapwood and heartwood.

Wood formation (xylogenesis) in vascular plants is a complex biological process which includes major developmental steps (i) cell division from secondary vascular cambium, (ii) cell expansion (cell elongation and differentiation), (iii) secondary cell wall deposition, (iv) programmed cell death and (v) heartwood formation in some trees (Plomion et al., 2001). Secondary xylem consists of heartwood, sapwood and a transition zone. Heartwood can be defined as the "often dark coloured, inner layers of the growing tree that have ceased to contain living cells, and in which the reserve materials (e.g. starch) have been removed" (Taylor et al., 2002). Therefore, heartwood lack physiological activity due to the absence of living cells and reserve materials. Heartwood is distinguished from sapwood by lower permeability and often decay resistance. Sapwood is the living part of the xylem, containing some living (parenchyma) cells and reserve materials (e.g. starch). The sapwood plays an important role in water conduction in plants (Taylor et al., 2002). The boundary of sapwood and heartwood is known as the 'transition zone' defined as "a narrow pale-coloured zone surrounding heartwood, containing living cells, usually devoid of starch, impermeable to liquids and moisture content less than sapwood and sometimes than heartwood". This zone is also known as the "white zone" and "dry zone" (Nobuchi & Harada, 1983). In this zone, ray and axial parenchyma cells are alive and convert primary metabolites (fat and starch) into secondary metabolites or heartwood deposits (Burtin et al., 1998; Magel, 2000). The death of both axial and ray parenchyma cells is resulting in heartwood. Ray parenchyma cells provide multiple roles and are involved in the wound response, carbohydrate storage, radial transport and heartwood formation. Parenchyma cells can also form protruding structures (tyloses) that block the conducting elements (vessels) prior to transitioning into heartwood (Chattaway, 1949). A multi-lamellate primary wall can be observed in developing tyloses. Tyloses are observed in eucalyptus species such as *E. oblique* and *E. miniata* (Foster, 1967).

Heartwood characteristics like durability, dimensional stability, pulpability and colour are attributed to the accumulation of compounds termed extractives. Eucalyptus species are endowed with polyphenolic compounds like flavandiols (condensed tannins), ellagitannins, gallotannins and kino. Tyloses contributes to the reduced permeability of heartwood which makes processing like drying and treating more difficult.

Literature reveals that ray parenchyma can remain alive for several (2-200) years, retaining their cellular intracellular structures (microtubules) and organelles (plastids, peroxisomes, nuclei) (Fukuda, 2000). The heartwood formation in young trees has not been studied in detail. There are inferences that some ray parenchyma cells stay alive for longer times in heartwood of young trees. This can lead to a gradual increase in the extractive content of heartwood with time. Identification of organelles of living parenchyma cells in heartwood can be performed by microscopy with specific stains.

Heartwood is formed in all durable species (angiosperms and gymnosperms). The genus eucalyptus is one of the angiosperms which contain tannins in the sapwood and in ray cells (Chattaway, 1952). Heartwood formation in eucalyptus species might be triggered by an increase of tannin in the ray parenchyma cells (Chattaway, 1952). These tannins are present in soluble form in ray cells and solidify with the addition of fixatives (phosphate buffered formalin, FAA) for microscopic visualization (Chattaway, 1952). Despite of many theories and hypothesis proposed, the exact biological process of heartwood formation is not understood.

METHODS

Wood material and fixation

Cores were obtained from 6-year old *E. bosistoana* trees in November 2015. The wood samples were fixed in a buffer comprising of cytoskeleton buffer stock solution (PME) (50 mM PIPES pH 7.2, 2 mM EGTA, 2 mM MgSO4), Triton x 100 (0.1%), DMSO (1%), formaldehyde (37%) and glutarldehyde (25%) under vacuum. Each core was subjected to fixation in 50 ml falcon tubes containing fixative solution. The tubes were placed in a portable vacuum pump for 15 min while inducing and releasing the pressure.

