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Australian Centre for International Agricultural Research

Final report

Enhancing the formation of heartwood in Sandalwood in Vanuatu

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1 Acknowledgments

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2 Executive summary

Why the work was done?

Sandalwood offers farming diversity and an economic opportunity for Vanuatu. Heartwood formation and sandalwood oil deposition, determines sandalwood value. This project explored the regulation of sandalwood heartwood formation through the identification and implementation of chemical treatments. Knowing the genes and enzymes that produce sandalwood oil provided a basic understanding. Missing was understanding gene regulation as sandalwood heartwood and oil yield fluctuate widely between trees. This work builds on earlier ACIAR work on sandalwood silviculture and breeding in Vanuatu.

What was achieved?

This preliminary work identifies chemicals that stimulate gene up-regulation in 10 in year-old trees to produce sandalwood oil. Two experiments were undertaken to reach this discovery.

The first experiment tested the application method as well as the treatments. Applied to non-sandalwood oil producing tree branches, the treatments included ethylene, auxin, cytokinin, methyl jasmonate, salicylic acid and a gibberellin inhibitor. A control to measure application method impact (drilling a hole in the branch) was included. Wounding and treatment application stimulated sandalwood oil and the amount increased overtime with no statistical difference between treatments. The sandalwood oil profile, however, changed with treatment. In *S. austrocaledonicum,* the major sandalwood oil components are the santalols and lanceol, and either one or the other is produced as they are from the same origin and produced by different enzymes. Initially, ethylene was the only treatment that stimulated santalol oil production. All other treatments, including the wounding-control, mainly formed lanceol. Overtime, santalol oil production increased.

In the second experiment, whole-tree application was tested to avoid wounding the tree. Two chemicals, ethylene as a foliage spray of ethrel, and the gibberellin-inhibitor, paclobutrazol as a root drench, were chosen for whole tree-treatment. Due to the concern with foliage absorption, on a follow-up trip, ethrel was applied as a root drench. Both treatments showed an increase in sandalwood oil production, with the paclobutrazol having the greatest effect. This may have been due to chemical absorption and needs further exploration.

What impacts has the project had or is it likely to have in the future?

- With wounding, Vanuatu sandalwood reacts to produce lanceol rather than the santalols. Santalols are the preferred component of sandalwood oil.
- Ethylene was the only treatment tested which sandalwood responded by immediately producing santalols when forming heartwood. This supported the assumption that the growth regulation process by which sandalwood oil is produced is a regulated senescence process
- Sandalwood oil production increased with a root drench of paclobutrazol or ethrel treatment. A foliar spray of ethrel also had a positive response but there was concern on efficacy of uptake.
- Enzyme measurement reflected the oil measurements and showed that this approach could be followed for quicker treatment-response assessment.
- At 10-years of age, *S. austrocaledonicum* is immature, that is true heartwood had not reliably formed. Projects need to plan for a rotation that takes into account the initiation of heartwood at 10-years of age.

What future actions might be required?

1. Larger, long-term trials to test the economic benefit of the treatments.
2. Education on heartwood formation in sandalwood as there is a misunderstanding that wounding can initiate heartwood. Wounding initiates stress-induced development and not the age-related development that forms true heartwood.
3. A better understanding on how heartwood formation and presence is modelled within a tree would assist in this education program.

3 Background

Sandalwood has been an important part of Vanuatu's economy. Extensive extraction and export of sandalwood between 1820 and 1850 was the region's first international commercial industry (Shineberg 1967). Since then, sandalwood export has been intermittent, reliant on natural populations re-establishing and growing to an acceptable commercial size before they can be harvested (Gilleson et al. 2008). Regrowth has supported a small commercial industry since the 1970s, making sandalwood a contributor to a modest, but important, cash economy in Vanuatu. When the gross domestic product per capita in Vanuatu is USD 2960, selling a mature sandalwood tree would make a significant impact to a household. As sandalwood can be harvested and stored without product deterioration, smallholders can schedule the delivery of their trees when the family requires cash.

Rural communities and plantation owners have embraced the establishment of Sandalwood trees. Being a high-value, low-volume, non-perishable product that is internationally in demand, it fits comfortably into Vanuatu's export capacity. Wild Sandalwood collection earns needed foreign exchange and demand outstrips supply. Creating a sustainable source of high oil-producing Sandalwood in Vanuatu would help satisfy global demand and provide needed economic sustenance to rural communities and newly established plantation companies

ACIAR has supported sandalwood expansion through focussed research effort in Vanuatu and other countries in the region. Research work demonstrated the financial benefits of planting sandalwood in Vanuatu (FST/2002/097, FST/2006/118) and this stimulated investments in planted sandalwood at both the commercial and smallholder scale. ACIAR has also supported germplasm improvement and its deployment to smallholder growers (FST/2006/118, FST/2008/010, ACIAR Technical Report No. 79). These research outputs combined with the wide distribution of the "Sandalwood growers guide" (ACIAR Monograph No. 151), has resulted in the improved growth rate and quality of sandalwood agroforestry in Vanuatu.

3.1 Oil composition

The amount of heartwood a sandalwood tree produces as it matures, and the sandalwood oil accumulation within this heartwood, determines its value. Physiologically, heartwood forms within a tree to protect its inner core from pests and disease by the laying down of protective chemicals, which in the case of sandalwood, is sandalwood oil. Heartwood chemical deposits occur in many tree species (Taylor et al 2002). Ecologically, these chemicals provide long-term protection against pathogens and microbial decay (Celedon and Bohlmann, 2017). In Sandalwood, the chemical complex consists of a number of chemical components called "sesquiterpenes" which form sandalwood oil.

Table 1: Commercial value of Sandalwood oil from different species (2014) to demonstrate the varying value between Sandalwood species (Chris Jones pers.comm.)

Sandalwood species	Value (US$ per Kg)	% value
S. album - Indian Sandalwood	$74	100
S. yasi	$45	61
S. austrocaledonicum	$38	51
S. macgregorii	$25	34
S. spicatum	$10	14
S. lanceolatum	$5	7

Species within the sandalwood genus all have defined geographical distributions and each varies in its Sandalwood oil profile. The change in components of the oil profile make

some species more favoured than other others (Table 1). Sandalwood oil profiles are analysed using Gas Chromatography. This analytical technique's mobile phase is a carrier gas, and once the sandalwood oil is injected and instantly vaporised, control of the flow rate of this carrier gas gives the clearest separation of the components in the sample (Figure 1).

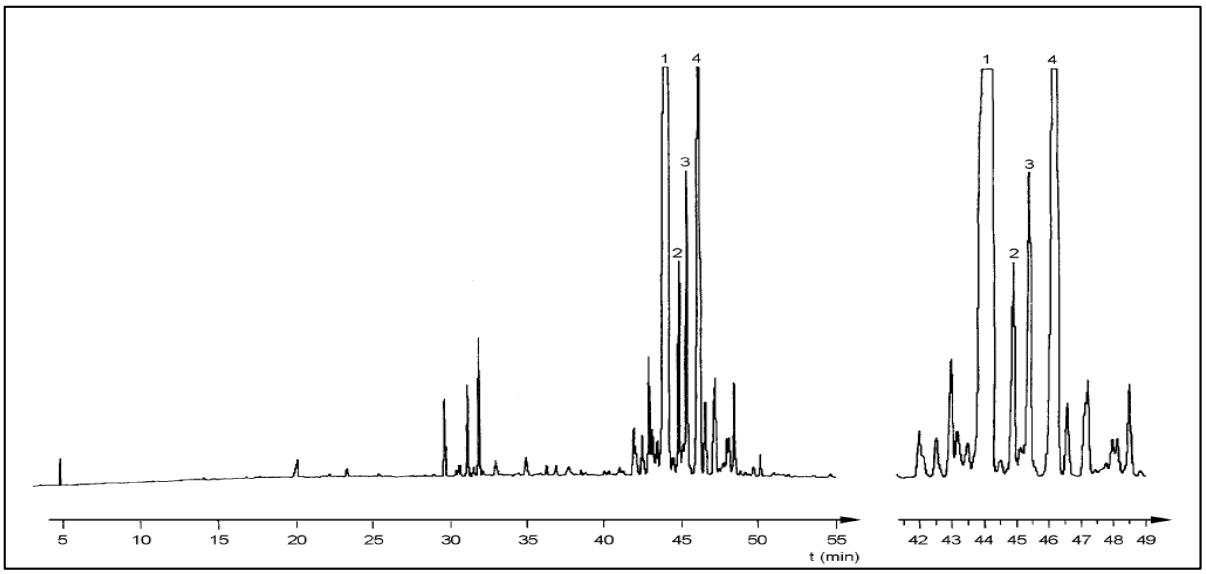

Figure 1: A chromatograph produced using Gas Chromatography to show the sandalwood components. The X axis is the gas retention time to show the separation order of the components and the Y axis shows the intensity enabling a quantity of each component to be calculated. From ISO 2002; IS 329 2004 S. album. The numbered peaks identify the santalols. The peaks between 30 and 36 minutes on the x axis represent the santalenes, precursors to the santalols

Commercial standards have been developed for two key Sandalwood commercial species *S. album* (titled Oil of sandalwood *Santalum album* L. or referred to as ISO 2002; IS 329 2004) and *S. spicatum* (titled Oil of Sandalwood or referred to as "AS 2112:2003") that defines Sandalwood oil to have a dominance of eight sesquiterpene compounds in its chemical profile (Table 2). Two of these compounds, "Z- α-" and "Z-β-santalol" determine the commercial quality of Sandalwood oil. Sandalwood oil sourced from a *S. album* must contain between 41-55% Z- α-santalol and 16-24% Z-β-santalol.

Table 2: Chromatic profile for Sandalwood oil (Australian standard AS 2112-2003. Oil of Sandalwood (Santalum spicatum (R.Br) A.DC.) and Oil of sandalwood (Santalum album L.) or referred to as ISO 2002; ISO 329 2004

Constituent	Proportion, percent			
	S. spicatum		*S. album*	
	Minimum	Maximum	Minimum	Maximum
Z-α-Santalol	15	25	41	55
epi-α-Bisabolol	2	12.5		
Z-β-Santalol	5	20	16	24
epi-β-Santalol	0.5	3.5	present	
Z-α-trans-Bergamotol	2	10	present	
E,E-Farnesol	2.5	15		
Z-Nuciferol	2	15		
Z-Lanceol	2	10		

As yet, no sandalwood oil standard has been developed for *S. austrocaledonicum*. Page et al (2010) referred to α and β santalol, cis-Nuciferol and (Z)-β-Cureumen-12-ol and their variation when assessing trees across the islands.

These different components in the sandalwood oil are synthesised by enzymes which are formed by genes (Figure 2). From the origin component produced by the plant, farnesyl pyrophosphate, the gene, SaSSy, produces the enzyme santalene synthase which produces four santalenes (α-santalene, β-santalene, epi-β-santalene and α-exo-bergamotene). The gene SaCYP736A167 produces a P450 enzyme which converts the santalenes into the valuable santalols, (Z)-α-santalol, (Z)-β-santalol, (Z)-epi-β-santalol and (Z)-α-exo-bergamatol. These are the main components of *S. album* sandalwood oil (Jones et. al 2011).

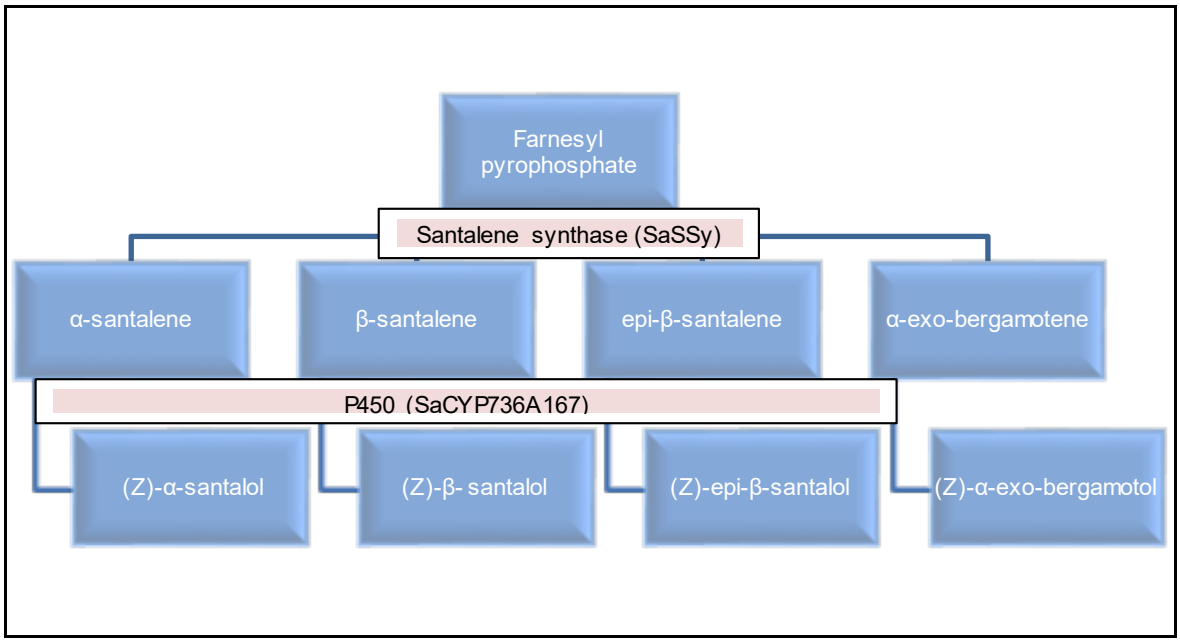

Figure 2: The chemical precursor for the sesquiterpenes is farnesyl pyrophosphate (FPP). The gene, SaSSy produces the enzyme santalene synthase which produces four santalenes (α-santalene, β-santalene, epi-β-santalene and α-exo-bergamotene). The gene SaCYP736A167 produces a P450 enzyme which converts the santalenes into the santalols, (Z)-α-santalol, (Z)-β-santalol, (Z)-epi-β-santalol and (Z)-α-exo-bergamatol.

3.2 Heartwood initiation

Sandalwood oil accumulates within the heartwood as the tree matures. Heartwood initiates from the base of the tree bole and spreads up the tree, with a small expansion downwards into the roots (Figure 3).

A juvenile tree consists of sapwood only. The scientific literature states that a tree is mature when there is a near constant proportion of heartwood to the total tree diameter (Pinto et al., 2004). Past Indian reports refer to sandalwood trees (*S. album*) initiating heartwood around age 10, whilst stating that heartwood formation could be delayed between 14 to 46 years. Even in mature trees the amount of heartwood was found to be highly variable (Rai, 1990; Haffner 1993; Venkatesan et al 1995).

The continued production of heartwood as the tree ages gives rise to the hypothesis that if heartwood is initiated its development will continue through the life of the tree. Heartwood formation requires a diversion of resources from new growth to the laying down of Sandalwood oil within the

Figure 3: Diagram of a Sandalwood tree showing the heartwood initiation at the base of the tree and its spread through the tree as it matures (red arrows).

core of the tree. The result is that Sandalwood tree growth regresses as the tree matures (Barbour et al., 2012; Figure 4).

Sandalwood heartwood formation is unusual (Celedon and Bohlman, 2018). Whilst most of the active genetic material (RNA) is in the sapwood and transition zone between the sapwood and heartwood, and there is ten-fold less of RNA in the heartwood, this heartwood RNA contained the gene transcripts related to the sandalwood oil biosynthetic pathway. This indicates that, whilst cells are dying in the heartwood, other cells remain alive and active producing the sandalwood oil. This accounts for the continuing increase

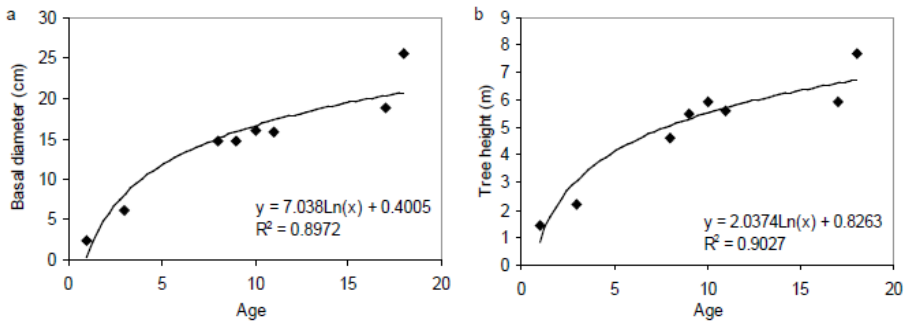

Figure 4: Diameter (a) and height (b) of Sandalwood grown with the long-term host Cathormium umbellatum at a 1:1 ratio starting with 462 Sandalwood stems per hectare (Barbour et al., 2012). This graph shows rapid Sandalwood growth with a gradual slowing from approximately 10 years onwards.

of heartwood sandalwood oil content over time.

Whilst the biosynthetic process of Sandalwood oil production is now understood (Jones et al., 2011; Celedon et al., 2016), the 'signal' that initiates heartwood production and the deposit of sandalwood oil is unknown. The genes themselves that produce the enzymes are all present in every sandalwood tree, so there is another factor that is controlling the genes to make the enzymes for the production of sandalwood oil.

3.3 Silvicultural management of heartwood production

Silvicultural management to promote heartwood production is a novel concept. This Project aims to increase the impact of previous ACIAR research investments by improving the value of Sandalwood plantings through heartwood management

Rapid growth in the first years of a sandalwood tree are important to determine the ultimate tree size and total amount of heartwood that can be produced (Figure 4). Ideal is for the tree to optimise its growth potential during the juvenile stage before the maturation process diverts resources into heartwood and sandalwood oil production. With the assurance that there is a reliable practical treatment to initiate maturation, landholders will be able to focus on tree growth, and then manipulate the trees to switch to heartwood oil production when they are preparing for tree harvest.

Heartwood formation is not an easily predictable pre-programmed event, but one that initiates by (presently) unknown internal or external signals. Heartwood formation is beneficial to tree survival for three main reasons:

A. Structural support is provided as the tree grows in stature with heartwood cell walls thickening and cells filling with viscous secondary metabolites
B. Nutrient and water demand reduce with the conversion of living sapwood with a high respiration rate into dead heartwood. The primary role of sapwood in a tree is to conduct water from the roots to the crown (Garner, 1995) with the amount of foliage on a tree often strongly correlating with the amount of sapwood (Berthier et al., 2001;

Ryan 1989). Costs are associated with maintaining sapwood in a tree with the respiring parenchyma consuming considerable amounts of energy (Ryan et al., 1995).

C. Disease and insect resistance increase within the inner core of the tree bole with removal of nutrients attractive to pests and the synthesis of deterrent secondary metabolites. Sapwood contains energy reserves such as carbohydrates with few toxic metabolites, so it is generally susceptible to infection when wounded (Hillis 1987; Ryan 1989). When programmed senescence progresses, many tree species recycle their nutrients (Bamber and Fukazawa, 1985) and these heartwood-forming cells lose their ability to conduct water and lose moisture. As a result, pathogens find heartwood less attractive as it lacks needed nutrients and moisture for their sustained establishment (Scheffer and Cowling, 1966).

Senescence, and the repartitioning of growth substances before the tissues die, is a possible growth regulation process that forms heartwood. Heartwood formation occurs when a series of 'messages' are received that stimulate plant hormone production and a growth regulation response. Chemicals that mimic the plant regulations responses, particularly involved in senescence, will be the focus of this Project.

3.4 Treatment selection

The treatments chosen focussed on manipulating plant regulation, particularly senescence. Senescence is the process of aging. Plants have both stress-induced and age-related developmental aging. Heartwood formation is an age-related senescence process.

Important plant regulation signalling compounds following plant stress and that activate defence responses are ethylene, jasmonate and salicylate (Reymond and Farmer 1998; Vlot et al. 2009; Zhu 2014; Dar et al. 2015). Ethrel is a systemic plant growth regulator belonging to the phosphonate family. It is readily absorbed by the plant and releases ethylene which is a natural plant hormone. Ethylene influences directly several physiological processes (ripening, maturation etc.) and stimulates the production of endogenous ethylene. Methyl jasmonate is a methyl ester widely distributed in the plant kingdom. It was first detected as a fragrant compound present in Jasmine oil. Methyl jasmonate is known to regulate plant growth in response to environmental stresses. Salicylic acid is found in plants with a role in plant growth and development, photosynthesis, transpiration, ion uptake and transport. It can play a role in plant resistance to pathogens by inducing the production of pathogenesis-related proteins.

Exogenous application of ethylene, jasmonate and salicylate and their analogues can elicit defence responses in plants (Enyedi et al. 1992; Xu et al. 1994; Benhamou and Bélanger 1998; Kouzai et al. 2018) including their contribution to the production of heartwood and extractives in trees (Shain and Hillis 1973; Lv et al. 2019). Examples provided are with *Quercus serrata* (Moungsrimuangdee et al. 2011), two coniferous species *Pseudotsuga menziesii* and *Sequoiadendron giganteum* (Hudgins and Franceschi 2004), the hardwood hybrid species *Populus tremula x tremuloides* (Lesniewska et al. 2017) and *Acacia auriculiformis* (Baqui et al. 1984) with varying responses. In the Sandalwood genus, stem injection of 2 and 3% ethephon into 3 year-old *S. album* trees had a stimulatory effect on heartwood development relative to water injections (Yun 2012) with 3% ethephon resulting in greater oil content and santalol content.

