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Note

Foliar application of methyl jasmonate does not increase terpenoid accumulation, but weakly elicits terpenoid pathway genes in sandalwood (*Santalum album* L.) seedlings

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Abstract The sesquiterpenoid rich essential oils of sandalwood (*Santalum album* L.) stems and roots are a highly sought commodity in the fragrance industry. Plantations of sandalwood are being established in northern Australia, however the valuable heartwood essential oils do not accumulate in substantial amounts before 10 years, while commercially viable harvests do not normally take place for at least 15 years. Inducing essential oil accumulation at an earlier stage, or increasing oil yield in mature trees, may have the potential to enhance the oil productivity of plantations. In this study, we investigated the effects of foliar application of methyl jasmonate on less than one-year-old sandalwood seedlings. Essential oil accumulation was unaffected in both stems and roots. However, at the gene transcript level, several key genes early in the biosynthesis of sandalwood oil components were induced in both leaves and stems. These results suggest that terpenoid biosynthesis in *S. album* does indeed respond to foliar application of methyl jasmonate, however the effects are small and the full biosynthesis of santalols is likely to be developmentally regulated.

Key words: *Santalum album*, Sandalwood oil, terpene induction, methyl jasmonate, gene expression.

Sandalwood, *Santalum album*, is a small hemiparasitic tree which accumulates a mixture of sesquiterpene olefins and alcohols in the ray parenchyma of mature xylem (Jones et al. 2008). These sesquiterpenes, predominantly α- and β- santalols, constitute sandalwood oil, which is highly valued in the perfume and fragrance industry. Sandalwood trees do not normally begin to yield fragrant heartwood until approximately 10 years of age (Jones et al. 2007) suggesting that oil accumulation is developmentally controlled and heavily dependent on age. Sandalwood grows relatively slowly and even similarly aged and spaced plantation trees vary widely in the amount of heartwood oil they contain. At harvest, entire trees are uprooted as the lower part of the stems and roots contain the highest amounts of heartwood essential oils. *Santalum album* plantations in northern Australia are usually scheduled for harvest after 15 years, although as trees age oil contents increase (Barbour et al. 2010). Long rotation times and slow onset of heartwood development has motivated growers to seek a means to induce early sesquiterpene production in plantation sandalwood. Ideally such a process would involve a simple treatment that could be applied to leaf surfaces or through other convenient means such as irrigation in nursery facilities or plantations.

Foliar application of methyl jasmonate (MeJ) has been shown to induce several different secondary metabolite biosynthetic pathways in both angiosperms and gymnosperms, with the terpenoid pathways often being highly induced (Ament et al. 2004; Miller et al. 2005; Rodriguez-Saona et al. 2001; Wang et al. 2010; Zulak et al. 2009). In the present work, initial investigation into the foliar application of MeJ was carried out on nine-month-old sandalwood saplings in the glasshouse (Figure 1A). Changes in terpenoid accumulation in roots and stems were monitored using gas chromatography-mass spectrometry (GC-MS). Concurrently, genes specific to terpene biosynthesis, along with candidate genes for pathogen defence and signalling were monitored for changes in their relative abundance by means of quantitative real-time polymerase chain reaction (qRT-PCR). By measuring these metabolic and transcript

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Figure 1. Photograph of the *Santalum album* plants during harvest time (March 2011). 1A shows a group of 9 month old plants in the greenhouse. Host plants (*Alternanthra nana*) have been trimmed and are just visible above the soil line. 1B shows the cleaned roots prior to destructive harvest with host plants (*Alternanthra nana*) visible in the bottom right. (Photographs by Chris Jones).

parameters over a 24-day time course, potential effects of exogenously applied MeJ on sandalwood could be quantified. It was hypothesised that foliar application of MeJ would result in an increase in the abundance of terpenoid and pathogenic defence related genes, followed by a detectable increase in accumulation of santalenes and santalols, the major constituents of sandalwood oil in both roots and stems.