The fixed wood specimens were washed several times with water. The length of the cores was measured with a ruler and split into 1 cm pieces. Radial- and cross-sections (10-20 µm thick) were prepared from each of these pieces using a sledge microtome. The measurements were repeated on a 2nd core, showing similar results.

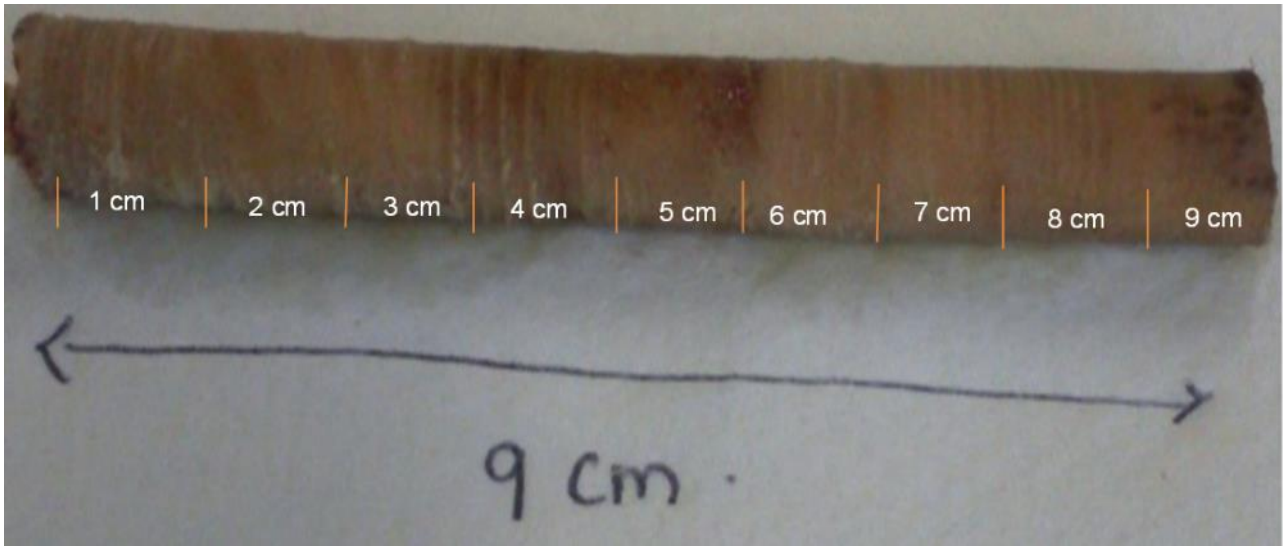

Figure 1: Vacuum fixed core from 6 year-old *E. bosistoana*

Microscopy

Conventional histochemical staining

A Nikon eclipse 50i fluorescence microscope equipped with transmitted light and a filter cube comprising of dichromatic mirror, exciter and barrier filters was used for the study.

The following colour-based stains were used:

- Potassium iodide (0.1%) – to stain starch grains in amyloplasts black

After staining, sections were mounted in glycerol and observed with a fluorescence microscope.

Confocal microscopy

A Leica SP5 confocal microscope system operating on a DMI6000 inverted microscope and equipped with 20 x NA 0.7 and 63 x NA 1.3 glycerol immersion lenses (Leica, Wetzlar, Germany) was used for confocal microscopy (Collings, 2015). High resolution Z stack images (1,024 pixels square) were recorded with 4-fold line averaging and 1.00 µm step size. Two different lasers that could provide excitation at 488 nm and 633 nm, and a transmitted light detector were used for immunolabelling. Fluorescence images of nuclei were collected using two different lasers i.e. 488 nm with emission from 500 nm - 533 nm to observe the green fluorescein-fluorescence from nuclie through microscope, while red Cy5-fluorescence from nuclei was excited at 633 nm, with emission set to 650 nm - 700 nm for capturing the images.