Besides ethylene, jasmonate and salicylate, three other treatments were chosen that represent the auxins, cytokinins and a gibberellin-inhibitor. In some instances, the application of an auxin can stimulate ethylene production however it is mostly known to encourage the elongation of shoots. Cytokinins stimulate cell division in plants. Among other actions, it spurs plant growth, sets blossoms, and improves fruit quality. Paclobutrazol acts by inhibiting gibberellin biosynthesis, reducing inter-nodal growth to give stouter stems, increasing root growth, causing early fruit-set and increasing seed-set in plants.

3.5 Treatment application

Application methodology of these chemicals into the tree requires consideration. The cambium tissue (immediately under the bark and the growing tissue of the tree bole) plays an active role in heartwood formation (Figure 5).

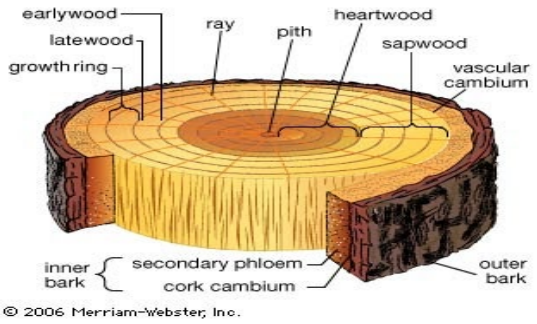

Figure 5: A cross section of a maturing bole (trunk) of a tree showing the outer bark, the living tissue of cambium and phloem making up the vascular cambium, sapwood and heartwood, with the pith in the centre. The rays are living tissues that connect the cambium through the sapwood to the heartwood. Source: Archistruct: Learning Architecture from Structure. Notes - Structure of Wood

When wounding a sandalwood tree (from mechanical damage, sun-scorch or pruning), the cambium tissue immediately elicits a stress-response. Similar to human wounds, the "protection" chemicals, or extractives, deposit around the circumference of the wound to prevent infection penetrating further into the tree (Hillis, 1987). Although stress-wound response "protection" chemicals share some chemical characteristics with heartwood formation chemicals, this stress response is not true heartwood (Shigo and Hillis 1973) and nor is the biosynthetic pathway that makes these "protective chemicals" necessarily the same (Magel 2000). In *S. album*, both heartwood formation and the wounding stress response produce compounds that contribute to the generation of sandalwood oil (Barbour et al., 2010).

Drilling through the bark to apply the chemical treatments will cause a stress-wound response. A control of wounding and with no treatment has been added so that the difference between a stress-induced or age related stimulus of heartwood formation can be discerned.

Figure 6: Eight year old *S. album* tree showing the effect of wounding and the response of the tree to protect the tree by laying down stress-induced heartwood (Barbour et al., 2012)

4 Objectives

The project aimed to identify practical methods to increase heartwood development in Vanuatu Sandalwood. This was to be achieved by accomplishing the following objectives:

1. To synthesise the current literature on heartwood formation in Sandalwood
2. To establish research and site relationships for Sandalwood heartwood stimulation experiments in Vanuatu
3. To test and identify compounds that stimulate heartwood formation in tree branches
4. To explore non-invasive treatment applications that stimulate whole-tree oil production
5. To promote an understanding of heartwood formation and silviculture techniques to stimulate its production in Vanuatu through close collaboration with the Vanuatu Forest Department

The expected outcome was to deliver to Sandalwood growers in Vanuatu are able to harvest their Sandalwood trees earlier and with reliable levels of Sandalwood oil.

5 Methodology

5.1 Consultation

Stakeholder meeting 1: Vanuatu Forestry Department

Discussed Project plan:

- Review of literature
- Experiment 1 on branches
- Experiment 2 on whole trees

The assistance was offered as well as the use of the old ACIAR vehicle.

Stakeholder meeting 2: Summit Estate

Discussed Project plan and the following questions asked:

Q1. How were we going to manage the genetic variation?

Treatments for the first experiment applied within one tree so there is no genetic effect within the replicate. Each tree would be assessed for its heartwood status prior to treatment.

Q2. Why did we want older trees?

Ten-year old trees are preferred as the treatments are to stimulate heartwood formation which requires a level of maturation before the process can occur.

5.2 Trial site selection

Cyclone 'Pam' passed through Vanuatu in 2015 prior to the start of the experiment, damaging many trees. This created a high level of background wounding making statistical analysis and interpretation challenging.

Both Experiment 1 and 2 occurred within the same trial site on Efate, Vanuatu. The trial site is located on 'Summit Estate', approximately 5km north-east of Port Vila. Site access is from Port Vila, via the Mele Road, turning left into Devils Point Road and then right onto the 'Summit Estate' turn-off (17°40'55.69"S, 168°13'46.54"E, Altitude 223m) (Figure 4).

Sandalwood trial site 868ATH was established in May 2007 as part of an ACIAR research project, "Identification of optimum genetic resources for establishment of local species of Sandalwood for plantations and agroforests in Vanuatu and Cape York Peninsula, FST/2002/097" (Dickinson and Page, 2007).

The original trial design was in rows 3 metres apart. Within each row, Sandalwood was planted every 6m with a long-term host between each Sandalwood and a short-term host planted 1.5 metres from the Sandalwood.

The long-term hosts were either *Canarium indicum* (Nangai); *Casuarina equisetifolia* (Sheoak); *Micromelum minutum* (Lime Berry) or *Pterocarpus indicus* (New Guinea rosewood). The short-term host was *Cajanus cajan* (Pigeon pea). Host survival was uneven (Figure 7).

Figure 7: Location of Sandalwood host trial, Port Vila (868ATH) used in Experiment 1 and 2 showing the region (top) and 'Summit Estate' and the position of the trial site (bottom).

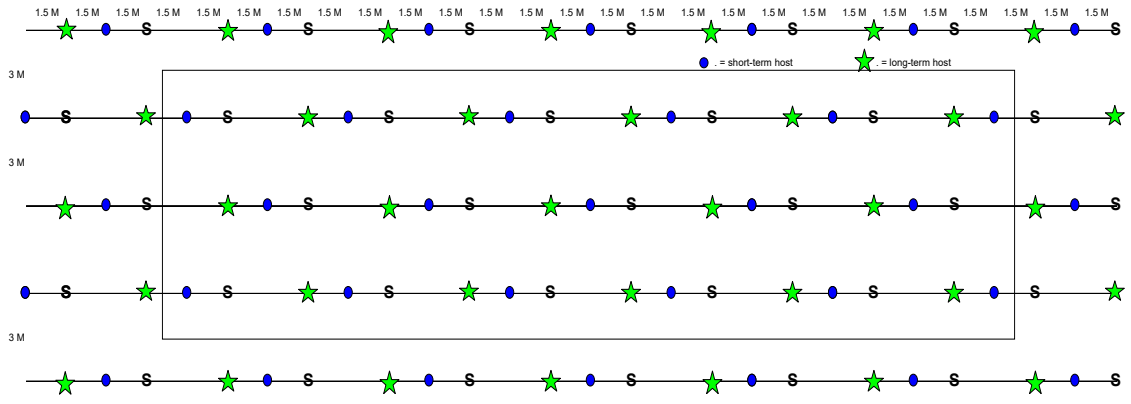

Figure 8: Layout of 868ATH trial site at planting. ★ is the long-term host, ● is the short-term host with **S** being Sandalwood

5.3 Experiment 1 – branch treatments

The first experiment tested a wide range of chemicals on their ability to upregulate sandalwood oil production in *S. austrocaledonicum*. Fifteen 10 year old trees were selected and grouped into three harvest times of 8, 16 and 32 weeks. Each tree was clearly marked with a spray painted ring at breast height (130 cm from the ground) and numbered 1–15.

Each tree had bark-to-bark wood cores extracted from the main stem at 20cm above ground level using a Haglof 5.15mm 2T increment borer (Figure 9a). After bark removal, the cores were wrapped and labelled for transport back to Perth. These cores were assessed for the presence of heartwood and the extent of variation in sandalwood oil (sesquiterpene content) across trees (Appendix 1). Silicon sealed the core holes in the tree stem.

Primary canopy tree branches (> 4cm diameter) were mechanically drilled producing holes at least 25 cm apart along the branches and of the same width and depth (6 mm x 32 mm) as the dowel inserted into the drilled hole (Figure 6b). The tree branch was labelled and treatments positions recorded (Appendix 2). Drilling shavings were captured in an envelope to be checked for Sandalwood oil prior to treatment. The dowel was soaked in one of seven inoculating solutions:

a) BAP = 6-benzylaminopurine (10 ppm) – a synthetic cytokinin
b) Ethrel = ethephon (10 ppm) - produces ethylene
c) IBA = indole-3-butyric acid (10 ppm); - a synthetic auxin
d) MeJ = methyl jasmonate (10 ppm));
e) MeS = methyl salicylate (10 ppm);
f) Paclo = paclobutrazol (4g/l);
g) Wounding control = distilled water

Figure 9: A. Taking a core from the base of the tree prior to branch treatment. **B**. Placing the treated dowel into the drill hole in the tree.

Branches from four randomly selected trees (biological replicates) were harvested at 8, 16 or 32 weeks after inoculation. The branch was cut 2 cm distant from either side of the wound site, and the branch cut in half (approximately 5 g total). One small section, used for RNA isolation, was immediately snap frozen in liquid nitrogen for transport back to Perth and stored at -80°C until analysis. The remaining sample was air dried and kept at ambient temperature until oil extraction and analysis by GC.

5.4 Experiment 2 - canopy and root treatments

The second experiment tested the non-invasive application of two plant growth regulators, ethrel and paclobutrazol, on heartwood oil production. To determine an appropriate sample size a power analysis was conducted using the R software package (R Core Team 2016; for full details see R script, available as Supplementary Material) before data collection using a moderate effect size (0.25) and a power of 0.80 (giving an 80% probability a type II error will not be committed). Based on the output, a sample size of 25 was selected as the minimum number of trees per treatment. Within the plantation, a maximum of 73 trees were available. This number allowed a maximum of two treatment applications which were selected on the basis of results from the first experiment.

A preliminary experiment was undertaken by Dr Tony Page in Queensland testing the Ethrel concentrations (Appendix 1). The treatment chosen was based on the level of defoliation.

Plantation trees were randomly selected for application of either

I. Ethrel as a canopy spray (n=24). Ethrel treatment comprised of 1L at a 0.1% concentration applied to each tree as a canopy spray
II. Paclobutrazol as a root drench (n=24). Paclobutrazol was applied as a root drench with 2g a.i. in 2L of water for each tree.control trees
III. Ethrel drench was an additional nine trees (n=9) treated with ethrel as a root drench.
IV. Control of no treatment (n=23)

Two treatment applications were applied: one at the commencement of the experiment (15th December 2017) and another 6 months later (18th June 2018).
Bark-to-bark wood cores were collected at 20 cm above ground level at 12 months (2018-12-12) after the first treatment. These wood cores were air dried and kept at ambient temperature until oil extraction and analysis by GC.

5.5 Oil extraction and analysis

Heartwood and sapwood distinction was determined by colour (sapwood- pale/yellow; heartwood- dark/brown or red), and samples of each were coarsely ground for oil extraction. Each sample between (~ 0.1-2.0 g) of wood shavings or ground wood cores was mixed with 10 ml of hexane, spiked with 10 mM of isobutyl benzene as an internal standard (Sigma Aldrich, Louis, MO, USA), and periodically shaken over 2 days. Samples were centrifuged at 1000 g for 10 minutes and the clarified hexane transferred into Gas Chromatography vials for analysis with GC-FID (for quantification) and GC-MS (for qualitative analysis). To determine the amounts of sesquiterpenes, the relative response factors were calculated for known standards (α-bisabolol, farnesol cis-nerolidol) as previously described in Moniodis *et al.* (2017).
Gas chromatography conditions were as follows: stationary phase; DB-Wax column (30 m long, 0.25 mm ID, 0.25 µM film thickness, Agilent Technologies, Santa Clara, CA, USA). Carrier gas was helium at 1 mL/min and pulsed pressure set at 172 kPa for 0.5 min. The injector was operated in pulsed split mode (1:10), with the injector temperature maintained at 250°C. Oven was programmed at 40°C for 1 minute, then raised at 10°/min to 130°C. This was followed by a steady temperature increase by 2°C/min to 200°C, and 20°C/min

to 240°C for a further 10 min. Scan mode was used over the range of 40-250 m/z. Run conditions for GC-MS were similar to that of GC-FID, except the detector was opened 5 min after injection.

5.6 Total RNA extraction and cDNA synthesis

Benchtops and equipment were treated with RNaseZAP™ (Sigma Aldrich, Louis, MO, USA) prior to extraction; as were the glassware, spatulas and the mortar and pestle used for grinding tissue. Treatment was followed by oven drying at 180°C for 16 hours. Plasticware (tips and tubes) were certified as RNase- and DNase-free (Life technologies, California, USA).

Wood tissue was finely powdered in liquid nitrogen using a mortar and pestle, and then stored at -80 °C prior to extraction. Total RNA was extracted from ~ 300 mg of powdered wood tissue using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich, Louis, MO, USA) and protocol A, including the on-column DNase I digest for removing traces of DNA. . Modifications to the protocol included: adding 1000 µl of buffer per 100 mg of tissue, increasing the initial incubation time in lysis buffer to 8 min, adding an extra wash step for wash solutions 1 and 2 for dark colour samples, and combining the lysate to improve recovery amounts since low yields occur from the wood-tissue.

RNA was quantified by measuring the absorbance at 260 nm with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). The purity of RNA was assessed at an absorbance ratio of $OD_{260/280}$ and $OD_{260/230}$. Only samples with an $OD_{260/280}$ nm absorption ratio > 1.9 were used for downstream applications. Due to the low amount of recoverable RNA, RNA integrity was not verified using agarose gel electrophoresis. Instead, product specificity was assessed by amplifying a full-length santalene synthase (> 1600 bp) from *S. austrocaledonicum* (Jones et al., 2011), as well as PCR products of selected reference and expression genes. Products were checked with 1% agarose gel electrophoresis and resulted in a single product with the desired length (~ 100 bp). A total of 1.4 µg of RNA was used to synthesise first-strand cDNA using SuperScript™ VILO™ MasterMix according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The synthesised cDNA was diluted to a final concentration of 10 ng/µl and stored at -80 °C until RT-qPCR analysis.

5.7 Candidate reference genes

Sequence and expression information from a transcriptomic study on *Santalum album* (Celedon *et al.* 2016) was used to select candidate reference genes, since it is known that expression levels of reference genes can differ under environmental conditions (Radonić *et al.* 2004). A total of 468 transcripts were found to be stably expressed across sap wood, transition zone and hardwood tissue in four *S. album* trees (log_2fold-change < 1, p-value < .05, FDR < .05). Analyses using the R package DESeq2 and applying a Wald test of log^2 fold change above or below a threshold were done.

Eight candidate reference genes, showing high expression in all tissues with annotations typical of "housekeeping genes", were selected to test their stability and suitability for expression studies in *S. austrocaledonicum* (Table 3). The PrimerQuest tool (Integrated

DNA Technologies, Illinois, USA) was used to design primers for these analyses The validity of these primers was evaluated prior to conducting RT-qPCR (Table 2), using ten randomly selected samples which included no wounding controls, wounded or chemically treated branches at either 8 or 16 weeks.

a) Since reference genes are used to normalise expression of target genes, their expression must be unaffected across the sample and test conditions to be compared. The following tables indicate the references genes tested (Table 3) and their suitability using various programs (Table X).

Table 3: Selection of candidate House-Keeping Genes (HKG's) for *S. austrocaledonicum*. Sequences based on a *S. album*

House-Keeping Gene	Predicted Sequence description
HKG1	Proteasome subunit beta type-1
HKG2	60S ribosomal protein L18-2 [Cucumis *melo*]
HKG3	Pre-mRNA-splicing factor SF2-like [*Cucumis sativus*]
HKG4	Pre-mRNA-splicing factor SLU7 [*Phoenix dactylifera*]
HKG5	Tubulin alpha-1 chain [*Panax ginseng*]
HKG6	Transcription initiation factor IIF subunit beta-like [*Cucumis melo*]
HKG7	Eukaryotic initiation factor 4A-3-like [*Vitis vinifera*]
HKG8	Translation initiation factor IF2/IF5 isoform 1 [*Theobroma cacao*]
HKG9	Eukaryotic translation initiation factor 3 subunit H [*Vitis vinifera*]

The stability of selected reference genes was tested using these approaches:

1. BestKeeper (Version 1) as an excel-based software (Pfaffl et al., 2004),
2. geNorm (qbase+) (Vandesompele et al., 2002),
3. NormFinder (Version 0953) algorithm used as an Excel add-in (Andersen et al., 2004) and the online RefFinder platform (http://www.leonxie.com/referencegene.php) was used to assess the output of these software packages (Xie et al., 2012).

Based on results from software outputs and primer efficiencies, HKG1 and HKG7 were selected as reference genes for this study

Table 4: Results from analysis of candidate House-Keeping Genes (HKG's) for *S. austrocaledonicum* using output of four programs. Sequences based on an *S. album*

	GE Norm	BestKeeper	RefFinder	NormFinder
	Stability value (M)	SD	Geomean of ranking values	Stability value
HKG8	0.357	0.770	1.860	0.257
HKG1	0.380	0.860	2.110	0.223
HKG7	0.389	0.630	2.780	0.409
HKG4	0.484	1.060	3.830	0.192
HKG5	0.573	1.270	4.140	0.086
HKG6	0.633	0.820	4.950	0.476
HKG2	0.710	0.710	4.920	0.706
HKG3	1.311	3.060	8.000	2.251

b) Selection of genes for expression studies and primer efficiency experiment
Limited sequence information for *S. austrocaledonicum* meant that design of most primers was based on sequence information available for *S. album*. There appears to be enough sequence similarity between these two species (and possibly other sandalwood species) to test gene expression levels. We selected the following sequences:

i) SaHMGCoA reductase (key regulatory enzyme in cytosolic MVA pathway)
ii) SaDXR (key regulatory enzyme in plastidic MEP/DXP pathway)
iii) SaFPPS (farnesyl pyrophosphate synthase; produces FPP, the precursor to sesquiterpenes)
iv) SauSSy (santalene synthase; S. austrocaledonicum)
v) SauBIS (bisabolene synthase, S. austrocaledonicum)
vi) SauSTPS (sesquiterpene synthase, S. austrocaledonicum)
vii) SaCYP76F39QV1 (E-santalol hydroxylase, S. album)
viii) SaCYP736 (Z-santalol hydroxylase, S. album)

PCR efficiency is a key indicator of the performance of a qPCR assay and is needed when fold changes are calculated in a quantitative analysis (Svec et al., 2014). For each gene, a standard curve with five concentrations was made using 4-fold dilution steps. A plot of Cq value versus log concentration was constructed giving a negative linear slope. The PCR efficiency was consistently high for selected genes (90%> and <110%) except HKG8 (84%)PCR efficiency was estimated by constructing a standard curve involving a series of samples with known amounts of target template. The sample was constructed by a 5-point serial dilution of a concentrated stock solution using 4-fold dilution steps (0.05, 0.05, 0.25, 5 and 25 ng template concentrations). Amplification efficiency ranged from 95 to 101 % of all selected primers, which falls within the accepted range of 90 to 110 %. In the absence of interfering substances, DNA is amplified with at least 90 % amplification efficiency (Svec et al 2014) (Figure 10).