To detect the variation of extractives fluorescence emission spectral images from each radial sections were collected with a 405 nm laser using a scanning fluorescence emission wavelength from 480 nm - 520 nm (λ scanning). Images were recorded at lower resolution (512 pixels square)

using 4-fold line averaging. Series of images were collected using fluorescence and transmitted light detectors. All images were captured at the same imaging conditions.

Sample preparation for immunolabelling

Fixed tissue sections were exposed to FAA solution (37% formaldehyde, 100% ethanol and 100% glacial acetic acid) overnight before immunolabelling. Radial sections were extracted with 1% triton solution for 1 h. Tissues were further washed with PBS followed by acetone treatment for 20 min at -20°C in a freezer to extract extractives. Sodium borohydride treatment was performed for 10 min at room temperature to reduce background fluorescence. Wood tissues were washed with PBS and blocked by incubation buffer (BSA) for 20 min. Sections were then incubated with primary antibodies for nuclie immunolabelling for 60 min at room temperature. Sections were then washed thrice with PBS and incubated with two secondary antibodies goat anti-rabbit Cy5 (GARb Cy5) (1:200 dilution) and sheep anti rabbit-fluorescein (SARb-F) (1:100 dilution) in incubation buffer for 60 min at room temperature. Sections were washed with PBS and mounted in glycerol.

Antibodies used:

- For nuclie labelling, sections were labelled with anti-histone H3 antibody (1:200 dilution) (Nic-Can et al., 2013). Goat anti-rabbit Cy5 (GARb Cy5) (1:200 dilution) and sheep anti rabbit-fluorescein (SARb-F) (1:100 dilution) were used as secondary antibodies (Table 1).

- Controls were run in the absence of primary antibodies. Secondary antibodies were included.

Table 1: Antibodies used for labelling of nuclei

Target	Supplier	Host	Dilution	Fluorochrome
Primary antibodies				
Anti-histone H3 antibody, CT	Sigma	Rabbit	1:200	NA
Secondary antibodies				
Rabbit	Jackson immunoresearch	Sheep	1:100	Fluorescein
Rabbit	Jackson immunoresearch	Goat	1:200	Cy5

Image modifications

Images were modified using standard brightness and contrast settings in Photoshop (version CS4, Adobe Systems, San Jose, CA, USA) and ImageJ (FIJI installation of version 1.47v, National Institute of Health, Bethesda, MD, USA). Image intensities were quantified from image stacks in ImageJ by selecting a region (usually parenchyma cells) with quantification using the 'Plot Z axis profile' function (Collings, 2015).

RESULTS

Heartwood formation in 6 year-old *E. bosistoana* was monitored by visualising the physiological changes in parenchyma cells along the radial profile of a bark to bark core. Nuclei are indicators of living cells and have been visualised by immuno-labelling in radial sections (Figure 2). The presence of starch in parenchyma cells was visualised using KI staining in radial sections (Figure 3). Formation of tyloses was observed in transverse sections without staining (Figure 4).

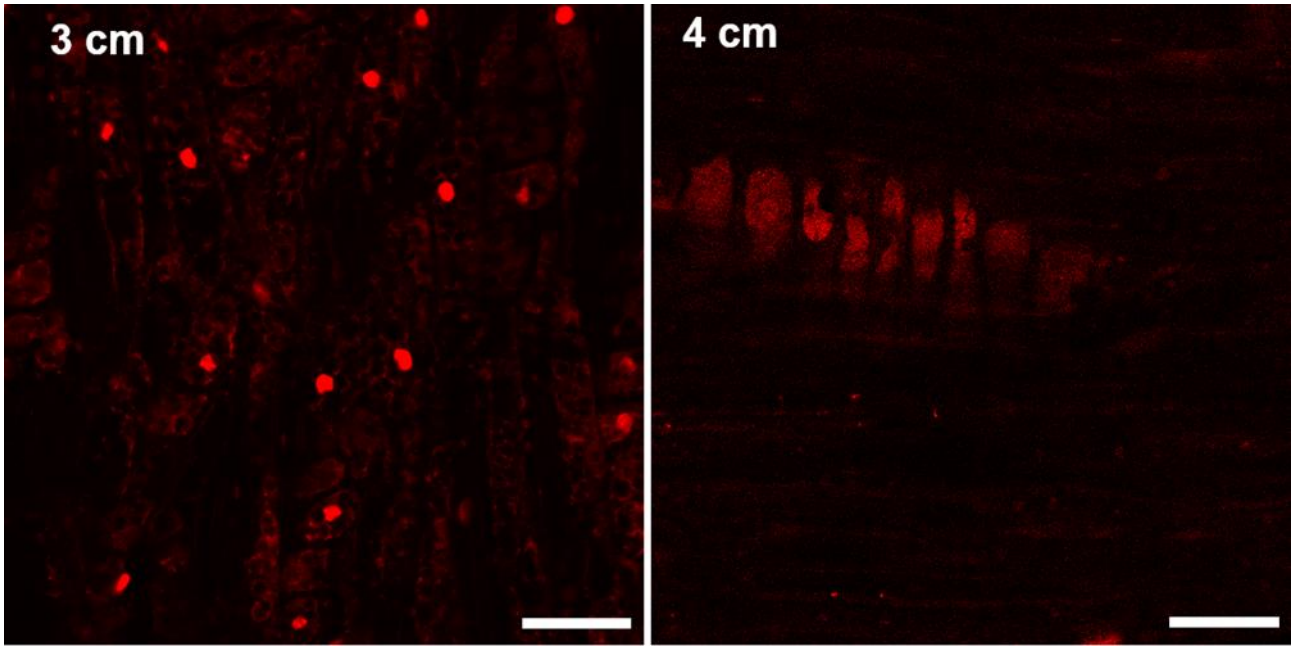

Figure 2: Radial sections from a 6 year-old *E. bosistoana* stem labelled for histone to visualise nuclei (red 'dots') in parenchyma cells. Nuclei were observed at the outside of the stem (left) but not at the centre (right). Scale bar 50 µm.

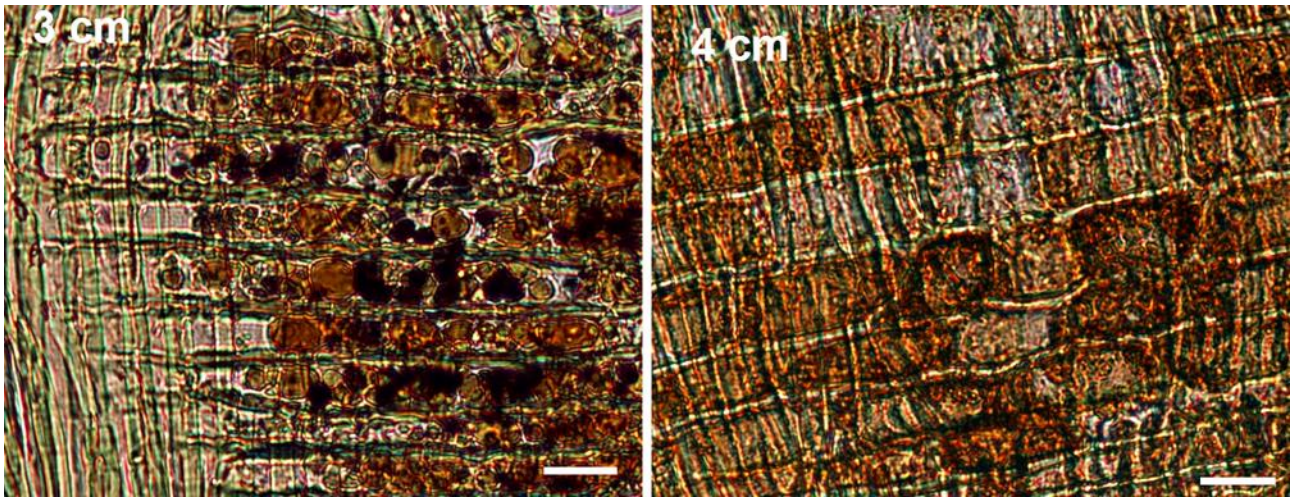

Figure 3: Radial sections of a 6 year-old *E. bosistoana* stem stained with KI, highlighting starch (amyloplasts) black. Amyloplasts were present in parenchyma cells at the outside of the stem (left) but not at the centre (right). Scale bar 10 µm