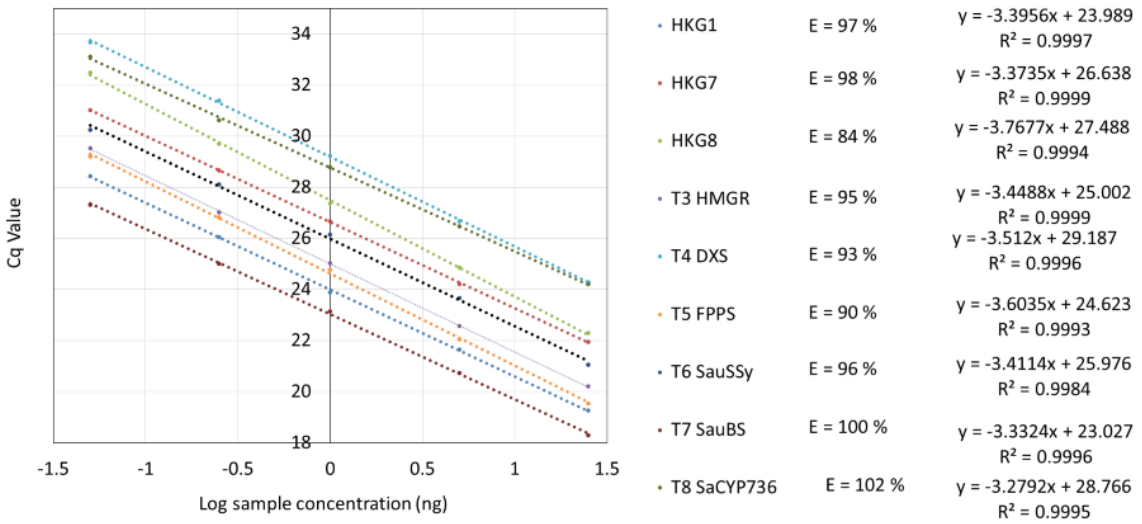

Figure 10: PCR Efficiency experiment to indicated performance of a qPCR assay. For each gene, a standard curve with five concentrations was made using four-fold dilution steps. A plot of Cq value versus log concentration was constructed giving a negative linear slope

Table 5. SYBR green qPCR efficiency of selected housekeeping and expression genes for *S. austrocaledonicum*

Gene	Slope (m)	Y-intercept	Correlation coefficient	Efficiency E = [10(1/-m)-1] x 100%
HKG1	-3.3956	23.989	0.9997	97
HKG7	-3.3735	26.638	0.9999	98
HKG8	-3.7677	27.488	0.9994	84
HMGR	-3.4488	25.002	0.9999	95
DXS	-3.512	29.187	0.9996	93
FPPS	-3.6035	24.623	0.9993	90
SauSSy	-3.4114	25.976	0.9984	96
SauBS	-3.3324	23.027	0.9996	100
SaCYP736	-3.2792	28.766	0.9995	102

Real-time quantitative PCR (RT-qPCR) was performed on an ABI 7500 real-time system. The cDNA of each sample representing one biological replicate was diluted to a working concentration of 10 ng/µl for the RT-qPCR analysis. The melt temperature was 55°C and product contained between 98 and 113 base pairs (Table 3). The 10 µl reaction contained 2.5 µl of diluted cDNA, 5 µl of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, California, USA), and 2.5 µl of diluted primer mix which contained 320 nM of primers for each reaction. The cycling parameters were as follows: 50 °C for 2 min (UDG activation), 95 °C for 2 min and 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 1 min. The 5-fold standard curve, which determined the PCR efficiency, was used to convert the quantification cycles (C_q-values) into the relative quantities (relative gene expressions). The relative quantity of target transcripts was measured against HKG1 and HKG7 gene transcripts as the internal standard by calculating $2^{-\Delta\Delta CT}$. Triplicate assays were performed for each sample. A dissociation step was performed at 1.6 °C/sec at 95 °C for 15 sec, 1.6 °C/sec at 60 °C for 1 min and 0.15 °C/sec at 95 °C for 15 sec.

5.8 Statistical analysis

All statistical analyses used the R software package (R Core Team 2016). A shapiro-wilcox test was first used to check whether components followed a normal distribution and P values >0.05 indicated they did not significantly deviate from normality. Differences were accepted as significant if $P < 0.05$. Parametric (ANOVA, Tukey's test) and non-parametric statistics (Friedman's, Kruskall-Wallis) were used for continuous variables normally and not normally distributed respectively. In Experiment 1, using branch samples, the Friedman test was used because the study was a non-randomised block design and for combining all weeks, a Kruskal-Wallis test was used.

For each qPCR treatment, reactions were set up with three biological replicates. For the treatments, changes in relative transcript abundance were expressed as the ratio between treatments and its specific control collected at the same time and grown under the same conditions. A mean value of the triplicate was used for the calculation. The ratio with a P-value <=0.05 was adopted as significant for down- or up-regulation (Duan et al, 2010 in wounding, gene expression folder).

6 Achievement against activities and outputs/milestone

Objective 1: To synthesise the current literature on heartwood formation in sandalwood

no.	Activity	outputs/ milestones	completion date	comments
1.1	Collate current results and literature	Finalise publication of recent outputs from research group - A	April 2017	The following papers were drafted and four submitted and accepted for publication Moniodis J, Jones CG, Renton M, Plummer JA, Barbour EL, Ghisalberti EL, Bohlmann J (2017) Sesquiterpene variation in West Australian sandalwood (*Santalum spicatum*). Molecules 22, 940. Moniodis J, Renton M, Jones CG, Barbour EL and Byrne, M (2017) Genetic and environmental parameters show associations with essential oil composition in West Australian sandalwood (*Santalum spicatum*). Australian Journal of Botany 66(1) 48-58 https://doi.org/10.1071/BT17116. Celedon, J and Bohlmann J (2018) An extended model of heartwood secondary metabolism informed by functional genomics. Tree Physiology 38 (3) 311–319. Burgess, TI Howard K Steel E, Barbour EL (2018) To prune or not to prune; pruning induced decay in tropical sandalwood. Forest Ecology and Management 430 204-218 Tungngoen K, Moniodis J, Flematti G, Burgess TI, Plummer JA, Barbour EL, Finnegan ( ) Oil production in *Santalum album* is enhanced by wounding and fungal infection. In preparation
1.2		Delay publication of review - A	January 2020	With the publication of the outstanding heartwood model review from Celedon and Bohlmann, the delivery of the review was delayed till after the completion of this work. Draft being finalised for submission.

Objective 2: To establish research and site relationships for sandalwood heartwood stimulation experiments in Vanuatu

no.	Activity	outputs/ milestones	completion date	Comments
2.1	Establish research and site relationships for sandalwood heartwood stimulation in Vanuatu	Site and research team established - PC	March 2017	Key relationships were established with the Vanuatu Forestry Department and Summit Estate. Dr Tony Page and Dr Liz Barbour travelled to Vanuatu to discuss the project, the role of the Forestry Department and Summit Estate and how the results will be communicated across Vanuatu.

Objective 3: To test and identify compounds that stimulate heartwood formation in tree branches

no.	Activity	outputs/ milestones	completion date	Comments
3.1	15 trees selected, measured, clearly identified and a core removed from the base of the tree	Base line of heartwood and sandalwood oil production from the experimental trees established	7-10 March 2017	Complete PC
3.2	15 tree branches treated with 7 treatments. Each treatment position labelled, a diagram and picture made of the tree	Exp 1: 15 trees treated at day 0	7-10 March 2017	Complete PC
3.3	Cores from the base of the tree were brought back to Perth for oil analysis	Base line of heartwood and sandalwood oil production from the experimental trees established	10th March 2017	Complete PC and A
3.4	8 weeks: Four replicates harvested and samples taken for oil analysis and gene regulation assessment. Samples brought back to Perth for oil and gene upregulation analysis	Exp 1: 8 week samples collected and analysed	Trip 15-21 May 2017.	Complete PC and A
3.5	16 weeks: Four replicates harvested and samples taken for oil analysis and gene regulation assessment. Samples brought back to Perth for oil and gene upregulation analysis	Exp 1: 16 week samples collected and analysed	Trip 23-26th July 2017	Complete PC and A
3.6	32 weeks: Four replicates harvested and samples taken for oil analysis and gene regulation assessment. Samples brought back to Perth for oil and gene upregulation analysis	Exp 1: 32 week samples collected and analysed	Trip 11-16th December 2017	Complete PC and A
3.7	Experiment 1 assessment	Exp1: Cores from base of pollarded trees taken	Trip	Complete PC
3.8	Exp1 samples sent to Perth for analysis	Cores received in Perth for analysis		Complete A

Objective 4: To explore non-invasive treatment applications that stimulate whole-tree oil production

no.	Activity	outputs/ milestones	completion date	Comments
4.1	Experiment 2 trees treated	Experiment 2 initiated – pacloburazol drench, foliar spray of ethrel and control	15th December 2017	Drench complete. Poor weather caused concern that the foliar treatment would be washed from the leaves PC
4.2	Second treatment with an additional treatment Experiment 2	Exp 2: Drench of ethrel	18th June 2018	Drench complete PC
4.3	Experiment 2 assessment	Exp 2: Cores taken from base of trees	12th December 2018	Complete PC
4.4	Exp 2 samples sent to Perth for analysis	Cores received in Perth for analysis	6th January 2019	Complete A

Objective 5: To promote an understanding of heartwood formation and silviculture techniques to stimulate its production in Vanuatu through close collaboration with the Vanuatu Forest Department.

no.	Activity	outputs/ milestones	completion date	Comments
2.1	Sponsorship of regional attendance of the Sandalwood Regional Forum, Port Vila, Vanuatu	Local representation at conference	11-13 November 2019	Complete PC
2.2	Presentation of paper on "Enhancing the formation of heartwood in Sandalwood in Vanuatu"	Overview of project to encourage discussion	11 November 2019	Complete PC
2.3	Field trip to experimental site	Further discussion of research outcomes	12 November 2019	Complete PC

PC = partner country, A = Australia

7 Key results and discussion

7.1 Branch wounding and treatment (Experiment 1)

7.1.1 Experimental tree heartwood assessment

Fifteen *Santalum austrocaledonicum* sandalwood trees selected for the first experiment. Twelve out of fifteen tree cores contained detectable levels of oil in their heartwood, while Trees 5, 7 and 10 did not contain oil. The presence or absence of oil could be observed in the photographs of the cores (Figure 13). Total oil content (% by weight) among ten-year-old plantation trees ranged from 0 to 2.2% ± 0.8%, with Tree 6 having the highest overall oil content. Tree 6 also had the largest diameter. Results were consistent with literature describing variation in oil content within and among sandalwood species. Relatively low yields were indicative of juvenile trees.

While majority of the oil was detected in the heartwood, low levels of sandalwood oil was also measured in the sapwood (Figure 12) which could also have included transition zone wood as samples were visually divided (Figure 13). Interestingly, Tree 15 was the only tree that had higher oil content in 'sapwood' regions rather than heartwood. The trunk of this tree appeared to be damaged.

Heartwood Oil Composition

Heartwood oil composition across the twelve *S. austrocaledonicum* trees containing oil showed variation in their composition (Table 6). The levels of total santalol (α- and β-santalol) ranged from 2 to 38%, with an average of 7% ± 7%. High standard deviations are indicative of the large tree-to-tree variation. Tree 6, with the highest oil content, had the highest santalol concentration of 38%. Tree 2 also had relatively high levels of α- and β-santalol (28%). Tree 12 had the lowest combined santalol concentration of 2%. Trees 8 and 11 had 80% similarity in their oil profile (data not shown). As expected, α- and β-santalol concentrations showed a strong correlation (> 90%) as they are co-produced (refer Figure 2 biosynthetic pathway).

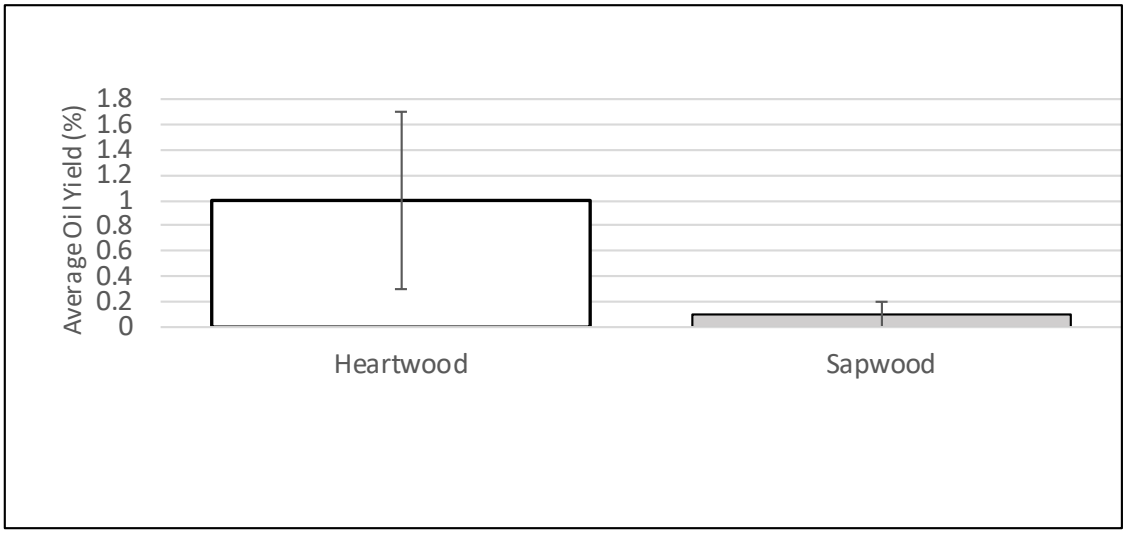

Figure 11: Mean oil yield in heartwood and sapwood of fifteen plantation *S. austrocaledonicum* trees

Tree	~ core length (cm)	Photo Dividing Sapwood and Heartwood for Oil Analysis	Oil detected in Sapwood or Heartwood	Oil Yield in Heartwood (%)	Oil Yield in Sapwood (%)	Total Oil Yield (%)
Tree 1	8.8		✓	0.2	0.14	0.36
Tree 2	9.6		✓	0.2	0.14	0.34
Tree 3	9.7		✓	0.2	0.01	0.20
Tree 4	10.7		✓	0.6	0.01	0.58
Tree 5	10.5		x	0.0	0.00	0.00
Tree 6	14.5		✓	2.2	0.01	2.22
Tree 7	9.5		x	0.0	0.00	0.00
Tree 8	10.5		✓	1.4	0.09	1.47
Tree 9	11.2		✓	1.0	0.02	0.97
Tree 10	8.3		x	0.0	0.00	0.00
Tree 11	10.7		✓	1.4	0.10	1.50
Tree 12	12.6		✓	1.6	0.04	1.65
Tree 13	10.8		✓	1.3	0.05	1.37
Tree 14	9.4		✓	1.2	0.01	1.19
Tree 15	11.0		✓	0.6	0.41	0.97

Figure 12: Cores of 15 *S. austrocaledonicum* trees showing heartwood/sapwood used for oil analysis. Oil yields (%) using GC-FID are presented

Other sesquiterpenes appear to be more dominant in these younger trees than expected in more mature trees. Lanceol was the most dominant sesquiterpene overall (Figure 13); however, oil composition is expected to change as these trees age, when santalol is expected to become more dominant. Variation in oil composition in *S. austrocaledonicum* (Table 6) is consistent with variations seen in literature reports.

Table 6: Heartwood oil composition (%) of fifteen 10-year-old *S. austrocaledonicum* plantation trees

Compound Name	R.I.	Lit R.I. *	Tree Number 1	2	3	4	5*	6	7*	8	9	10*	11	12	13	14	15
α-santalene	1575	1575[A]	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
epi-β-santalene	1636	1637[A]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-santene	1650	1651[A]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-bisabolene	1729	1724[A]	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.3	0.3	0.0	0.2	0.5	0.3	0.2	0.0
α-bisabolol	2209	2207[A]	0.0	2.1	2.5	3.0	0.0	0.7	0.0	1.8	1.7	0.0	1.6	1.8	1.0	1.3	1.3
Z-α-santalol	2336	2344[A]	3.7	19.3	7.2	11.2	0.0	23.1	0.0	5.5	9.7	0.0	5.2	1.0	4.5	2.5	1.2
Z-α-exo-bergamotol	2348	2344[A]	4.3	6.0	5.4	4.2	0.0	8.5	0.0	4.9	5.0	0.0	4.0	2.2	3.4	4.6	2.35
Farnesol	2353	2342[A]	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.5	0.0	0.0	0.5	0.4	0.5	0.4	0.0
epi-β-santalol	2403	2406[A]	0.0	0.0	0.0	0.9	0.0	1.5	0.0	0.7	0.0	0.0	0.7	0.4	0.7	0.6	0.0
z-β-santalol	2418	2423[A]	1.2	8.8	3.9	4.8	0.0	15.2	0.0	2.5	7.9	0.0	3.1	1.0	3.7	3.0	2.7
E-α-exo-bergamotol	2422	2401[B]	8.4	2.3	7.4	1.4	0.0	3.8	0.0	6.8	5.2	0.0	5.9	4.4	3.8	7.7	2.4
z-β-curcumen-12-ol	2481	2478[A]	20.5	0.0	0.0	0.0	0.0	8.0	0.0	14.4	0.0	0.0	0.0	0.0	0.0	15.6	0.0
Z-lanceol	2486	2486[A]	0.0	37.4	46.8	53.7	0.0	10.5	0.0	29.9	36.3	0.0	39.5	55.3	33.8	28.2	43.5
Z-nuciferol	2514	2513[A]	14.1	11.9	18.0	9.9	0.0	5.8	0.0	11.1	10.1	0.0	13.7	12.5	19.3	9.8	23.0
TOTAL (%)			**52**	**88**	**91**	**90**	**0**	**78**	**0**	**78**	**76**	**0**	**74**	**79**	**71**	**74**	**76**

* Trees with no detectable oil by GC-MS or GC-FID .

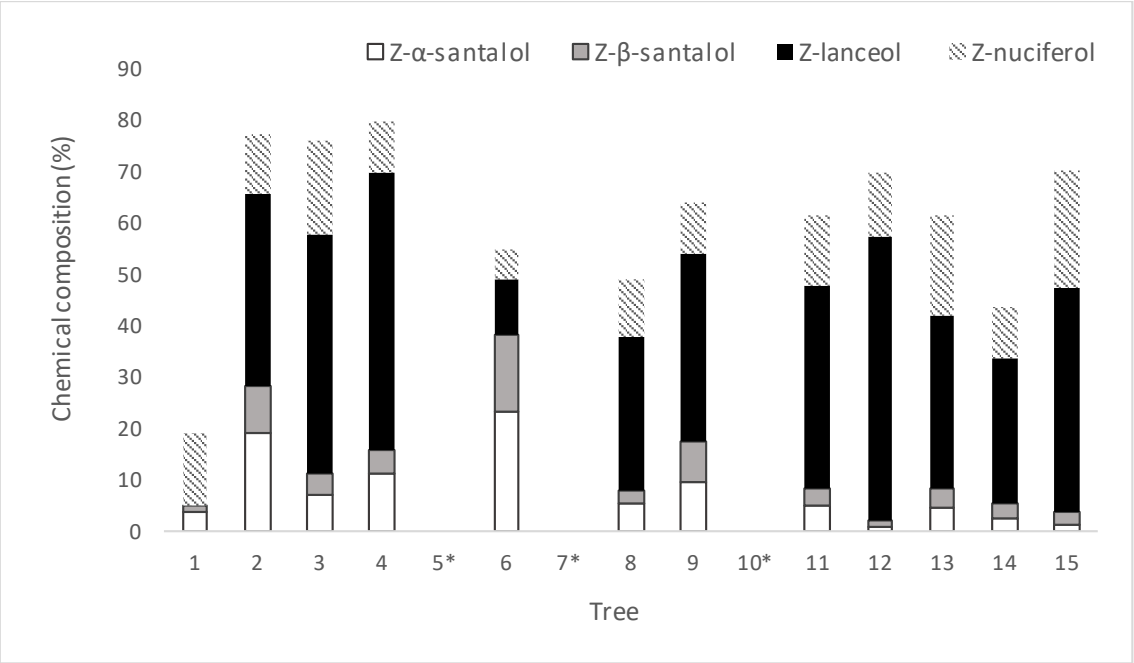

Figure 13: Comparison of four major sesquiterpenols in *S. austrocaledonicum* heartwood samples

Sapwood Oil Composition

Of the trees with detectable oil in their sapwood and transition zones, lanceol was again the most dominant sesquiterpene. The exception was Tree 6, which contained α- and β-santalol (Figure 13). Four trees contained no detectable oil in sapwood regions.

Comparison of the major oil components in heartwood and sapwood showed similar proportions of sesquiterpenes, with lanceol being slightly more dominant and nuciferol significantly less dominant in sapwood than heartwood (Figure 15).