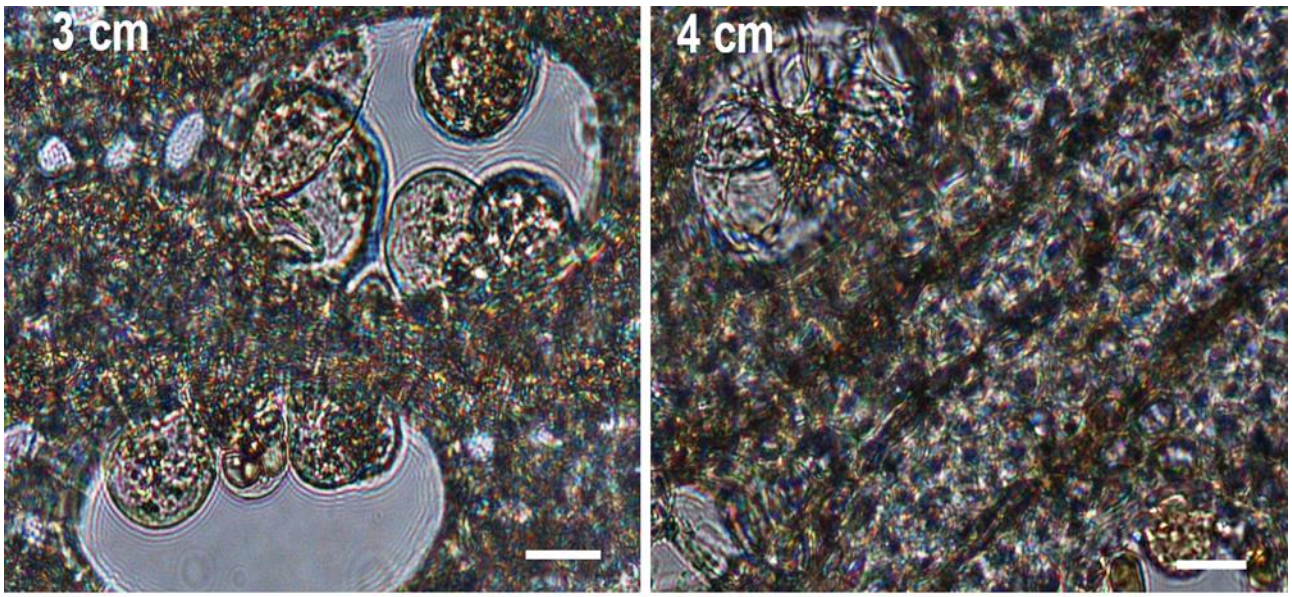

Figure 4: Transverse sections of 6 year-old *E. bosistoana* stems showing formation of tyloses in vessels. Developing tyloses (left) and fully blocked vessels in the centre of the stem. Scale bar 10 µm.

The physiological changes through the radial profile of the 6 year-old *E. bosistoana* tree are summarised in Table 2.

Starch

Starch grains were observed in axial and ray parenchyma cells in the outer parts of the stem. The parenchyma cells in the centre of the stem were devoid of starch. A similar observation was reported by Nakaba and co-workers for *Robinia pseudoacacia* (Nakaba et al. 2008; 2012). The parenchyma cells are a reservoir of metabolic products including starch, fat and protein (Pruyn et al., 2012). This is consistent with the current understanding of heartwood formation with the removal of reserve carbohydrates being an essential factor making the stem less attractive for decaying organisms.