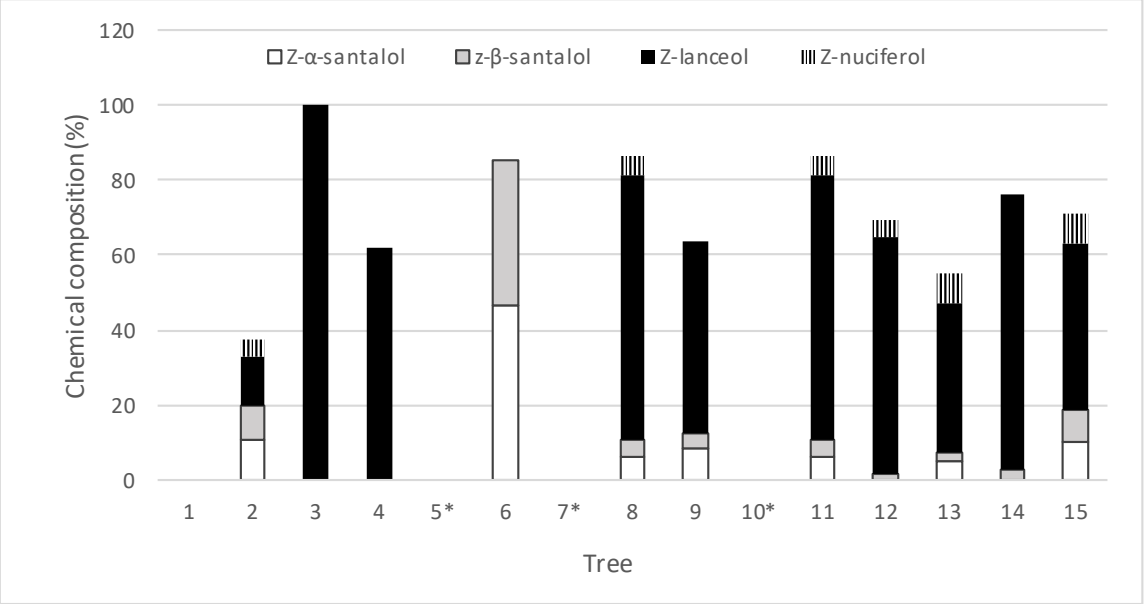

Figure 14 - Comparison of four major sesquiterpenols in *S. austrocaledonicum* sapwood samples

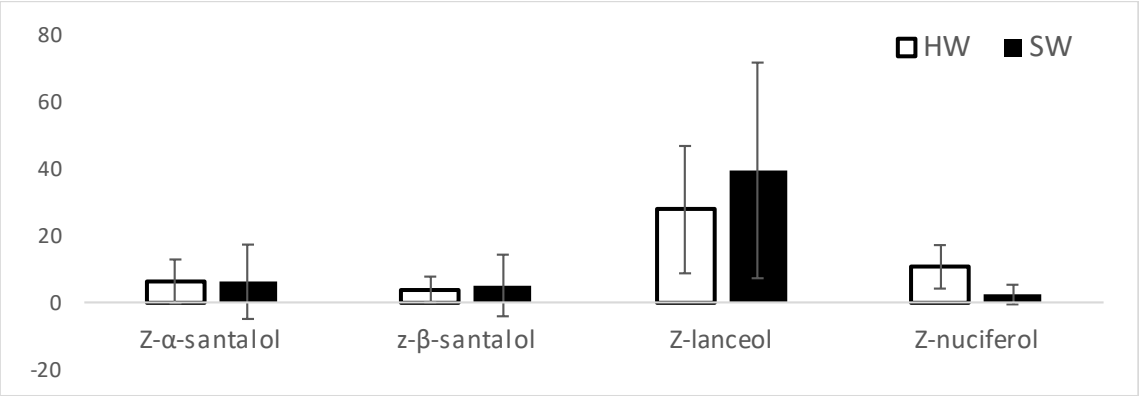

Figure 15 - Heartwood and sapwood proportions of major sesquiterpenes in *S. austrocaledonicum* plantation trees

7.1.2 Branch treatment response

The branches of the fifteen selected trees were confirmed to have no oil prior to inoculation with dowels with the seven treatments

Total sandalwood oil production

All treatments (1-7) induced production of sesquiterpenes by 8 weeks after treatment (Figure 16). The large standard deviations indicate large tree-to-tree variation. The treatments did not have significantly different effects at this time point. These results show that sesquiterpene production in branches can be stimulated by either wounding or chemical treatment.

Oil composition in branches after treatment

Twenty-one components detected in the oil profile of *S. austrocaledonicum* account for 85-100% of the total oil profile. Oil composition between treatments showed significant differences (Figure 14).

At eight weeks (first harvest), only with ethrel treatment were α-, β-, epi-β-santalene and the corresponding santalols (α- and β-santalol and bergamotol) detected (Figure 14 A,B,E,F). Total santalol content of 36.9% ± 2.1% SE in ethrel treated branches was highest compared to other treatments, including the wounding only site at week 8.

Methyl salicylate, 6-benzylaminopurine, indole-3-butyric acid, paclobutrazol and the water (wounding) control produced lanceol as a major component, with some santalol induction apparent in some trees (Figure 14).

As can be seen from the biosynthetic pathways shown (Figure 15a), either the santalols or lanceol are produced. These results indicate that the lanceol response is a wounding stress-related response. This is further supported by sandalwoods response to methyl jasmonate. No branches treated with methyl jasmonate produced santalols or bergamotol at this time point. Interestingly, Ethrel stimulated α and β-santalol production above lanceol (Figure 15b)

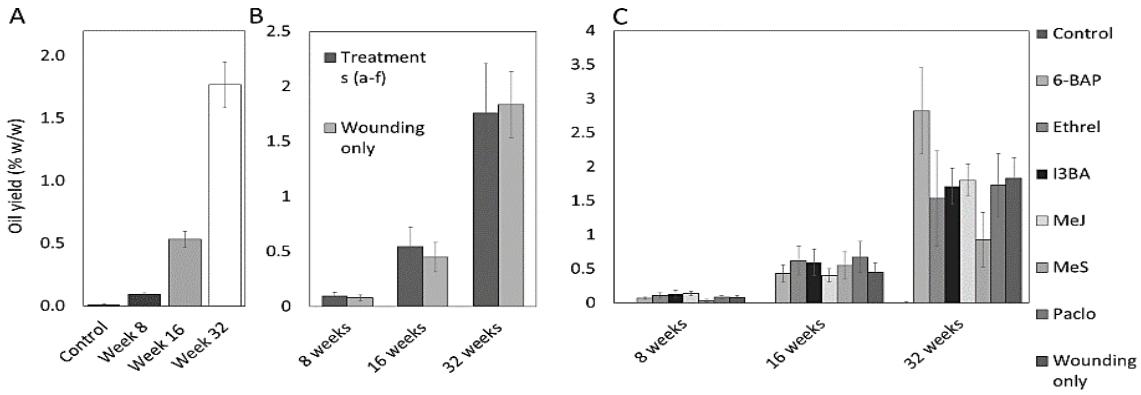

Figure 16: Wounding induces oil production in *S. austrocaledonicum* branches. Mean oil yield in percentage (%w/w) at 8, 16 and 32 weeks (n = 32). Data corresponds to means ± SE (n=32). **A** Mean oil yield in percentage (%w/w) and **B** total oil yield in g/L at 8, 16 and 32 weeks (n = 32). Data corresponds to means ± SE. **C** shows effect of treatments.

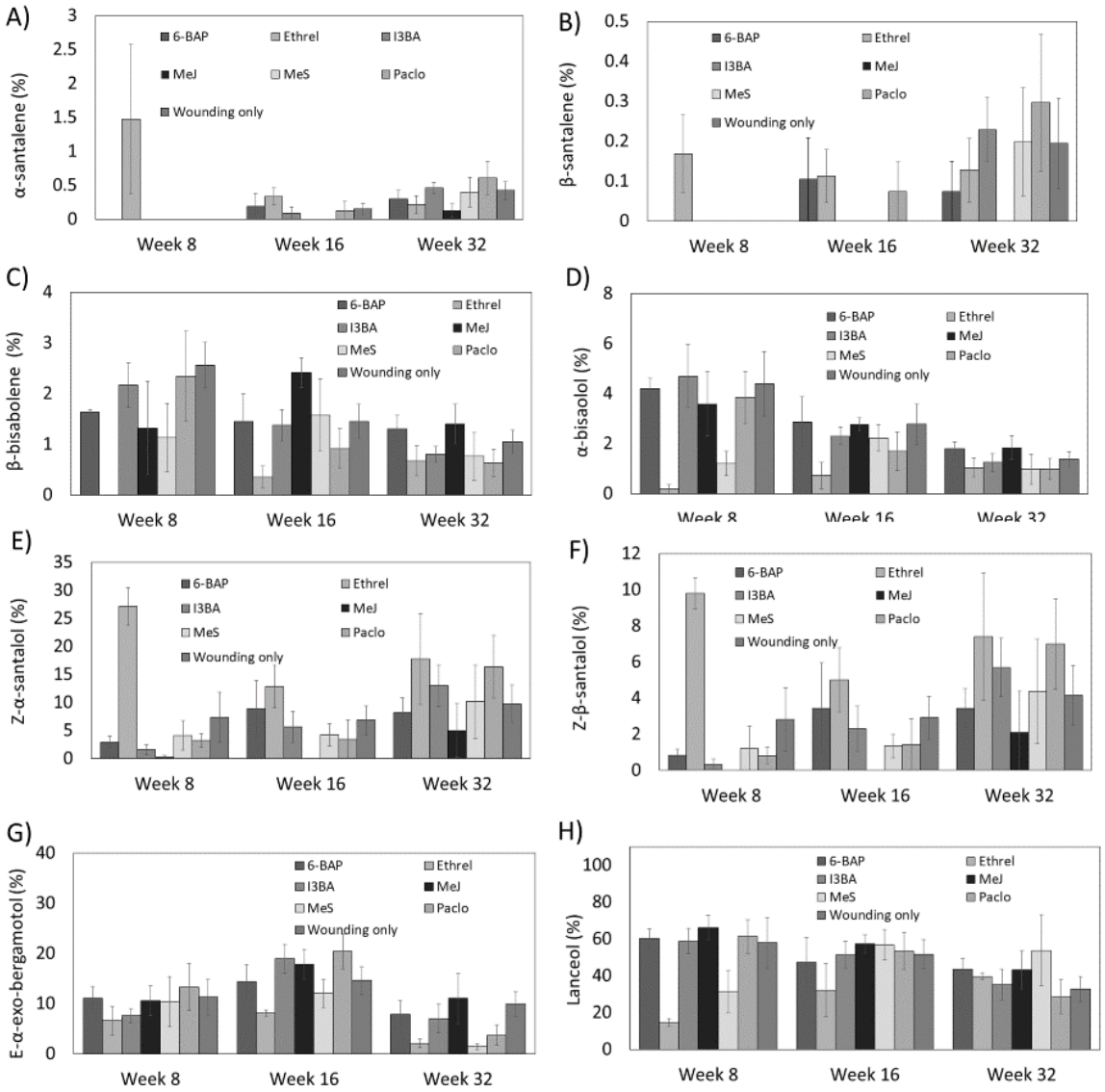

Figure 17: Oil production in *S. austrocaledonicum* branches. Mean oil yield in percentage of individual components: A) α-santalene B) β-santalene C) β-bisabolene D) α-bisabolol E) *Z-α*-santalol *F*) *Z*-β-santalol G) *E- α-exo*-bergamotol and H) lanceol. Data corresponds to means ± SE. Asterisks (*P <0.05*) indicate values that are significantly different.

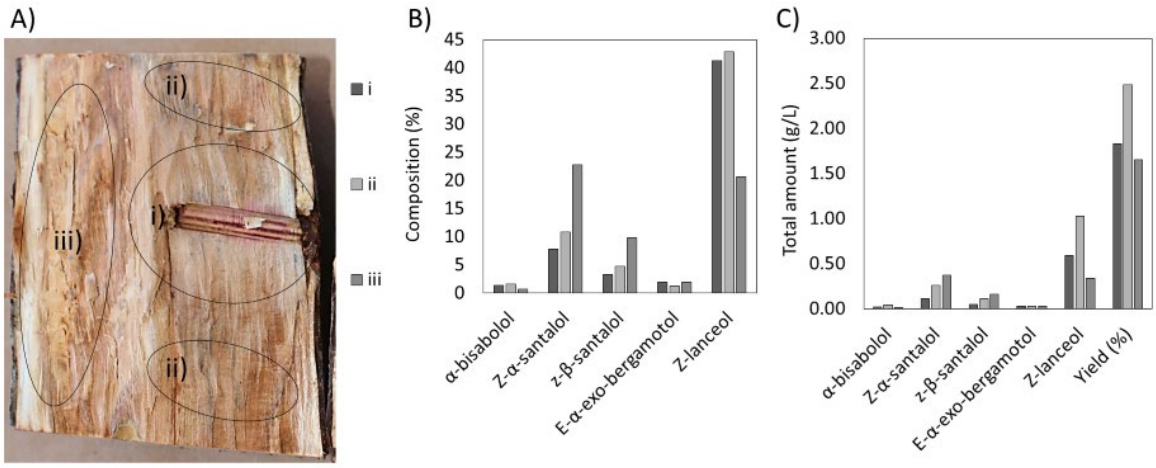

Figure 18: Example branch at 32 weeks showing differences in oil profile around wound site (Individual with MeS treatment.) The lanceol content is higher nearer the wound site than further away (iii). The santalols are higher further away from the wound site.

At week 16, santalol detection was with all treatments except Methyl jasmonate, which contained no detectable trace.

At week 32, Methyl jasmonate treatment had the lowest santalol content with ethrel and paclobutrazol showing the highest (Figure 16). Ethrel treatment was the highest with an average 26.7% ± 3.6% SE santalol content. Branch extracts from Methyl jasmonate treatments consistently contained the lowest levels of santalol content, which averaged at 2.48% ± 1.1% SE over all sampling points (Figure 18). The amount of α, β-, epi-β-santalene and the corresponding alcohols and bergamotol (santalols) all tended to increase between sampling points. Large standard deviations are indicative of the natural variability of *S. austrocaledonicum* oils.

Lanceol content mirrors the santalol trend in response to different treatments. When santalol was high, lanceol was low and *visa versa*. At 8 weeks, lanceol was highest at Methyl jasmonate sites (66.1% ± 6.5% SE) and significantly lower than ethrel sites (P=0.006) (14.8% ± 2.0% SE). Lanceol in ethrel extracts were also significantly lower (P<0.05) than the wounding only site, BAP, IBA and paclobutrazol at week 8 (Table 3).Treatments with Methyl jasmonate contained the highest average amounts of lanceol compared to all treatments. Across all treatments, lanceol was the most dominant oil component at 8 weeks (50.1% ± 4.5% SE); 16 weeks (50.0% ± 3.6% SE) and 32 weeks (39.5% ± 3.6% SE). The amount of Z-lanceol increased with each sampling time, but to a lesser extent than other components. As a result, the proportion of Z-lanceol in the oil profile tended to decrease overtime (Figure 2). This data indicates that lanceol is compound (sesquiterpene) produced as an initial wound response.

A principle component analysis (PCA) showed differences in total oil composition between ethrel and MeJ treatments at 8 weeks (Figure 18). Ethrel treatments appear to group towards the Z-a-santalol axis and MeJ appear to cluster around the Z-lanceol axis except for one replicate in both treatments (Figure 20B). The PCA demonstrates that Z-α-santalol and Z-lanceol influence much of the variability and are the main contributors for the oil extract composition after treatment in *S. austrocaledonicum* branches.

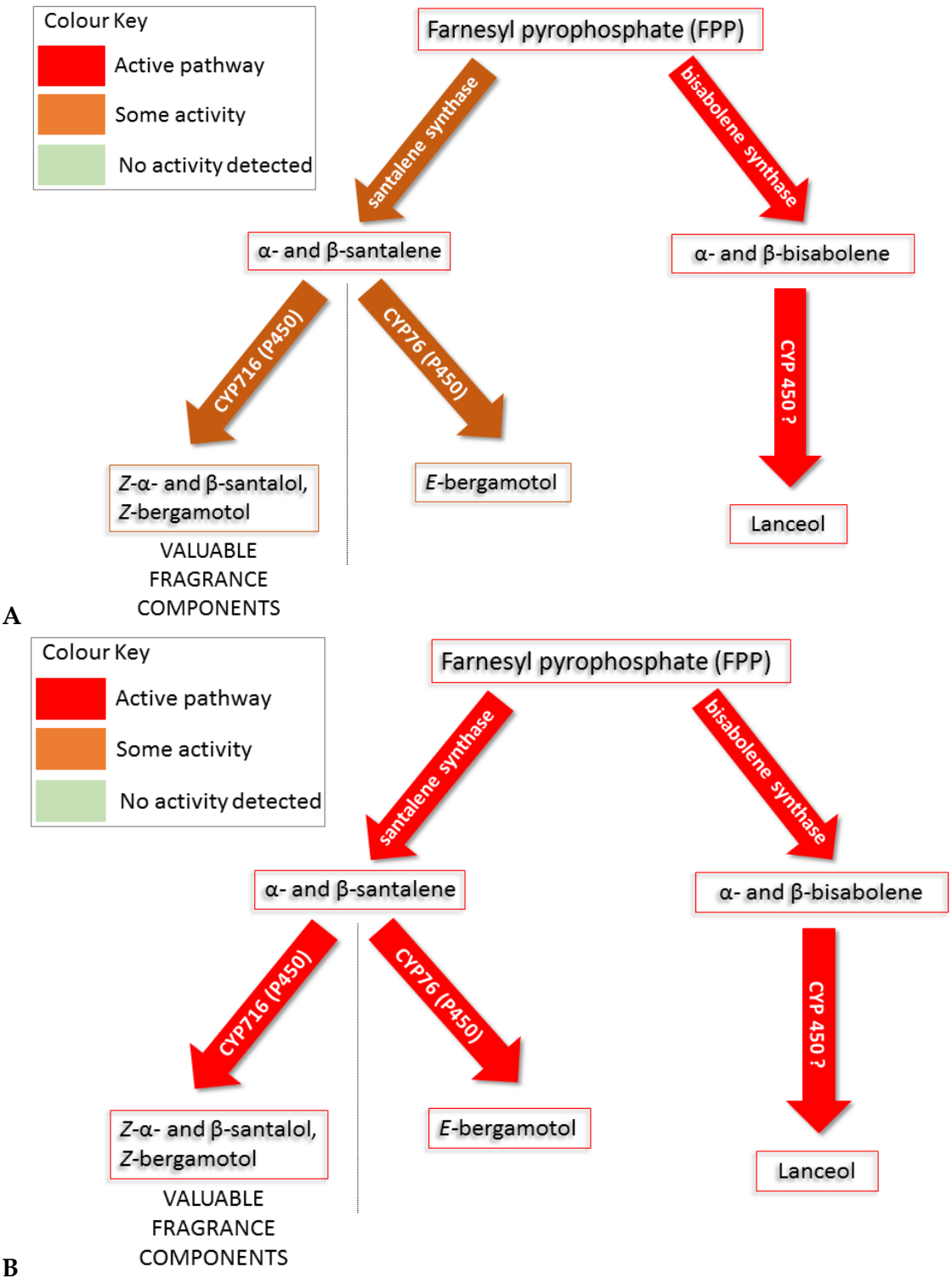

Figure 19 - Major pathways active 8 weeks after chemical treatment A) Methyl salicylate, deionised water, 6-benzylaminopurine, indole-3-butyric acid and B) Ethrel

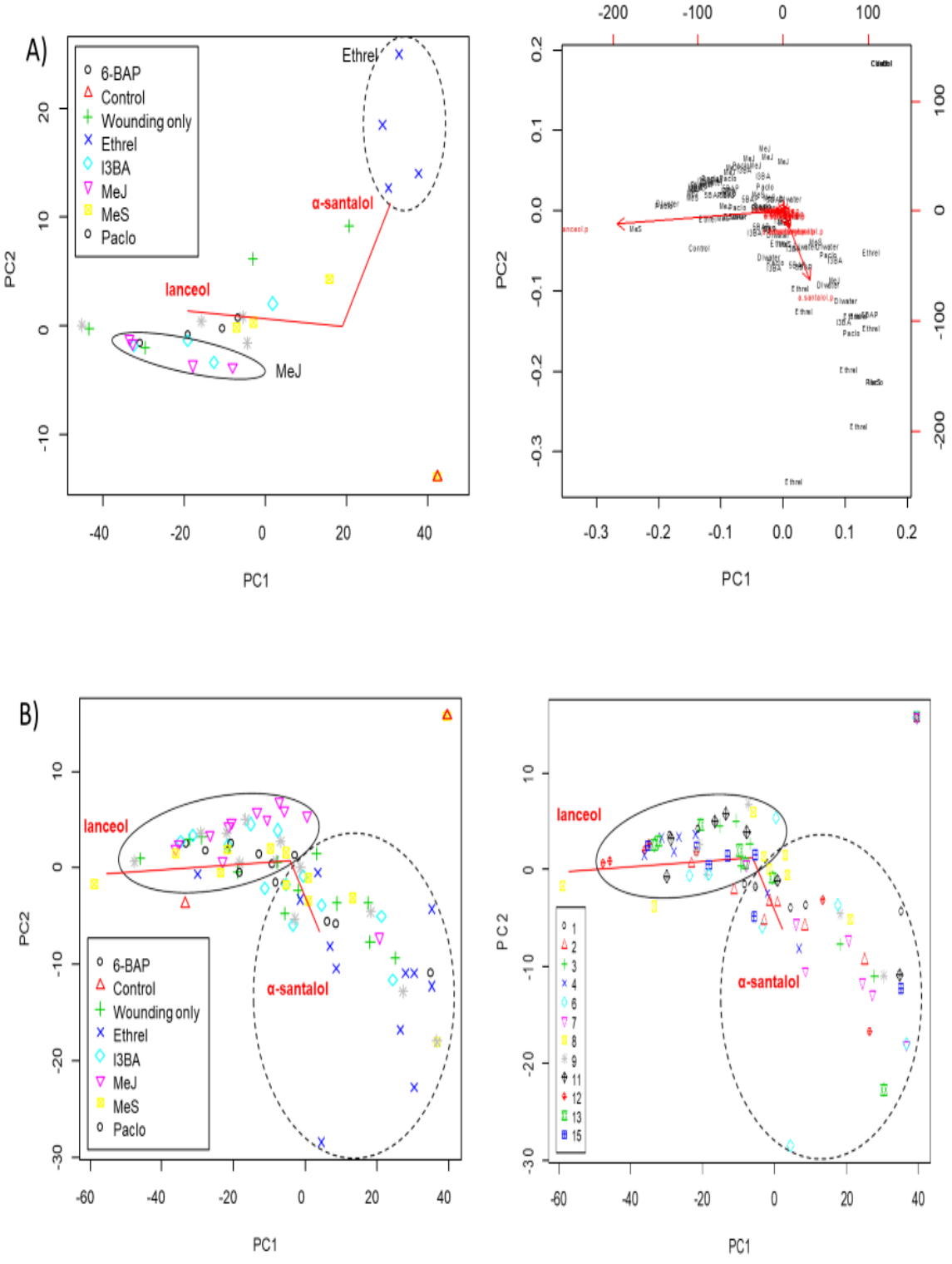

Figure 20: Two-dimensional principal component analysis (PCA) ordination scores of *S. austrocaledonicum* harvested from chemically treated branches at **A)** 8 weeks explaining 0.895+0.06238 of the total variance and **B)** All weeks combined. Each point represents an individual tree, and points close together are similar in terms of composition. The first three components represent 99% of the total variance.

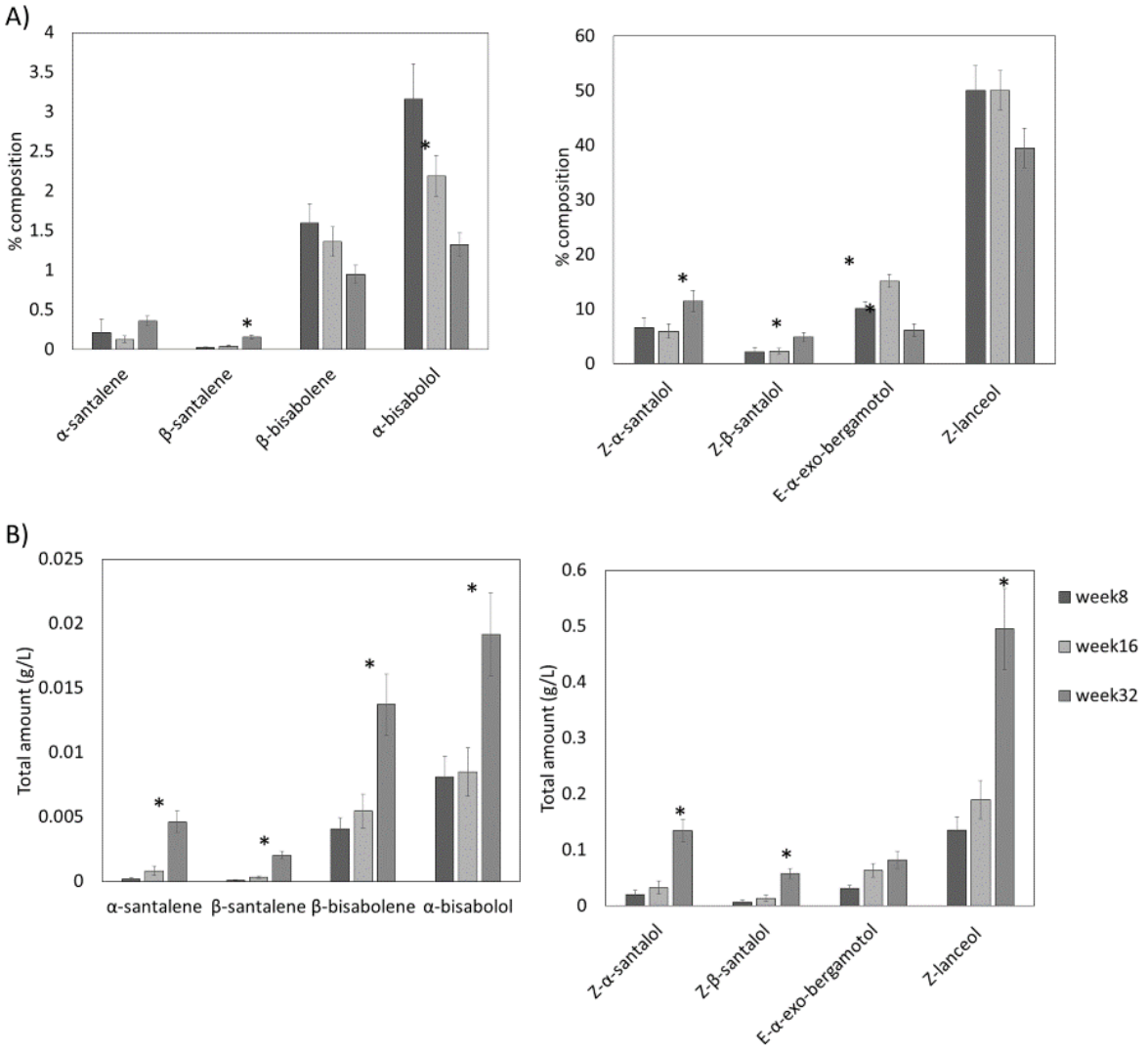

Figure 21: Branch oil extracts **A)** percentage composition data showing how components change in their contribution to profile **B)** Total amount of each compound generally increases with time. Asterisks (P <0.05) indicate values that are significantly different

Table 7: Friedman test to detect significant differences between treatments (dependent variable), and i) yield ii) total α-santalol percentage and iii) total *Z*-lanceol percentage (independent variables). A Friedman nemenyi post-hoc test was used to find which treatments were significant at 8, 16 and 32 weeks of inoculating branches (MeJ = methyl jasmonate, control = no wounding, 6BAP = 6-benzylaminopurine). Significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, n.s., not significant

Time (weeks)	Independent	Dependent	Chi-squared	DF	P-Value	Post.hoc test results (Friedman nemenyi)	P-value
8	Yield	Treatment	17.416	7	0.01***	MeJ-control	0.01**
16	Yield	Treatment	11.417	7	0.12	n.s	n.s
32	Yield	Treatment	15.75	7	0.03*	Control-6BAP	0.05.
8	α-santalol	Treatment	16.487	7	0.02**	Control-ethrel	0.02*
16	α-santalol	Treatment	11.512	6	0.07	Ethrel-MeJ	0.05.
32	α-santalol	Treatment	12.642	7	0.08	n.s	n.s
8	*Z*-lanceol	Treatment	19.454	7	0.007***	Control-MeJ	0.03*
16	*Z*-lanceol	Treatment	4.8333	7	0.68	n.s	n.s
32	*Z*-lanceol	Treatment	6.3333	7	0.50	n.s	n.s

7.1.3 Gene expression

Investigation of the expression profile of terpene synthase genes in wounded branches at 8 and 16 weeks explored their link with oil induction. Only three known sequences were from *S. austrocaledonicum* exclusively (Jones et al., 2011). The remainder of the genes were isolated from *S. album*

- SaDXS (1-Deoxy-d-xylulose 5-phosphate synthase). A key regulatory enzyme in plastidic MEP/DXP pathway. The very low level expression of SaDXS was variable across treatments with no significant difference between the chemical treatment, wounding control and control with no treatment.

- *Sa*HMGR (key regulatory enzyme in cytosolic MVA pathway). There was minimal upregulation of this gene in the sapwood. Treatments both wounding and with chemicals showed a low level of upregulation for this gene. Only at 8 weeks was there a significant difference between the control and other treatments seen with methyl jasmonate treatment.

- *Sa*FPPS (Farnesyl pyrophosphate synthase). There was minimal upregulation of this gene in the sapwood. Treatments both wounding and with chemicals upregulated a low expression of this gene ensuring that the santalenes components of the sandalwood oil were produced.

- SauSSy (santalene synthase; *S. austrocaledonicum*). There was no upregulation of this gene in the sapwood. However with wounding and treatment, the gene had a high level of up-regulation to a maximum of 130-fold. Notably ethrel and paclobutrazol showed the highest activity. There was a significant difference between SauSSy transcripts between MeJ and ethrel, which broadly reflects the santalene composition in oil extracts from branches at week 8

- SauBIS (bisabolene synthase, *S. austrocaledonicum*). There was no upregulation of this gene in the sapwood. Treatments both wounding and with chemicals upregulated a high expression of this gene with an average of 160- and 225-fold greater abundance

- *SaCYP736 (*Z-santalol hydroxylase, *S. album*) There was no upregulation of this gene in the sapwood. Treatments both wounding and with chemicals upregulated a low expression of this gene

- SaCYP76 (Z-santalol hydroxylase) S. album. There was minimal upregulation of this gene in the sapwood. Treatments both wounding and with chemicals upregulated a low expression of this gene

- SaSesqui (α-humulene/γ-cadinene/β-elemene synthase). There was minimal upregulation of this gene in the sapwood. Only salicylic acid and methyl jasmonate upregulated gene expression.

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Wounding induced a significant increase in the abundance of *Sa*HMGR, *Sa*FPPS, , *Sau*SSy, SauBIS and *Sa*CYP736 transcripts relative to the control of HKGs 1 and 7 (Figure 22).

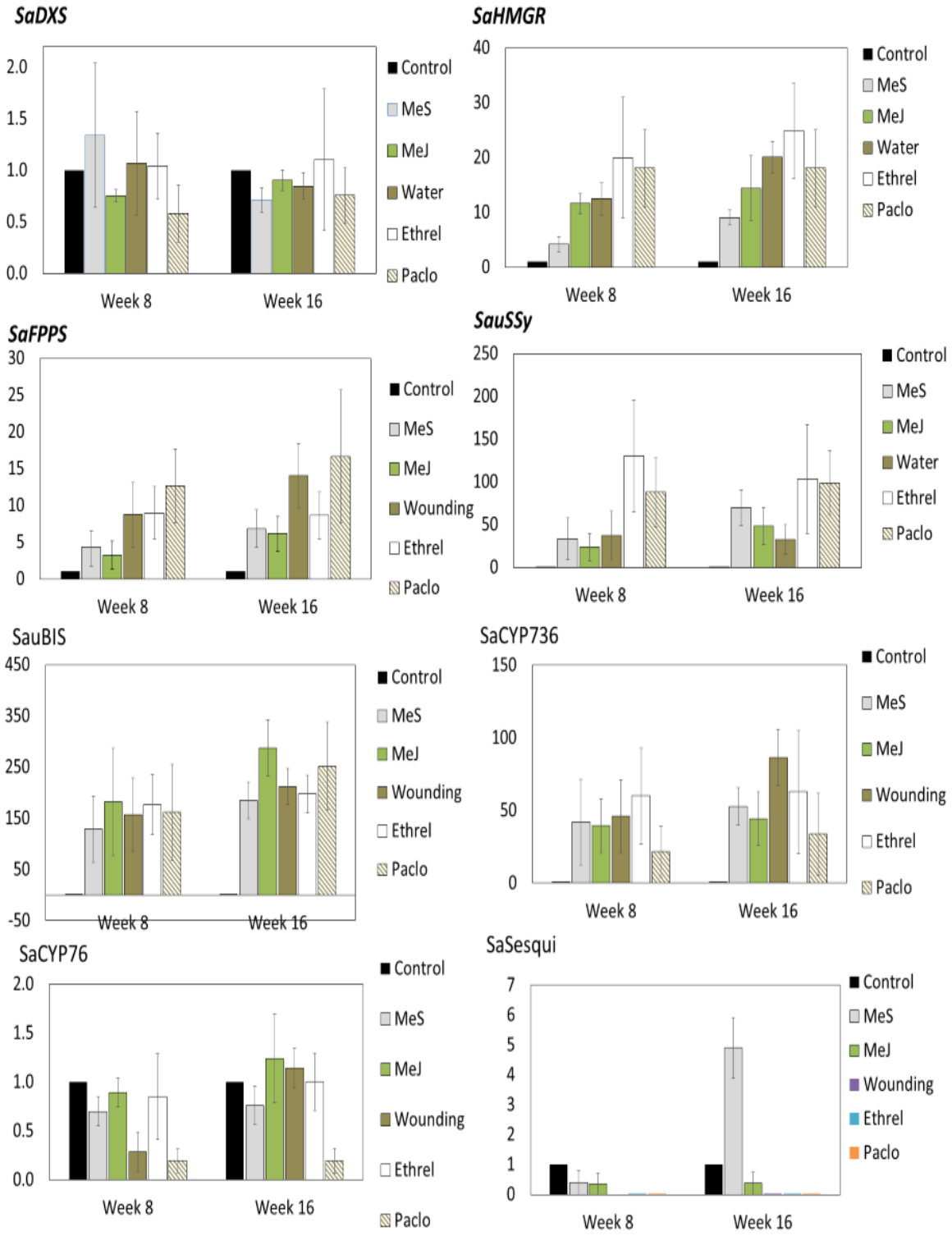


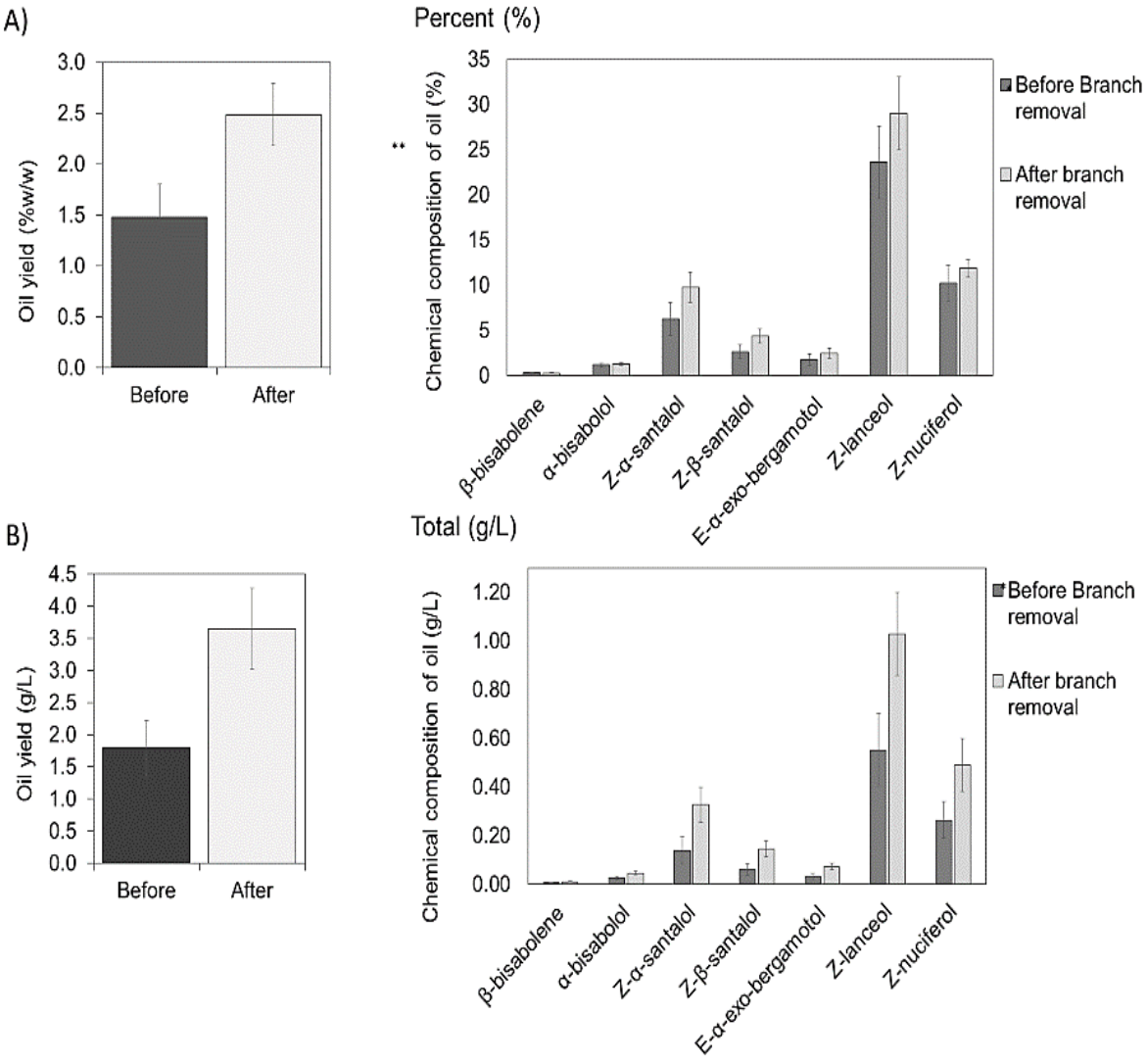
Figure 22: Figure 6. Gene expression results after 8 and 16 weeks of treatment. SaDXS (1-Deoxy-d-xylulose 5-phosphate synthase); SaHGMR is the key regulatory enzyme in cytosolic MVA pathway; SaFPPS (farnesyl pyrophosphate synthase) produces FPP, the precursor to sesquiterpenes; SauSSy (santalene synthase) S. austrocaledonicum; SauBIS (bisabolene synthase) S. austrocaledonicum; SaCYP736 (Z-santalol hydroxylase) S. album; SaCYP76 **(**Z-santalol hydroxylase) S. album; SaSesqui (α-humulene/γ-cadinene/β-elemene synthase)

7.1.4 Experimental tree heartwood assessment after treatment

After the experiment was completed and 8-12 months after treatment and pollarding, tree cores taken from a similar position showed that oil content range had increased to between from 0.4% to 4.1%, with a mean of 2.5% ± 0.3% SE.

The increase of 1.1% on average in less than a year was statistically significant (P=0.005). Additionally, the three trees that produced no oil prior to branch removal all contained heartwood and oil and a heartwood to sapwood ratio to an average of one-third the length of the core after pollarding.

Figure 23: Heartwood oil samples from trunks of fourteen 11-year-old trees before treatment on branches and subsequent removal.



Statistically when the tree was 11 years old and had been previously wounded by coring, a relationship was identified between stem diameter at ground level and oil content (%w/w) was significant (P=0.01) and marginally significant at breast height (P=0.06) (Table 7). Across all trees, the heartwood volume, heartwood:sapwood ratio and stem

diameter at breast height had increased significantly (P<0.05) following treatment and branch removal.

Detected were the 21 oil components as major contributors to the oil profile of *S. austrocaledonicum* accounting for 72-95% of the total profile (Table 6). Similar to the branches, sesquiterpenoid composition showed differences in amounts of individual components, with lanceol being the major contributor across individuals ranging from 5.9 to 62%, with a mean content of 23 ± 4.3% SE before and 29 ± 4.2% SE after branch removal (Table 7).

Percentage Composition	Oil composition	santalene	Total α- and β-santalol	santalol	β-bisabolene	α-bisabolol	E-α-*exo*-bergamotol	lanceol	nuciferol	Yield (%)
				All trees environmental						
After branch removal (pollard and/or time effect)	n.s.	α: n.s. β: n.s.	n.s.	α: 0.07. β: 0.07.	n.s.	n.s.	n.s.	0.05.	n.s.	0.005**
Main host species	0.001**	n.s.	n.s.	n.s.	0.05.	0.001**	n.s.	0.001**	n.s.	n.s.
				Morphological						
Tree height	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Stem diameter at ground level	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.01*
Stem diameter (at 1.37 cm)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.06.

Total amount (g/L)	Oil composition	santalene	Total α- and β-santalol	santalol	β-bisabolene	α-bisabolol	E-α-*exo*-bergamotol	lanceol	nuciferol	Yield (g/L)
				All trees environmental						
After branch removal (pollard and/or time effect)	0.02*	α: 0.02* β: 0.005**	0.0004***	α: 0.00*** β: 0.00***	0.01*	0.0000***	0.02*	0.0000***	0.002**	0.0000***
Main host species	0.03*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
				Morphological						
Tree height	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Stem diameter at ground level	0.04*	n.s.	n.s.	n.s.	n.s.	0.02*	n.s.	n.s.	n.s.	n.s.
Stem diameter (at 1.37 cm)	0.04*	n.s.	n.s.	n.s.	n.s.	0.01*	n.s.	n.s.	n.s.	n.s.

		Heartwood (HW) and Sapwood (SW) Characteristics after branch removal (1 year later)					
	Statistics	Stem diameter at ground level	Stem diameter (at 1.37 cm)	HW length	HW volume	SW length	SW volume	HW:SW ratio
After branch removal (pollard and/or time effect)	t-test Wilcox	n.s.	0.01*	0.002**	0.002**	0.004**	0.003**	0.001**

Table 8: Association between heartwood oil composition and environmental and morphological features of *Santalum austrocaledonicum* using linear regression (ANOVA), Adonis, Wilcox t-test and Kruskal-Wallis tests 14 individuals of *S. austrocaledonicum* before and after branch removal one year following branch treatments. Parametric tests were used for normally distributed continuous variables, and non-parametric for all others. Significant differences are indicated: *, P<0.05; **, P<0.01; ***, P<0.001, n.s., not significant

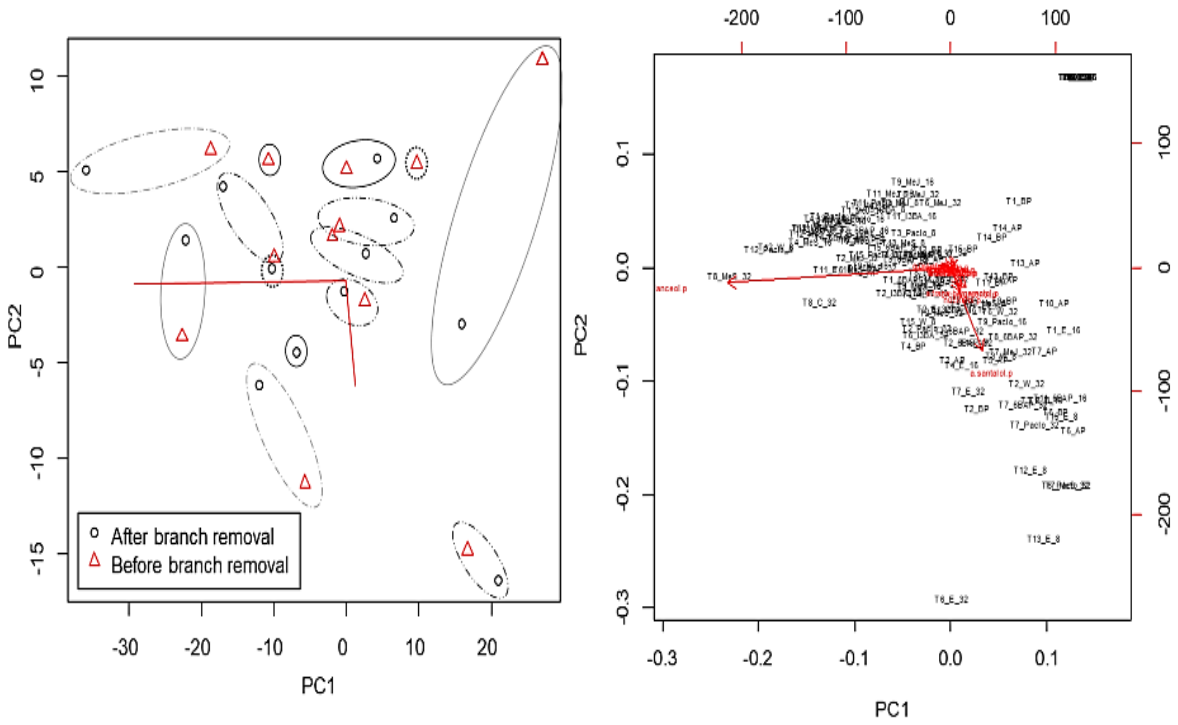


Figure 24: Two-dimensional principal component analysis (PCA) ordination scores of *S. austrocaledonicum* harvested from trees that were chemically treated in their branches at A) Each point represents an individual tree, and points close together are similar in terms of composition. B) Represents all trees and their chemical analysis showing the trend towards lanceol.