Nuclei

The disappearance of nuclei was monitored in each section of the core as an indication of cell death of parenchyma cells (Marty, 1999). Presence of nuclei was observed in the outer parts of the stem. No indication of nuclei was found in the centre of the stem (4-6 cm). Similar observations were reported for *Populus sieboldii* x *P. grandidentata* (Nakaba et al., 2012). Labelling of nuclei was more prominent in axial parenchyma cells around vessels. In relation to starch, the nuclei disappeared after the mobilisation of starch grains. The order of disappearance of starch is consistent with the death of parenchyma cells resulting in heartwood formation.

Tyloses

Tyloses were found in the centre of the stem. Vessels were open in the outer parts of the stem. This is consistent with conductive sapwood fulfilling the function of water transport in a tree. Initial formation of tyloses was observed after mobilisation of starch but before the death of the parenchyma cells (i.e. nuclei present) (3 cm and 7 cm).

Wood types

The observed physiological changes are consistent with the formation of true heartwood (i.e. no living parenchyma cells) at the centre of young (6 year-old) *E. bosistoana* tree. The transition zone, in which sapwood is transformed into heartwood, was found to be a reasonable narrow zone with the loss of conductivity, removal of research carbohydrates (starch) and the deposition of extractives occurring in close proximity (~1 cm in radial width).

Table 2: Physiological changes in parenchyma cells through a 6 year-old *E. bosistoana* stem.

Position	1 cm	2 cm	3 cm	4 cm	5 cm	6 cm	7 cm	8 cm
Starch (KI stain)	Yes	Yes	Yes (some)	No	No	No	Yes (some)	Yes
Nuclei (histone labelling)	Yes	Yes	Yes	No	No	No	Yes	Yes
Tyloses	No	No	Yes	Yes	Yes	Yes	Yes	No
Tissue type	**Sap wood**	**Sap wood**	**Transition zone**	**Heart wood**	**Heart wood**	**Heart wood**	**Transition zone**	**Sap Wood**

Extractives

The fluorescence emission spectra after excitation at 405 nm of parenchyma and fibre cells of sapwood and heartwood are given in Figure 5. The fluorescence emission spectra from parenchyma cells differed from those of fibres. Fibres were fluorescing at higher wave number than parenchyma. This is an indication of different chemical composition of the 2 cell types. When comparing heartwood with sapwood the fluorescence emission spectra of heartwood emitted at lower wavenumbers. This is consistent with the presence of reddish compounds in heartwood (Koch & Kleist, 2001). The signal was present in parenchyma as well as fibre cells, indicating the presence of extractives in both cell types. However, more extractive should be present in the parenchyma cells. The latter is in line with Chattaway's (1952) observation that the genus eucalyptus contain tannins in the sapwood and in ray cells.

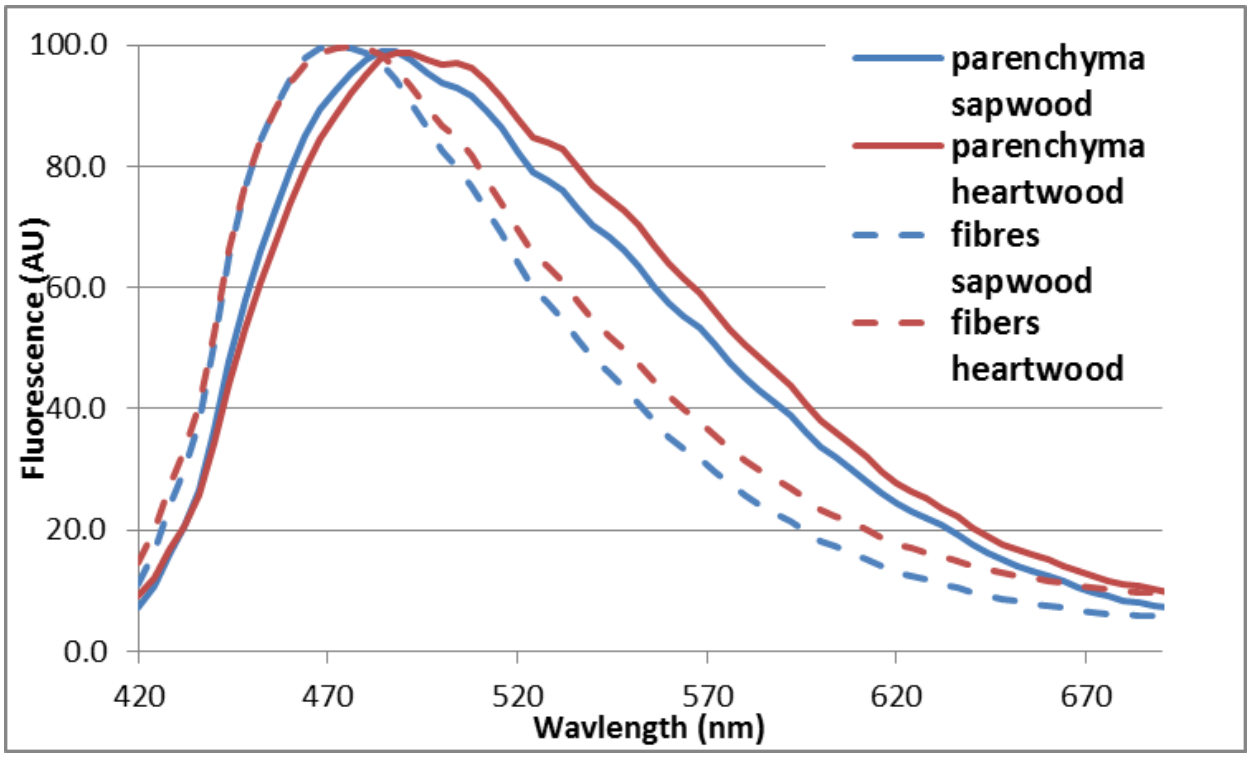

Figure 5: Fluorescence emission spectra from parenchyma (solid line) fibre (dashed line) cells in sapwood (blue) and heartwood (red) from a 6 year-old *E. bosistoana* stem using 405 nm excitation.

CONCLUSION

- True heartwood was found in 6 year-old *E. bosistoana*. This rejects the hypothesis of a prolonged transition from sapwood to heartwood, i.e. the possibility of a continued deposition of extractives at the centre of the stem. Previously reported data on extractive contents in young trees would have been consistent with this mechanism.

- The loss of conductivity in sapwood and the transition into heartwood (programmed cell death of parenchyma) was reported to be independent processes in trees (Ziegler, 1968). In 6 year-old *E. bosistoana*, these two processes were occurring in close proximity, i.e. absence of non-conducting sapwood.

REFERENCES

Burtin, P., Jay, A. C., Charpentier, J. P., & Janin, G. (1998). Natural wood colouring process in Juglans sp.(*J. nigra, J. regia* and hybrid *J. nigra* 23× *J. regia*) depends on native phenolic compounds accumulated in the transition zone between sapwood and heartwood. *Trees, 12*(5), 258-264.

Chattaway, M. M. (1949). The Development of Tyloses and Secretion of Gum in· Heartwood Formation. *Australian Journal of Biological Sciences, 2*(3), 227-240.

Chattaway, M. M. (1952). The sapwood-heartwood transition. *Australian Forestry, 16*(1), 25-34.

Collings, D. A. (2015). Optimisation approaches for concurrent transmitted light imaging during confocal microscopy. *Plant Methods, 11*(40), 1-11.

De Filippis, L., & Magel, E. (2012). Identification of biochemical differences between the sapwood and transition zone in *Robinia pseudoacacia* L. by differential display of proteins. *Holzforschung, 66*(4), 543-549.

Decombeix, A.-L., Meyer-Berthaud, B., Rowe, N., & Galtier, J. (2005). Diversity of large woody lignophytes preceding the extinction of Archaeopteris: New data from the middle Tournaisian of Thuringia. *Review of Palaeobotany and Palynology, 137*(1), 69-82.