Total santalol content ranged from ranged from 2.8 to 36% with an average of 9.0 ± 1.4% SE before and 14.1 ± 1.3% SE after 12 months. High standard deviations are indicative of the large tree-to-tree variation. While the proportional contribution of oil components did not change significantly following treatment and branch removal, the total amount of individual sesquiterpenes (g/L) including the santalenes, santalols, β-bisabolene, α-bisabolol, *E*-α-*exo*-bergamotol, lanceol and nuciferol increased significantly ($P<0.05$) (Table 7).

The tree with the highest oil content (and largest core diameter), also had the highest total santalol content of 36%. As expected, α- and β-santalol amounts showed a strong correlation ($R^2 > 0.9$) as they are co-produced (Celedon et al., 2016, Diaz-chavez et al., 2013). Lanceol was the most dominant sesquiterpene overall. Results are consistent with literature reports describing variation in oil content in *S. austrocaledonicum* trees (Bottin *et al.* 2007; Page *et al.* 2010b).

A principle component analysis (PCA) revealed high levels of correlation between sesquiterpene components, with lanceol and α-santalol accounting for 92% of the observed variance (Figure 24). When oil profiles were clustered based on tree (genetic) or treatments (environment) significant overlap in terpenoid profiles made it difficult to differentiate. Oil composition, β-bisabolene and α-bisabolol (%) was significantly associated with main host species, but not to tree height or diameter (Table 7), warranting further investigation into environmental contributors to oil profiles. It was evident that by 32 weeks oil production was still active and that composition may continue to change.

Table 9: Table 3. Sesquiterpene composition in *S. austrocaledonicum* trunks of 14-year-old trees before and after treatment course

			Before Branch Removal				After Branch Removal			
Heartwood	R.I.	R.I. (lit)	Mean (%)	SE	Min	Max	Mean (%)	SE	Min	Max
α-santalene	1572	1575[A]	0.125	0.04	0.00	0.55	0.133	0.03	0.00	0.424
α-exo-bergamotene	1585	1586	0.010	0.01	0.00	0.09	0.011	0.01	0.00	0.077
epi-β-santalene	1634	1637[A]	0.060	0.02	0.00	0.29	0.088	0.02	0.00	0.306
β-santalene	1648	1651[A]	0.065	0.03	0.00	0.33	0.088	0.02	0.00	0.289
Unknown1	1656	??	0.459	0.14	0.00	2.07	0.243	0.02	0.10	0.388
β-bisabolene	1722	1724[A]	0.295	0.07	0.00	0.97	0.280	0.04	0.10	0.571
β-bisabolol	2140	??	0.726	0.13	0.00	1.44	0.924	0.05	0.55	1.163
α-bisabolol	2204	2207[A]	1.126	0.23	0.00	2.78	1.244	0.16	0.38	2.523
Z-α-santalol	2335	2344[A]	6.353	1.95	0.00	23.27	9.760	1.73	1.92	24.592
Z-α-exo-bergamotol	2346	2344[A]	2.178	0.55	0.00	7.01	2.988	0.44	1.03	7.083
Farnesol	2347	2342[A]	1.390	0.26	0.00	3.10	1.667	0.14	1.08	2.758
epi-β-santalol	2400	2406[A]	0.413	0.11	0.00	1.31	0.604	0.12	0.08	1.594
z-β-santalol	2415	2401[B]	2.689	0.84	0.00	10.39	4.355	0.81	0.87	11.377
E-α-exo-bergamotol	2418	2423[A]	1.879	0.69	0.00	9.39	2.474	0.55	0.36	8.425
Unknown3	2430	??	4.128	0.84	0.00	9.47	4.324	0.74	0.25	9.207
Unknown4	2462	??	1.092	0.22	0.00	2.17	0.896	0.11	0.00	1.595
Z-β-curcumen-12-ol	2479	2478[A]	7.266	1.56	0.00	16.97	9.292	1.32	3.01	18.418
Z-lanceol	2485	2486[A]	23.305	4.25	0.00	49.28	29.018	4.21	5.94	62.437
Z-nuciferol	2510	2513[A]	10.105	2.15	0.00	23.61	11.887	1.02	6.45	21.168
Unknown5	2542	??	1.596	0.52	0.00	4.82	3.095	0.30	1.33	5.097
Unknown6	2764	??	7.315	1.64	0.00	21.44	9.651	1.24	2.90	17.999
HW-Area %			72.575	10.52	0.00	95.19	93.022	0.38	88.60	94.341
Yield percent (%)			1.393	0.34	0.00	3.94	2.486	0.32	0.45	4.072

Heartwood	R.I.	R.I. (lit)	Mean (g/L)	SE	Min	Max	Mean (g/L)	SE	Min	Max
α-santalene	1572	1575[A]	0.0026	0.0014	0.00	0.02	0.0049	0.0012	0.00	0.02
α-exo-bergamotene	1585	1586	0.0003	0.0002	0.00	0.00	0.0005	0.0002	0.00	0.00
epi-β-santalene	1634	1637[A]	0.0013	0.0007	0.00	0.01	0.0032	0.0008	0.00	0.01
β-santalene	1648	1651[A]	0.0015	0.0008	0.00	0.01	0.0033	0.0008	0.00	0.01
Unknown1	1656	??	0.0080	0.0026	0.00	0.04	0.0105	0.0021	0.00	0.02
β-bisabolene	1722	1724[A]	0.0058	0.0016	0.00	0.02	0.0107	0.0017	0.00	0.02
β-bisabolol	2140	??	0.0159	0.0046	0.00	0.05	0.0415	0.0082	0.00	0.09
α-bisabolol	2204	2207[A]	0.0232	0.0065	0.00	0.08	0.0484	0.0080	0.01	0.10
Z-α-santalol	2335	2344[A]	0.1324	0.0578	0.00	0.83	0.3486	0.0730	0.08	0.96
Z-α-exo-bergamotol	2346	2344[A]	0.0477	0.0177	0.00	0.25	0.1120	0.0216	0.03	0.28
Farnesol	2347	2342[A]	0.0317	0.0091	0.00	0.09	0.0773	0.0160	0.01	0.18
epi-β-santalol	2400	2406[A]	0.0085	0.0032	0.00	0.05	0.0211	0.0046	0.00	0.06
z-β-santalol	2415	2401[B]	0.0568	0.0257	0.00	0.37	0.1557	0.0338	0.03	0.44
E-α-exo-bergamotol	2418	2423[A]	0.0351	0.0129	0.00	0.17	0.0777	0.0134	0.01	0.18
Unknown3	2430	??	0.1000	0.0298	0.00	0.27	0.2258	0.0635	0.01	0.61
Unknown4	2462	??	0.0233	0.0062	0.00	0.06	0.0361	0.0064	0.00	0.10
Z-β-curcumen-12-ol	2479	2478[A]	0.1789	0.0538	0.00	0.49	0.4789	0.1241	0.04	1.23
Z-lanceol	2485	2486[A]	0.5056	0.1521	0.00	1.97	1.0709	0.1783	0.16	2.15
Z-nuciferol	2510	2513[A]	0.2461	0.0793	0.00	1.00	0.5166	0.1125	0.04	1.70
Unknown5	2542	??	0.0496	0.0180	0.00	0.18	0.1457	0.0337	0.02	0.36
Unknown6	2764	??	0.1590	0.0446	0.00	0.50	0.4721	0.1190	0.04	1.44
HW-Area %			72.575	10.52	0.00	4.20	93.022	0.38	0.57	7.52
Yield (g/L)			1.6333	0.4322	0.00	4.20	3.8616	0.6344	0.57	7.52

			Before Branch Removal				After Branch Removal			
Sapwood	R.I.	R.I. (lit)	Mean (%)	SE	Min	Max	Mean (%)	SE	Min	Max
α-santalene	1572	1575[A]	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.14
α-exo-bergamotene	1585	1586	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
epi-β-santalene	1634	1637[A]	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.11
β-santalene	1648	1651[A]	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.12
Unknown1	1656	??	0.00	0.00	0.00	0.00	0.15	0.06	0.00	0.70
β-bisabolene	1722	1724[A]	0.00	0.00	0.00	0.00	0.14	0.06	0.00	0.75
β-bisabolol	2140	??	0.00	0.00	0.00	0.00	0.36	0.19	0.00	2.32
α-bisabolol	2204	2207[A]	0.00	0.00	0.00	0.00	0.93	0.29	0.00	3.30
Z-α-santalol	2335	2344[A]	7.51	3.89	0.00	48.99	13.79	2.20	0.00	30.00
Z-α-exo-bergamotol	2346	2344[A]	1.91	0.97	0.00	11.36	3.38	0.70	0.00	8.10
Farnesol	2347	2342[A]	0.14	0.08	0.00	0.91	0.90	0.25	0.00	2.65
epi-β-santalol	2400	2406[A]	0.05	0.05	0.00	0.67	0.22	0.13	0.00	1.58
z-β-santalol	2415	2401[B]	3.03	1.58	0.00	20.03	5.81	0.90	0.00	14.13
E-α-exo-bergamotol	2418	2423[A]	0.00	0.00	0.00	0.00	7.06	1.60	0.00	19.19
Unknown3	2430	??	0.00	0.00	0.00	0.00	3.59	0.66	0.00	8.46
Unknown4	2462	??	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Z-β-curcumen-12-ol	2479	2478[A]	4.03	1.39	0.00	12.72	9.45	1.10	0.00	17.60
Z-lanceol	2485	2486[A]	38.17	9.86	0.00	100.00	26.95	3.69	0.00	49.27
Z-nuciferol	2510	2513[A]	2.15	0.89	0.00	9.23	10.43	1.34	0.00	18.14
Unknown5	2542	??	0.00	0.00	0.00	0.00	2.59	0.58	0.00	5.54
Unknown6	2764	??	0.00	0.00	0.00	0.00	5.59	1.12	0.00	12.76
HW-Area %			56.97	10.42	0.00	100.00	91.36	7.05	0.00	100.00
Yield percent (%)			0.09	0.05	0.00	0.74	0.03	0.01	0.00	0.13

Sapwood	R.I.	R.I. (lit)	Mean (g/L)	SE	Min	Max	Mean (g/L)	SE	Min	Max
α-santalene	1572	1575[A]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
α-exo-bergamotene	1585	1586	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
epi-β-santalene	1634	1637[A]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
β-santalene	1648	1651[A]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Unknown1	1656	??	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
β-bisabolene	1722	1724[A]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
β-bisabolol	2140	??	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
α-bisabolol	2204	2207[A]	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.002
Z-α-santalol	2335	2344[A]	0.022	0.013	0.000	0.185	0.010	0.003	0.000	0.037
Z-α-exo-bergamotol	2346	2344[A]	0.006	0.004	0.000	0.057	0.003	0.001	0.000	0.011
Farnesol	2347	2342[A]	0.001	0.001	0.000	0.016	0.001	0.000	0.000	0.003
epi-β-santalol	2400	2406[A]	0.001	0.001	0.000	0.012	0.000	0.000	0.000	0.002
z-β-santalol	2415	2401[B]	0.009	0.005	0.000	0.074	0.004	0.002	0.000	0.018
E-α-exo-bergamotol	2418	2423[A]	0.000	0.000	0.000	0.000	0.005	0.003	0.000	0.037
Unknown3	2430	??	0.000	0.000	0.000	0.000	0.003	0.001	0.000	0.009
Unknown4	2462	??	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Z-β-curcumen-12-ol	2479	2478[A]	0.017	0.010	0.000	0.145	0.006	0.002	0.000	0.021
Z-lanceol	2485	2486[A]	0.076	0.048	0.000	0.689	0.014	0.002	0.000	0.036
Z-nuciferol	2510	2513[A]	0.014	0.011	0.000	0.159	0.006	0.001	0.000	0.020
Unknown5	2542	??	0.000	0.000	0.000	0.000	0.002	0.001	0.000	0.010
Unknown6	2764	??	0.000	0.000	0.000	0.000	0.003	0.001	0.000	0.010
HW-Area %			72.575	10.52	0.00	4.20	93.022	0.38	0.57	7.52
Yield (g/L)			0.146	0.093	0.000	1.336	0.060	0.015	0.000	0.213

Investigating the sandalwood oil components in greater detail, differences in yield before and after branch treatment and pollarding showed that the yield (g/l) significantly increased. This increase was due to the increase in production of lanceol, which also showed to increase significantly.

Table 10: Sandalwood oil components before and after branch treatment and pollarding. Significant differences are indicated: *, P<0.05; **, P<0.01; ***, P<0.001, n's., not significant

Independent	Mean (before pollard)	Mean (after pollard)	T-test (testing difference)	P-Value
Yield (%)	1.47	2.34	t = 1.8846, df = 14	0.08
α-santalol (%)	6.29	9.31	t = 1.4158, df = 14	0.17
β-santalol (%)	2.65	4.13	t = 1.5638, df = 14	0.14
β-bisabolene (%)	0.28	0.29	t = -0.2191, df = 14	0.82
E-α-trans-bergamotol (%)	1.76	2.32	t = 0.74744, df = 14	0.46
lanceol (%)	23.60	31.15	t = 1.6697, df = 14	0.11
Yield (g/L)	1.790	3.647	t = 2.8595, df = 14	0.01**
α-santalol (g/L)	0.139	0.326	t = 1.9501, df = 14	0.07
β-santalol (g/L)	0.059	0.145	t = 1.9801, df = 14	0.06.
β-bisabolene (g/L)	0.006	0.010	t = 2.1006, df = 14	0.05.
E-α-trans-bergamotol (g/L)	0.033	0.072	t = 2.2976, df = 14	0.03*
lanceol (g/L)	0.552	1.028	t = 2.9745, df = 14	0.01**

7.2 Non-invasive treatment of ethrel and paclobutrazol

7.2.1 Sandalwood oil yield

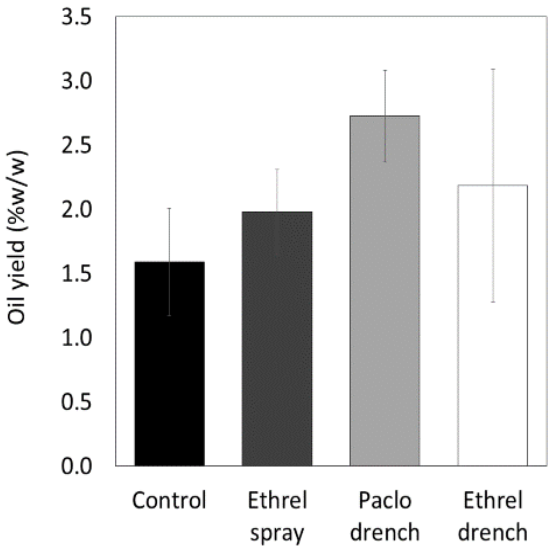


Figure 25: Mean yield percentage (%w/w) of heartwood oils from *S. austrocaledonicum* trees treated with a foliar ethrel spray (n=24), paclobutrazol (paclo) drench (n=24) or an ethrel drench (n=9). Control (n=23). Data corresponds to means ± SE. Asterisks (*P <0.05*) indicate values that are significantly different from the control.

Total oil content in heartwood extracts was highest in the trees treated with the paclobutrazol root drench with a mean of 2.7% ± 0.4% SE (Figure 25) followed by the ethrel drench 2.2% ± 0.9% SE, ethrel spray 2.0% ± 0.3% SE and the control group having the lowest mean of 1.6 ± 0.4% SE. Due to the large variation in oil yield, statistically significant results were only detected between the control and paclobutrazol ($P<0.05$).

When these results are expressed as a yield (g/l), the highest yield was with the ethrel spray (3.55 g/l) followed by the paclobutrazol drench (3.26 g/l) and the ethrel drench (2.95 g/l). All of these yileds were above the control of 2.38 g/l.

Since the greatest value to plantation managers is an increase in yield and heartwood volume, differences between stem diameter, heartwood volume (cm^2), sapwood volume (cm^2) and the HW:SW ratio were calculated. The HW:SW ratio was lowest in the control group, however due to variation, this was not significant ($P>0.05$).

These preliminary results indicate that a non-invasive application of chemicals can influence yields and should be further explored including different concentrations, range of chemicals and mode and number of applications.

7.2.2 Oil composition

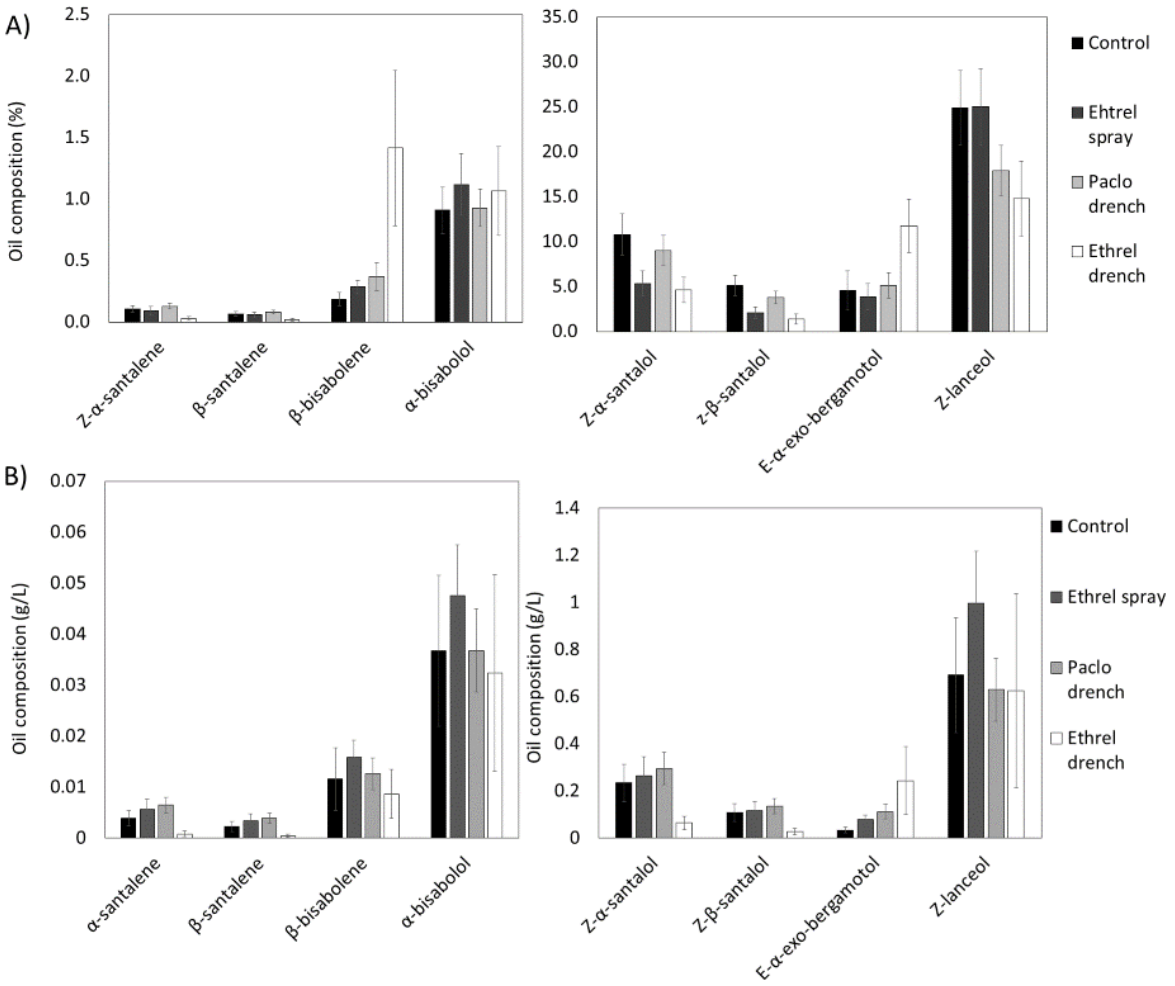


Figure 26. Mean A) composition (%) or B) total amount (g/L) of major oil components in the heartwood oil extracts of *S. austrocaledonicum* trees treated with a foliar ethrel spray (n=24), paclobutrazol (paclo) drench (n=24), an ethrel drench (n=9) or control (n=24). Data corresponds to means ± SE. Asterisks ($P<0.05$) indicate values that are significantly different from the control.