Feng, Z., Wang, J., Rößler, R., Kerp, H., & Wei, H.-B. (2013). Complete tylosis formation in a latest Permian conifer stem. *Annals of Botany, 111*(6), 1075-1081.

Foster, R. (1964). Fine structure of tyloses. *Nature, 204*, 494-495.

Foster, R. (1967). Fine structure of tyloses in three species of the Myrtaceae. *Australian Journal of Botany, 15*(1), 25-34.

Fukuda, H. (2000). Programmed cell death of tracheary elements as a paradigm in plants. *Plant Molecular Biology, 44*(3), 245-253.

Groover, A. T. (2005). What genes make a tree a tree? *Trends in Plant Science, 10*(5), 210-214.

Hillinger, C., Höll, W., & Ziegler, H. (1996). Lipids and lipolytic enzymes in the trunkwood of *Robinia pseudoacacia* L. during heartwood formation. *Trees, 10*(6), 366-375.

Koch, G., & Kleist, G. (2001). Application of scanning UV microspectrophotometry to localise lignins and phenolic extractives in plant cell walls. *Holzforschung, 55*(6), 563-567.

Magel, E. A. (2000). Biochemistry and physiology of heartwood formation. *Experimental Biology Reviews*, 363-376.

Marty, F. (1999). Plant vacuoles. *The Plant Cell, 11*(4), 587-599.

Meyer-Berthaud, Soria, A., & Decombeix, A. L. (2010). The land plant cover in the devonian: a reassessment of the evolution of the tree habit. In M. Vecoli, G. Clément & Meyer-Berthaud. (Eds.), *The terrestrialization process: Modelling complex interactions at the biosphere-geosphere interface* (pp. 57-68). London, England: The Geological Society.

Murmanis, L. (1975). Formation of tyloses in felled Quercus rubra L. *Wood Science and Technology, 9*(1), 3-14.

Nakaba, S., Begum, S., Yamagishi, Y., Jin, H.-O., Kubo, T., & Funada, R. (2012). Differences in the timing of cell death, differentiation and function among three different types of ray parenchyma cells in the hardwood *Populus sieboldii*× *P. grandidentata*. *Trees, 26*(3), 743-750.

Nakaba, S., Yoshimoto, J., Kubo, T., & Funada, R. (2008). Morphological changes in the cytoskeleton, nuclei, and vacuoles during cell death of short-lived ray tracheids in the conifer *Pinus densiflora*. *Journal of Wood Science, 54*(6), 509-514.

Nic-Can, G., Hernández-Castellano, S., Kú-González, A., Loyola-Vargas, V. M., & De-la-Peña, C. (2013). An efficient immunodetection method for histone modifications in plants. *Plant Methods, 9*(1), 1.

Nobuchi, T., & Harada, H. (1983). Physiological features of the 'white zone' of sugi (*Cryptomeria japonica* D. Don)-cytological structure and moisture content. *Journal of the Japan Wood Research Society, 29*, 824-832.

Plomion, C., Leprovost, G., & Stokes, A. (2001). Wood formation in trees. *Plant Physiology, 127*(4), 1513-1523.

Pruyn, M. L., & Spicer, R. (2012). Parenchyma. *eLS*, 1-8.

Simpson, M. G. (2010). *Plant systematics* (2nd ed.). San Francisco, CA: Academic Press.

Stewart, C. M. (1966). Excretion and heartwood formation in living trees. *Science, 153*(3740), 1068-1074.

Taylor, A. M., Gartner, B. L., & Morrell, J. J. (2002). Heartwood formation and natural durability- A review. *Wood and Fiber Science, 34*(4), 587-611.

Van Lierde, J. (2013). *What causes natural durability in Eucalyptus bosistoana timber? a dissertation submitted in partial fulfilment of the requirements for the degree of Bachelor of Forestry Science with Honours.* B For Sci (Hon), University of Canterbury, Christchurch, New Zealand.

Ziegler, H. (1968). Biological aspects of heartwood formation. *Holz Roh-Werst*, *26* , 61-68.