The major components of the sandalwood oil were Z-α-santalol, z-β-santalol, E-α-exo-bergammotol and z-lanceol (Figure 26). Mean santalol content was highest in the control group, followed by paclobutrazol and interestingly, significantly higher than the ethrel drench (P=0.004) (Figure 26). The β-santalol content was significantly higher in control and paclobutrazol treatments compared to the ethrel drench (P=0.03), which seemed to have a negative impact on santalol content. This result is opposite to the branch-induction experiment. Only a small number of components including β-bisabolene and *E*-α-*exo*-bergamotol were significantly different between treatments (Table 11), with a larger number of terpenoid profiles showing significant overlap and no distinct clustering based on treatments (data not shown). Although these results are preliminary, they do indicate that oil profile can also be manipulated via a non-wounding route and warrants further experimentation, particularly in older plantation trees.

Table 11: Sesquiterpene composition of *Santalum austrocaledonicum* heartwood extracts treated with four treatments, paclobutrazol drench, ethrel spray and ethrel drench.

Retention Index			Paclobutrazol drench				Ethrel spray				Ethrel drench				Control			
Component	R.I.	R.I. (lit)	Mean	SE	Min	Max	Mean	SE	Min	Max	Mean	SE	Min	Max	Mean	SE	Min	Max
Z-α-santalene	1572	1575[A]	0.13	0.02	0.00	0.39	0.09	0.03	0.00	0.71	0.03	0.02	0.00	0.13	0.11	0.03	0.00	0.42
Z-α-exo-bergamotene	1585	1586	0.01	0.00	0.00	0.07	0.01	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.07
epi-β-santalene	1634	1637[A]	0.07	0.01	0.00	0.23	0.05	0.02	0.00	0.44	0.02	0.01	0.00	0.09	0.07	0.02	0.00	0.26
β-santalene	1648	1651[A]	0.08	0.02	0.00	0.24	0.06	0.02	0.00	0.48	0.02	0.01	0.00	0.08	0.07	0.02	0.00	0.27
Unknown1	1656	??	0.75	0.18	0.00	4.14	0.44	0.10	0.00	1.87	2.19	0.64	0.25	5.33	0.18	0.04	0.00	0.63
β-bisabolene	1722	1724[A]	0.37	0.11	0.00	2.84	0.28	0.06	0.00	0.91	1.41	0.63	0.09	4.38	0.18	0.05	0.00	1.01
β-bisabolol	2140	??	1.06	0.18	0.00	4.49	0.82	0.12	0.00	2.32	1.16	0.38	0.00	3.44	0.55	0.10	0.00	1.38
α-bisabolol	2204	2207[A]	0.93	0.15	0.00	2.96	1.12	0.25	0.00	5.45	1.07	0.36	0.00	2.54	0.91	0.19	0.00	2.50
Z-α-santalol	2335	2344[A]	8.99	1.68	0.00	34.51	5.33	1.37	0.00	28.55	4.64	1.42	0.45	10.42	10.78	2.28	0.00	35.74
Z-α-exo-bergamotol	2346	2344[A]	2.50	0.41	0.00	9.23	1.59	0.32	0.00	6.41	0.98	0.45	0.00	3.89	2.69	0.57	0.00	7.84
Farnesol	2347	2342[A]	1.77	0.22	0.00	3.25	1.66	0.22	0.00	3.52	1.46	0.50	0.00	3.99	1.25	0.23	0.00	3.18
epi-β-santalol	2400	2406[A]	0.38	0.11	0.00	2.46	0.23	0.08	0.00	1.48	0.07	0.07	0.00	0.56	0.52	0.15	0.00	2.45
E-α-exo-bergamotol	2415	2401[B]	3.79	0.71	0.00	15.91	2.09	0.61	0.00	13.19	1.36	0.58	0.00	4.41	5.13	1.15	0.00	18.41
z-β-santalol	2418	2423[A]	5.12	1.41	0.00	28.99	3.87	1.43	0.00	27.47	11.75	2.95	1.46	27.08	4.54	2.17	0.00	37.06
Unknown3	2430	??	6.49	0.54	0.00	10.06	5.39	0.76	0.00	11.53	5.54	1.32	0.00	12.20	3.63	0.63	0.00	10.73
Unknown4	2462	??	0.57	0.11	0.00	1.98	0.60	0.12	0.00	1.99	1.50	1.25	0.00	10.20	0.41	0.11	0.00	1.99
Z-β-curcumen-12-ol	2479	2478[A]	15.45	1.61	3.96	41.25	10.73	1.33	0.00	21.73	13.77	1.95	6.54	25.34	8.56	1.16	0.00	19.81
Z-lanceol	2485	2486[A]	17.88	2.84	1.80	50.80	25.01	4.27	0.00	70.76	14.75	4.23	2.77	35.92	24.88	4.15	0.00	85.22
Z-nuciferol	2510	2513[A]	10.56	1.36	2.51	31.14	13.23	1.94	0.00	34.78	11.26	1.72	4.91	19.83	10.94	1.62	0.00	27.06
Unknown5	2542	??	3.65	0.42	0.00	7.30	3.17	0.49	0.00	6.97	3.90	0.92	0.00	6.40	2.29	0.37	0.00	5.51
Unknown6	2764	??	12.08	1.20	0.00	23.26	10.04	1.41	0.00	24.20	12.35	2.68	0.00	25.58	12.84	2.46	0.00	48.75
Total santalol			12.79	2.37	0.00	50.43	7.43	1.94	0.00	41.74	6.00	1.87	0.58	14.83	15.92	3.42	0.00	54.16
Oil yield (%w/w)			2.73	0.36	0.00	5.80	1.98	0.33	0.00	4.93	2.19	0.91	0.01	6.00	1.59	0.42	0.00	8.04

** [A] Valder et al., 2003

** [B] Braun et al., 2007

7.3 Discussion

The value of a sandalwood tree is in the amount of heartwood a tree contains. The quality of the oil sets the price. The more santalols dominating the sandalwood oil composition, the higher the price. *S. austrocaledonicum* produces trees with high levels of santalols and can demand top prices with the reputation its oil being the third most valuable of the *Santalum* species.

Harvesting of immature Sandalwood trees results in minimal heartwood formation, low sandalwood oil yields and low prices. If this heartwood has been stimulated by wounding, then this action will degrade the sandalwood quality. This will lead to a poor perception of the quality of sandalwood from Vanuatu.

This research showed that this group of 10-year old trees in Vanuatu were immature, that is they contained sapwood only or small amounts of heartwood. Three trees only contained sapwood and ten of the remaining twelve trees contained darkened wood with high levels of lanceol suggesting wounding played a major role in the presence of sandalwood oil.

The variation in heartwood formation is a concern. This project investigated how heartwood production could be reliably initiated so that when mature trees were harvested, they had a high proportion of heartwood.

The approach taken was to use a variety of plant-hormone related compounds that were explored for their ability to upregulate key genes for sandalwood oil production.

All treatments produced a range of sandalwood oil components which was reflected in an increase in sandalwood oil. Unexpected was the dominance of the sandalwood oil component, lanceol, which correlated to a wounding response. This wounding is referred to in the literature as a stress-induced developmental aging as compared to age-induced.

With cyclone Pam having passed through Vanuatu prior to the initiation of these experiments (2015), the research team was well aware and appreciated the wounding impact of the high velocity winds. This introduced a level of 'noise' in the results which was shown with the presence of sandalwood oil in the sapwood

The sandalwood oil biosynthesis pathway for the production of each of the sandalwood oil components shows that either the tree will produce lanceol or the santalols as both components are from the same source, and different enzymes form either lanceol or the santalols.

The only plant hormone-related compound that stimulated and directed sandalwood production to the santalols was ethrel which results in the production of the plant hormone ethylene. This is a plant hormone that controls the remobilisation of nutrients prior to cells dying, as is the case with fruit ripening. With all the other treatments, wounding (stress-induced developmental aging) appeared to be the driver of initial sandalwood oil production.

Interesting was the upregulation of different genes in the sandalwood oil biosynthetic pathway. It should be noted that the lanceol gene was not included in this up-regulation study. The greatest increase in up-regulation was with bisabolene synthase. This is a gene that makes bisabolene from the sandalwood oil precursor farnesyl pyrophosphate (FPP). There is another gene needed to synthesise this further into bisabolol. These two components are a very small part of the sandalwood profile (Table 6) and from the results are not linked to a wounding response.

The second-highest upregulation of a gene was santalene synthase and this gene up-regulation was also not linked to wounding. This is the gene that would make santalene from the sandalwood oil precursor farnesyl pyrophosphate (FPP). Santalene is the precursor that makes the most sought-after components of sandalwood oil, Z-α-and β-santalol and z-bergamatol, as well as E-bergamatol. The treatments that gave the highest up-regulation of this gene was ethrel and paclobutrazol.

The project explored the up-regulation of two genes that make different santalols from santalene, and they performed differently. The gene SaCYP736, that makes the preferred α-santalol, was not affected by the wounding whereas the gene SaCYP76 making E-α-santalol was upregulated with wounding. In this case the ethrel maintained an upregulation effect but not paclobutrazol to the same extent.

This was a first exploration into using the biosynthetic pathway genes to try and understand heartwood formation. It gives a glimpse of the usefulness of the technique to provide a deeper understanding on treatment response. This is particularly useful when dealing with long-term plant development responses.

Whole-tree treatment to stimulate heartwood formation is not a new concept in Vanuatu. Trees have been pollarded (the crown removed from the bole), branches from the crown cut, the trees bole restricted with wire as well as cutting into the base of the bole and cutting into the roots.

When using a chemical treatment, the question was if the chemical would be absorbed and sandalwood oil stimulation still occur? Whole-tree treatment, either as a drench at the base of the tree or as a foliar spray, avoids wounding.

Two chemicals were chosen for the final, whole-tree test. The first was ethrel which when the pH changes when taken up by the tree, releases the gaseous hormone ethylene. This plant hormone is known as the senescence hormone as due to its presence fruit will ripen and cut flower petals will die. During this ripening or death process, there is a re-mobilisation of nutrients from the dying or ripening tissues to other needing tissues. This chemical was shown to stimulate the santalol biosynthetic pathway in preference to the lanceol.

Because of this remobilisation activity of the ethylene, the second chemical chosen was paclobutrazol which is known to stunt plant growth by shortening the internode length between the leaves of the new growth. In situations where the plant sets the buds and then continues to elongate to set new leaves, paclobutrazol will minimise the new leaf growth to support flower development. Thus there is a redirection of plant assimilates towards the centre of the tree, and we were hoping this would result in greater heartwood formation.

The absorption of paclobutrazol through the roots has been studied in detail (Mari-no, 1981; Ziv and Ariel, 1991) and it is known that only if there is a high level of clay in the soil that may bind the paclobutrazol will translocation into the plant be limited. In the case of ethrel, this too has been shown to be readily absorbed by the plant and ethylene production stimulated. The release of ethylene is a simple base-catalysed reaction and does not involve enzyme activity on the part of the plant. Ethrel is stable in the acid form but breaks down to form ethylene at pH 3.5 or above. Most ethrel applied to plants eventually is converted to ethylene with examples given of half of the amount applied converted within 6 days (Abeles, 1973). There is little understood about the absorption of Ethrel through the roots.

Whole tree treatment with ethrel and paclobutrazol stimulated heartwood production. Although results are preliminary, they do indicate that oil profile can also be manipulated via a non-wounding route and warrants further experimentation, particularly in older trees.

If a plant development purpose of heartwood formation is to provide structural support, then rapid tree growth to produce a large tree could have a heartwood-stimulation effect. Additional, to provide the security that all sandalwood trees have commercial amounts of heartwood at harvest, a heartwood-stimulation treatment is needed.

This research project offers a solution. When trees are approximately 10 years of age, towards the end of their fast-growing juvenile stage, a treatment could be applied that switches heartwood formation in all trees. This would ensure that all trees have at least 10 years of heartwood formation before being harvested at approximately 20 years.

The chemical heartwood-stimulation treatment is simple. A ring around the base of the tree is cleared of vegetation and the chemical is applied in liquid form. This is taken up and translocated within the tree. Alternatively, as occurs in horticulture, the spraying of the trees could occur.

8 Impacts

8.1 Scientific impacts – now and in 5 years

This project demonstrated the use of the identified sandalwood oil biosynthetic pathway to understand heartwood formation. This together with using plant hormone-related treatments provided new insight into the regulation of sandalwood oil biosynthesis. This project tested, for the first time, the usefulness of understanding gene upregulation with different silviculture treatments. This was a beginning and the techniques need to be further explored to refine the results for commercial confidence.

8.2 Capacity impacts – now and in 5 years

The Vanuatu Forestry Department has been closely involved with this project, particularly Joseph Tungon, Michael Tabi and James Samuel who worked directly in the field with Dr Barbour and Dr Page contributing to the implementation of the experiments.

Joseph Tungeon and Michael Tabi are highly skilled in their knowledge of sandalwood with their regular engagement with this ACIAR project and others. They are a leading influence for the wide adoption of sandalwood across Vanuatu.

This project allowed the re-thinking of heartwood formation in sandalwood, and highlighted the risks of tree wounding:

- creating a low value oil (with a dominance of lanceol),
- damaging the cambium and radial parenchyma and stopping sandalwood oil production
- attracting diseases, particularly heartwood rot, which interfere with the steady accumulation of sandalwood oil in the heartwood

Close communication was also maintained with Summit Estate, particularly Lee Peterson. Their plantation practices have changed during this Project period and they are producing outstanding sandalwood plantations.

This Project work was shared at the Sandalwood Regional Forum 2019, Port Vila, Vanuatu both as a paper and field discussion.

8.3 Community impacts – now and in 5 years

8.3.1 Economic impacts.

The value of a sandalwood tree is in the amount of heartwood produced and the oil profile from the oils extracted from this heartwood.

A major issue is the variability of heartwood formation and this project addressed the stimulation of heartwood production. If all trees initiate heartwood at the same time, a greater uniformity between trees is expected.

8.3.2 Social impacts

With increased confidence in heartwood formation, more families will plant sandalwood trees to plan for future security.

8.3.3 Environmental impacts

More sandalwood trees will be planted with their increased value.

8.4 Communication and dissemination activities

Communication with the Vanuatu Forestry Department and Summit Estate was undertaken at project initiation, and during the project.

Project sampling was undertaken together with Vanuatu Forestry Department, leaders were Joseph Tungeon and Michael Tabi, and both were highly experienced with sandalwood establishment and experimentation.

This Project linked with Dr Tony Page who is presently managing the major sandalwood project in Vanuatu. This link was utilised throughout this project.

Sandalwood Regional Forum (11-14 November 2019) was supported by this Project by paying for the attendance of regional sandalwood researchers and managers.

Presenting a summary paper, Dr Liz Barbour highlighted the outcomes of this project at this conference. Further discussions were held at the trial site.

Two papers are in draft to be submitted to peer-reviewed journals. The first is the scientific work undertaken in this Project and the second is the review of sandalwood heartwood formation.

9 Conclusions and recommendations

The success with earlier ACIAR projects in Vanuatu have built an understanding of how to grow sandalwood from a seedling into a mature tree. Nursery production of sandalwood seedlings is reliable and their field establishment successful. The host relationship to optimise sandalwood growth has improved with the change in practice from total removal of all vegetation prior to establishment to sandalwood plantings preferably following an enrichment approach, and with greater success. Pruning techniques to coax leader shoot domination is a common approach showing great rewards, and reducing wounding through the removal of major branches. All in all, the knowledge to grow high quality sandalwood is evident.

Sandalwood production in Vanuatu now needs to address heartwood formation as this is the basis of tree value. It is clear, from the literature and results from this project, that stress-related ageing does not produce true heartwood. Quality age-induced heartwood needs time to form as it is linked to maturation. Once initiated, all evidence shows that its formation continues. To switch the tree from its high-growth juvenile state to a maturation state will slow overall tree growth so the timing of this conversion is critical for overall tree productivity, and the final amount of heartwood produced.

9.1 Conclusions

1. With wounding, Vanuatu sandalwood reacts to produce lanceol rather than the santalols. Santalols are the preferred component of sandalwood oil.
2. Ethylene was the only treatment tested on the branches which sandalwood responded by immediately producing santalols. This supported the assumption that the growth regulation process by which sandalwood oil is produced is a regulated senescence process
3. Sandalwood oil production increased with a root drench of paclobutrazol or ethrel treatment. A foliar spray of ethrel also had a positive response but there was concern on efficacy of uptake due to rain directly after treatment.
4. Gene upregulation measurement reflected the oil measurements and showed that this approach could be followed for quicker treatment-response assessment.
5. At 10-years of age, *S. austrocaledonicum* is immature, that is true heartwood had not reliably formed. Projects need to plan for a rotation that takes into account heartwood initiation at 10-years of age.

9.2 Recommendations

1. Larger, long-term trials established with the whole tree-treatments to test their economic benefit. The project outcomes suggest that the sandalwood tree should be grown rapidly for the first 10 years during the period the wood is juvenile. Stimulation of all trees to initiate age-related heartwood formation would be ideal at this 10 year mark, and then the trees are grown for a further 10 years for heartwood formation before harvest.

2. Education on heartwood formation in sandalwood is needed as there appears to be a misunderstanding that wounding can initiate heartwood. Wounding initiates stress-induced development and not the age-related development that forms true heartwood. This stress-induced heartwood formation will bias towards lanceol rather than the santalols. Any tree wounding is not advantageous to heartwood formation.

3. A better understanding of heartwood modelling within a tree would assist in this education program and provide a more realistic understanding of heartwood growth rate as a sandalwood tree ages.

4. Further exploration of the use of the gene upregulation measurement as a response to treatment. This would provide a clearer view of sandalwood oil production with heartwood formation.

10 References

10.1 References cited in report

Abeles F (1973) Ethyene in plant biology. USDA. Academic Press

Arbellay E, Fonti P, Stoffel M (2012) Duration and extension of anatomical changes in wood structure after cambial injury. *Journal of Experimental Botany* **63**, 3271–3277.

AS (2003) Oil of sandalwood [Santalum spicatum (R.Br) A.DC.]. AS2112-2003. In. (Standards Australia International, Sydney, Australia)

Azren PD, Lee SY, Emang D, Mohamed R (2019) History and perspectives of induction technology for agarwood production from cultivated Aquilaria in Asia: a review. *Journal of Forestry Research* **30**, 1-11. doi:10.1007/s11676-018-0627-4

Baldovini N, Delasalle C, Joulain D (2011) Phytochemistry of the heartwood from fragrant Santalum species: a review. *Flavour and Fragrance Journal* **26**, 7-26. doi:10.1002/ffj.2025

Baqui S, Shah JJ, Syamprasad G (1984) Effect of ethephon and amino ethoxy vinyl glycine on heartwood formation in *Acacia-auriculiformis* Cann. *Proceedings of the Indian Academy of Sciences-Plant Sciences* **93**, 77-82.

Barreto A (2017) Conservation of sandalwood (*Santalum album* L.) in East Timor - case study. Universidade de Évora.

Benhamou N, Bélanger RR (1998) Induction of systemic resistance to Pythium damping-off in cucumber plants by benzothiadiazole: ultrastructure and cytochemistry of the host response. *The Plant Journal* **14**, 13-21. doi:10.1046/j.1365-313X.1998.00088.x

Biggs AR (1987) Occurrence and location of suberin in wound reaction zones in xylem of 17 tree species. *Phytopathology* **177**, 718-725.

Bois E, Lieutier F (1997) Phenolic response of Scots pine clones to inoculation with Leptographium wingfieldii, a fungus associated with Tomicus piniperda. *Plant Physiology and Biochemistry* **35**, 819-825.

Bottin L, Isnard C, Lagrange A, Jeann Marc B (2007) Comparative molecular and phytochemical study of the tree species *Santalum austrocaledonicum* (Santalaceae) distributed in the New-Caledonian archipelago. *Chemistry & Biodiversity* **4**, 1541-1556.

Brand JE, Norris LJ, Dumbrell IC (2012) Estimated heartwood weights and oil concentrations within 16-year-old Indian sandalwood (*Santalum album*) trees planted near Kununurra, Western Australia. *Australian Forestry* **75**, 225–232.

Burgess TI, Howard K, Steel E, Barbour EL (2018) To prune or not to prune; pruning induced decay in tropical sandalwood. *Forest Ecology and Management* **430**, 204-218. doi:https://doi.org/10.1016/j.foreco.2018.08.009

Byun-McKay A, Godard KA, Toudefallah M, Martin DM, Alfaro R, King J, Bohlmann J, Plant AL (2006) Wound-induced terpene synthase gene expression in sitka spruce that exhibit resistance or susceptibility to attack by the white pine weevil. *Plant Physiology* **140**, 1009-1021. doi:10.1104/pp.105.071803

Celedon JM, Chiang A, Yuen M, Diaz-Chavez ML, Madilao LL, Finnegan PM, Barbour EL, Bohlmann J (2016) Heartwood-specific transcriptome and metabolite signatures of tropical sandalwood (*Santalum album*) reveal the final step of (*Z*)-santalol fragrance biosynthesis. *The Plant Journal* **86**, 289-299.

Chen X, Liu Y, Yang Y, Feng J, Liu P, Sui C, Wei J (2018) Trunk surface agarwood-inducing technique with *Rigidoporus vinctus*: An efficient novel method for agarwood production. *PLOS ONE* **13**, e0198111. doi:10.1371/journal.pone.0198111

Cheng QW, Xiong YP*, et al.* (2019) Callus of East Indian sandalwood co-cultured with fungus Colletotrichum gloeosporioides accumulates santalenes and bisabolene. *Trees-Structure and Function* **33**, 305-312. doi:10.1007/s00468-018-1758-0

Chung J-P, Chen K-H (2018) Developing an in vitro quasi-symbiotic culture system of Aquilaria malaccensis with potentially beneficial microbes on agarwood resinous formation. *Plant Cell, Tissue and Organ Culture (PCTOC)* **133**, 193-202. doi:10.1007/s11240-017-1372-7

Coakley T (2013) Sandalwood markets and threats. In 'Proceedings of the International Sandalwood Symposium 2012'. (Eds M Nageswara-Rao, JR Somji and D Harbaugh) pp. 71-81. (Lulu Press, Inc. Raleigh, NC, USA: East-West Center, University of Hawaii, Honolulu)

Copini P, den Ouden J, Decuyper M, Mohren GMJ, Loomans AJM, Sass-Klaassen U (2014) Early wound reactions of Japanese maple during winter dormancy: the effect of two contrasting temperature regimes. *Aob Plants* **6**, 8. doi:10.1093/aobpla/plu059

Cordero LDP, Kanninen M (2003) Heartwood, sapwood and bark content, and wood dry density of young and mature teak (*Tectona grandis*) trees grown in Costa Rica. *Silva Fennica* **37**, 45-54.

da Silva JAT, Kher MM, Soner D, Page T, Zhang X, Nataraj M, Ma G (2016) Sandalwood: basic biology, tissue culture, and genetic transformation. *Planta* **243**, 847–887. doi:10.1007/s00425-015-2452-8

Dar TA, Uddin M, Khan MMA, Hakeem KR, Jaleel H (2015) Jasmonates counter plant stress: A Review. *Environmental and Experimental Botany* **115**, 49-57. doi:10.1016/j.envexpbot.2015.02.010

Delvaux C, Sinsin B, Van Damme P, Beeckman H (2010) Wound reaction after bark harvesting: microscopic and macroscopic phenomena in ten medicinal tree species (Benin). *Trees* **24**, 941-951. doi:10.1007/s00468-010-0465-2

Du S, Yamamoto F (2007) An overview of the biology of reaction wood formation. *Journal of Integrative Plant Biology* **49**, 131-143. doi:10.1111/j.1744-7909.2007.00427.x

Ecker JR, Davis RW (1987) Plant defense genes are regulated by ethylene. *Proceedings of the National Academy of Sciences* **84**, 5202-5206. doi:10.1073/pnas.84.15.5202

Enyedi AJ, Yalpani N, Silverman P, Raskin I (1992) Signal molecules in systemic plant-resistance to pathogens and pests. *Cell* **70**, 879-886. doi:10.1016/0092-8674(92)90239-9

Eyles A, Davies NW, Mohammed C (2003) Wound wood formation in *Eucalyptus globulus* and *Eucalyptus nitens*: anatomy and chemistry. *Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere* **33**, 2331-2339. doi:10.1139/x03-149

Faizal A, Esyanti RR, Aulianisa EN, Iriawati, Santoso E, Turjaman M (2016) Formation of agarwood from Aquilaria malaccensis in response to inoculation of local strains of Fusarium solani. *Trees - Structure and Function*, 1-9. doi:10.1007/s00468-016-1471-9

Frankenstein C, Schmitt U, Koch G (2006) Topochemical studies on modified lignin distribution in the xylem of poplar (Populus spp.) after wounding. *Annals of Botany* **97**, 195-204. doi:10.1093/aob/mcj030

Gan S , Amasino RM. 1997. Making sense of senescence (molecular genetic regulation and manipulation of leaf senescence). Plant Physiology 113, 313–319.

Gillieson D, Page T, Silverman J (2008) 'An inventory of wild sandalwood stocks in Vanuatu.' Australian Centre for International Agricultural Research, Publication number: FR2008-08. Project Number FST/2006/118. ISBN: 978 1 921434 36 5, Canberra. (http://aciar.gov.au/publication/fr2008-08).

Gominho J, Pereira H (2005) The influence of tree spacing in heartwood content in *Eucalyptus globulus* Labill. *Wood and Fiber Science* **37**, 582-590.

Harju AM, Venalainen M, Laakso T, Saranpaa P (2009) Wounding response in xylem of Scots pine seedlings shows wide genetic variation and connection with the constitutive defence of heartwood. *Tree Physiology* **29**, 19-25. doi:10.1093/treephys/tpn006

Hettiarachchi DS (2013) The good, the bad and the ugly: A review on sandalwood quality. In 'Proceedings of the International Sandalwood Symposium 2012'. (Eds M Nageswara-Rao, JR Somji and D Harbaugh) pp. 207-216. (Lulu Press, Inc. Raleigh, NC, USA: East-West Center, University of Hawaii, Honolulu)

Hillis WE (1987) 'Heartwood and Tree Exudates.' (Springer-Verlag: Berlin)

Hudgins JW, Franceschi VR (2004) Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation. *Plant Physiology* **135**, 2134-2149. doi:10.1104/pp.103.037929

Igartua DV, Moreno K, Monteoliva SE (2017) *Acacia melanoxylon* in Argentina: heartwood content and its relationship with site, growth and age of the trees. *Forest Systems* **26**, 12. doi:10.5424/fs/2017261-10195

IS 329 (2004) Oil of Sandalwood [PCD 18: Natural and Synthetic Fragrance Materials]. In. (Bureau of Indian Standards: New Dehli)

ISO (2002) '3518:2002 (E) Oil of sandalwood (*Santalum album* L.).' International Organization for Standardization, Geneva.

Jain SH, Arya R, Kumar H (2007) Distribution of sandal (*Santalum album* L.), current growth rates, predicted yield of heartwood and oil content and future potential in semi arid and arid regions of Rajasthan, India. *Forests, Trees and Livelihoods* **17**, 261-266. doi:10.1080/14728028.2007.9752601

Knapic S, Pereira H (2005) Within-tree variation of heartwood and ring width in maritime pine (Pinus pinaster Ait.). *Forest Ecology and Management* **210**, 81-89.

Kouzai Y, Noutoshi Y, Inoue K, Shimizu M, Onda Y, Mochida K (2018) Benzothiadiazole, a plant defense inducer, negatively regulates sheath blight resistance in Brachypodium distachyon. *Scientific Reports* **8**, 17358. doi:10.1038/s41598-018-35790-w

Kumar ANA, Joshi G, Ram HYM (2012) Sandalwood: history, uses, present status and the future. *Current Science* **103**, 1408-1416.

Lee DJ, Burridge AJ, Page T, Huth JR, Thompson N (2018) Domestication of northern sandalwood (*Santalum lanceolatum*, Santalaceae) for Indigenous forestry on the Cape York Peninsula. *Australian Forestry* **82**, 1-9. doi:10.1080/00049158.2018.1543567

Lesniewska J, Ohman D, Krzeslowska M, Kushwah S, Barciszewska-Pacak M, Kleczkowski LA, Sundberg B, Moritz T, Mellerowicz EJ (2017) Defense Responses in Aspen with Altered Pectin Methylesterase Activity Reveal the Hormonal Inducers of Tyloses. *Plant Physiology* **173**, 1409-1419. doi:10.1104/pp.16.01443

Lev-Yadun S (2002) The distance to which wound effects influence the structure of secondary xylem of decapitated *Pinus pinea*. *Journal of plant growth regulation* **21**, 191-196. doi:10.1007/s003440010057

Lim PO , Kim HJ, Nam HG. 2007. Leaf senescence. Annual Review of Plant Biology 58, 115–136.

Liu Y, Chen H*, et al.* (2013) Whole-tree agarwood-inducing technique: An efficient novel technique for producing high-quality agarwood in cultivated *Aquilaria sinensis* trees. *Molecules* **18**, 3086-106. doi:10.3390/molecules18033086

López-Sampson A, Page T (2018) History of use and trade of agarwood. *Economic Botany* **72**, 107-129. doi:10.1007/s12231-018-9408-4

Lv FF, Li SS, Feng J, Liu PW, Gao ZH, Yang Y, Xu YH, Wei JH (2019) Hydrogen peroxide burst triggers accumulation of jasmonates and salicylic acid inducing sesquiterpene biosynthesis in wounded Aquilaria sinesis. *Journal of Plant Physiology* **234**, 167-175. doi:10.1016/j.jplph.2019.02.006

Mari-no,G (1981) The effect of Paclobutrazol on in vitro rooting, trans-plant establishment and growth of fruit plants. Plant Growth Reg. 7:237-246

Marpeau A, Walter J, Launay J, Charon J, Baradat P, Gleizes M (1989) Effects of wounds on the terpene content of twigs of maritime pine (Pinus pinaster Ait.) II. Changes in the volatile terpene hydrocarbon composition. *Trees-Structure and Function* **3**, 220-226.

Melcher E, Jungel P, Mollendorf B, Schmitt U (2003) Identification of a hydroxy substituted calamenene - a sesquiterpene associated with wound reactions in non-infected xylem of *Tilia* spp. *Phytochemistry* **62**, 707-713. doi:10.1016/s0031-9422(02)00362-x

Miranda I, Gominho J, Araujo C, Pereira H (2014) Family effects in heartwood content of Eucalyptus globulus L. *European Journal of Forest Research* **133**, 81-87. doi:10.1007/s10342-013-0741-y

Mohamed R, Jong PL, Zali MS (2010) Fungal diversity in wounded stems of Aquilaria malaccensis. *Fungal Diversity* **43**, 67-74. doi:10.1007/s13225-010-0039-z

Moniodis J, Jones CG, Renton M, Plummer JA, Barbour EL, Ghisalberti ELa, Bohlmann J (2017) Sesquiterpene variation in West Australian sandalwood (Santalum spicatum). *Molecules* **22**, 940. doi:10.3390/molecules22060940

Moungsrimuangdee B, Moriwaki H, Nakayama M, Nishigaki S, Yamamoto F (2011) Effects of injection of ethrel, methyl jasmonate, and salicylates and *Raffaelea quercivora* inoculation on sapwood discoloration in *Quercus serrata*. *Iawa Journal* **32**, 41-53. doi:10.1163/22941932-90000041

Moya R, Bond B, Quesada H (2014) A review of heartwood properties of Tectona grandis trees from fast-growth plantations. *Wood Science and Technology* **48**. doi:10.1007/s00226-014-0618-3

Nilsson M, Wikman S, Eklund L (2002) Induction of discolored wood in Scots pine (*Pinus sylvestris*). *Tree Physiology* **22**, 331-338.

Padmanabha HSA (2013a) Indian sandalwood market and trend. In 'Proceedings of the International Sandalwood Symposium 2012'. (Eds M Nageswara-Rao, JR Somji and D Harbaugh) pp. 82-92. (Lulu Press, Inc. Raleigh, NC, USA: East-West Center, University of Hawaii, Honolulu)

Padmanabha HSA (2013b) Sandalwood plantations in agroforestry systems. In 'Proceedings of the International Sandalwood Symposium 2012'. (Eds M Nageswara-Rao, JR Somji and D Harbaugh) pp. 186-195. (Lulu Press, Inc. Raleigh, NC, USA: East-West Center, University of Hawaii, Honolulu)

Page T, Potrawiak A, Berry A, Tate H, Tungon J, Tabi M (2010a) Production of Sandalwood (*Santalum austrocaledonicum*) for improved smallholder incomes in Vanuatu. *Forests, Trees and Livelihoods* **19**, 299-316.

Page T, Southwell I, Russell M, Tate H, Tungon J, Sam C, Dickinson G, Robson K, Leakey RRB (2010b) Geographic and phenotypic variation in heartwood and essential-oil characters in natural populations of *Santalum austrocaledonicum* in Vanuatu. *Chemistry & biodiversity* **7**, 1990-2006.

Page T, Tate H, Bunt C, Potrawiak A, Berry A (2012) 'Opportunities for the smallholder sandalwood industry in Vanuatu.' (Australian Centre for International Agricultural Research (ACIAR): Canberra)

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, 6. doi:10.1093/nar/29.9.e45

Popp MP, Johnson JD, Lesney MS (1995) Changes in ethylene production and monoterpene concentration in slash pine and loblolly-pine following inoculation with bark beetle vectored fungi. *Tree Physiology* **15**, 807-812. doi:10.1093/treephys/15.12.807

Radomiljac AM, McComb JA, McGrath JF (1999) Intermediate host influences on the root hemi-parasite Santalum album L. biomass partitioning. *Forest Ecology and Management* **113**, 143-153.

Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A (2004) Guideline to reference gene selection for quantitative real-time PCR. *Biochemical and biophysical research communications* **313**, 856-862.

Rashkow ED (2014) Perfumed the axe that laid it low: The endangerment of sandalwood in southern India. *Indian Economic and Social History Review* **51**, 41-70. doi:10.1177/0019464613515553

Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. *Current Opinion in Plant Biology* **1**, 404-411. doi:10.1016/s1369-5266(98)80264-1

Rhind JP (2014) 'Fragrance and wellbeing: Plant aromatics and their influence on the psyche.' (Singing Dragon: London)

Sangareswari M, Parthiban KT, Kanna SU, Karthiba L, Saravanakumar D (2016) Fungal microbes associated with agarwood formation. *American Journal of Plant Sciences* **7**, 1445-1452. doi:doi: 10.4236/ajps.2016.710138

Schmitt U, Liese W (1993) Response of xylem parenchyma by suberization in some hardwoods after mechanical injury. *Trees* **8**, 23-30. doi:10.1007/BF00240978

Shain L, Hillis WE (1973) Ethylene production in xylem of *Pinus radiata* in relation to heartwood formation. *Canadian Journal of Botany-Revue Canadienne De Botanique* **51**, 1331-1335. doi:10.1139/b73-166

Shigo AL (1984) Compartmentalization: A conceptual framework for understanding how trees grow and defend themselves. *Annual Review of Phytopathology* **22**, 189-214. doi:10.1146/annurev.py.22.090184.001201

Shineberg D (1967) 'They came for Sandalwood: A study of the sandalwood trade in the south-west Pacific 1830-1865.' (Melbourne University Press: Melbourne)

Subasinghe SMCUP (2013) Sandalwood Research: A Global Perspective. *Journal of Tropical Forestry and Environment* **3**, 1-8.

Sun Q, Rost TL, Matthews MA (2008) Wound-induced vascular occlusions in *Vitis vinifera* (vitaceae): Tyloses in summer and gels in winter. *American Journal of Botany* **95**, 1498-1505. doi:10.3732/ajb.0800061

Tan CS, Isa NM, Ismail I, Zainal Z (2019) Agarwood Induction: Current Developments and Future Perspectives. *Frontiers in Plant Science* **10**, 13. doi:10.3389/fpls.2019.00122

Taylor AM, Gartner BL, Morrell JJ (2002) Heartwood formation and natural durability - A review. *Wood and Fiber Science* **34**, 587-611.

Thomson LAJ (2006) *Santalum austrocaledonicum* and *S. yasi* (sandalwood) Santalaceae (sandalwood family). In 'Traditional Trees of Pacific Islands: their Culture, Environment and Use'. (Ed. CR Elevitch) pp. 675-694. (Permanent Agriculture Resources (PAR). ISBN 0-9702544-5-8. URL: http://www.traditionaltree.org: Holualoa)

Vek V, Oven P, Ters T, Poljansek I, Hinterstoisser B (2014) Extractives of mechanically wounded wood and knots in beech. *Holzforschung* **68**, 529-539. doi:10.1515/hf-2013-0003

Vlot AC, Dempsey DMA, Klessig DF (2009) Salicylic Acid, a Multifaceted Hormone to Combat Disease. *Annual Review of Phytopathology* **47**, 177-206. doi:10.1146/annurev.phyto.050908.135202

Xu Y, Chang PFL, Liu D, Narasimhan ML, Raghothama KG, Hasegawa PM, Bressan RA (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell* **6**, 1077-1085. doi:10.1105/tpc.6.8.1077

Yan TT, Yang S, Chen Y, Wang Q, Li GY (2019) Chemical profiles of cultivated agarwood induced by different techniques. *Molecules* **24**, 14. doi:10.3390/molecules24101990

Yang JM, Park S, Kamdem DP, Keathley DE, Retzel E, Paule C, Kapur V, Han KH (2003) Novel gene expression profiles define the metabolic and physiological processes characteristic of wood and its extractive formation in a hardwood tree species, Robinia pseudoacacia. *Plant Molecular Biology* **52**, 935-956. doi:10.1023/a:1025445427284

Ye ZH, Zhong R. 2015. Molecular control of wood formation in trees. J Exp Bot. 66(14):4119-31.

Yun DR (2012) Study on the mechanism of forming aromatic heartwood in *Santalum album* l. by ethephon stimulation. Guangzhou University of Traditional Chinese Medicine.

Zhang Z, Wei JH, Han XM, Liang L, Yang Y, Meng H, Xu YH, Gao ZH (2014) The sesquiterpene biosynthesis and vessel-occlusion formation in stems of *Aquilaria sinensis* (Lour.) gilg trees induced by wounding treatments without variation of microbial communities. *International Journal of Molecular Sciences* **15**, 23589-23603. doi:10.3390/ijms151223589

Zhu ZQ (2014) Molecular basis for jasmonate and ethylene signal interactions in Arabidopsis. *Journal of Experimental Botany* **65**, 5743-5748. doi:10.1093/jxb/eru349

Ziv, M. Ariel,(1991) Bud proliferation and plant regeneration in liquid-cultured Philodendron treated with Ancymidol and Paclobutrazol. Plant Growth Regulation 10 : 53-57

10.2 List of publications produced by project

11 Appendixes

11.1 Appendix 1: The effect of foliar application of different concentrations of ethrel on *Santalum album.*

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The use of ethrel to stimulate oil biosynthesis has been demonstrated in *Santalum austrocaledonicum* branches. This study was undertaken to examine the effect of different concentrations of ethrel applied as a foliar spray to *S. album*. Ethrel at 0.1, 0.5, 1, 2 & 4% was sprayed to leaf drip (upper and lower surfaces covered), with an average of 850mL applied per tree. The level of defoliation was assessed after 14 days with significant leaf drop recorded across all treatments. The percentage leaf drop was 96, 97, 98, 100, 100% for 0.1, 0.5, 1, 2 & 4% Ethrel respectively. This demonstrates that *Santalum album* is particularly sensitive to Ethrel application. Ethrel concentrations of 0.1 and 0.5% resulted in almost complete defoliation, but shoot tips remained green after two weeks. Trees treated with 1, 2 & 4% Ethrel had a more severe reaction with complete defoliation and necrosis of shoot tips.

Further studies with whole canopy treatment should be conducted with concentrations of up to 0.1% for *S. album* and *S. austrocaledonicum*.

Further observations will be carried out over the coming months to determine canopy recovery in each of the Ethrel treatments.

Methods

Fifteen trees of *Santalum album* were treated with one of five concentrations of Ethrel (3 trees each with 0.1, 0.5, 1, 2 & 4%) on 24th November 2017. A total of five litres of Ethrel was mixed for each concentration. Trees were sprayed until leaf drip on both sides of the leaves with the total volume applied per tree varying (Table 1) according to tree size.

Trees were assessed for level of defoliation 14 days after Ethrel application on 8th December 2017.

Table 12: Ethrel concentrations and the volumes applied for each treatment

Ethephon Concentration	Ethrel mL/5L	Vol. applied per 3 trees	Mean vol. tree^{-1}
0.1%	6.9	1.95L	650mL
0.5%	34.72	2.56L	850mL
1%	69.44	2.42L	806mL
2%	138.8	3.12L	1040mL
4%	277.7	2.7L	900mL
		Mean	**850mL**

Results

Ethel Concentration	Prior to application	14 days after application	Defoliation
0.1%			98%
0.1%			95%
0.1%			95%

Ethrel Concentration	Prior to application	14 days after application	Defoliation
0.5%			100%
0.5%			95%
0.5%			98%

Ethrel Concentration	Prior to application	14 days after application	Defoliation
1%			95%
1%			100%
1%			100%

Ethrel Concentration	Prior to application	14 days after application	Defoliation
2%			100%
2%			100%
2%			100%

Ethrel Concentration	Prior to application	14 days after application	Defoliation
4%			100%
4%			100%
4%			100%