Ninety-six five-month old sandalwood seedlings were donated from a commercial sandalwood grower (Tropical Forestry Services, Kununurra) and re-potted into 250 mm diameter free-draining pots using a coarse sand-compost based potting mixture. The seedlings arrived as co-plantings with the herbaceous *Alternanthra nana* R. Br., which is a popular choice of host plants to establish seedlings and young sapling trees of hemiparasitic sandalwood (Radomiljac et al. 1998). Host plants were trimmed to ca. 20 cm tall on a regular basis to limit the impact of resource competition with sandalwood. Saplings were watered daily to field capacity and fertilised fortnightly with Thrive (Yates, Melbourne) complete water-soluble fertiliser. The plants were maintained in the glasshouse over the summer months (November to January) before the experiment commenced in February (Figure 1A). Half of the plants (48) were moved to an adjacent glasshouse where they were treated with 0.1% MeJ solution (with 0.1% Tween 80 as a surfactant) sprayed over the foliage at a rate of approximately 20 ml per plant. Control plants were sprayed with a 0.1% Tween 80 only solution, at the same 20 ml per plant application rate as the MeJ treated plants. Treated plants were moved back to the original glasshouse after 24 h, but located four meters away from the untreated plants. Air flow in the glasshouse was predominantly upwards with little opportunity for mixing between treated and untreated plants.

Four plants (i.e. biological replicates) were removed and destructively sampled from both the treated and control cohorts at each time point. At four hours post treatment, leaf samples were taken from four biological replicates of both treated and untreated plants, and these same plants were later used for a destructive harvest after 24 h. Further sampling time points were 2 d, 4 d, 8 d, 16 d and 24 d. Mature and young leaves (roughly 20 g) were taken from each plant at harvest, frozen in liquid nitrogen and stored at −80°C. These leaves were used for the gene expression study only, as sandalwood does not accumulate any sesquiterpenes in their leaves. Stems (soil level to two thirds of the height of the seedling (ca. 80 cm)) were stripped of leaves and coarsely chopped before being flash frozen. Roots were gently washed of soil (Figure 1B) and dried on paper towel before being coarsely chopped and flash frozen. All material was labelled and stored at −80°C before proceeding to the extraction steps.

Samples were transferred to paper envelopes and

placed in a vacuum desiccator for 2 d. Most of the dried sample (2–3 g) was used for essential oil analysis using GC-MS. Sesquiterpene extraction was done over a week in 25 ml volumetric flasks using spectroscopic grade hexane (Jones et al. 2007). An aliquot was taken from these and analysed by GC-MS using single ion monitoring (SIM) at m/z=94; the dominant ion for most of the sesquiterpenes found in sandalwood oil. Concentrations of santalenes and santalols were calculated using an external standard curve from a dilution series of authentic sandalwood oil run under the same GC-MS conditions. Total sesquiterpenes were recorded as the sum of all detectable peaks matching the retention times of sandalwood oil. Due to the low concentration of sesquiterpenoids in these extracts, only santalenes and santalols could be detected using SIM. Samples were separated on a 30 m DB-WAX column (Agilent) of 0.25 mm diameter, 0.25 μm film thickness. Oven temperature was set to 40°C for 1 min, then increased at 10°C min^{-1} to 220°C where it was held for 25 min. MSD was set to 70 eV in SIM mode.

Leaf samples were ground in liquid nitrogen to a fine powder using a mortar and pestle, while root and stem samples were ground in a commercial Waring blender with liquid nitrogen in a stainless steel bowl. RNA extraction was done using methods described earlier (Jones et al. 2008, 2011). RNA quality was inferred by separation on a non-denaturing electrophoresis gel and by spectrophotometry. cDNA was synthesised from 3 μg of total RNA using the methods described by Zulak et al. (2009). Sixteen genes (including two housekeeping genes) were probed using primers derived from sequences identified in previously reported *S. album* xylem Sanger EST and 454 cDNA library (Jones et al. 2011). Selection of these genes was based on a hierarchy of known involvement in general terpenoid metabolism. General terpenoid metabolism genes which are early in the pathway, 3-hydroxy-3-methylglutary-CoA reductase (*hmgr*: Supplement S1), 1-deoxyxylulose-5-phosphate synthase (*dxs1*: Supplement S1) and isopentyl diphosphate isomerase (*ippi2*: Supplement S1), were used in the analysis (Cane 1990; Külheim et al. 2011) as well as genes known to be involved in sandalwood specialized sesquiterpenol biosynthesis (farnesyl diphosphate synthase (*SaFPPS*-HQ343293), santalene synthase (*SaSSy*-HQ343276), bisabolene synthase (*SaBS*-HQ343279), sesquiterpene synthase (*SaSTPS*-ACF24768), sesquisabinene synthase (*SaSQBS*-HQ384285)) (Jones et al. 2011, Moniodis et al. 2014). The cytochrome P450 gene, *SaCYP76F38v2* (KC533718), which is a member of the sandalwood CYP76F group of sesquiterpenol biosynthesis (Diaz-Chavez et al. 2013; Moniodis et al. 2014) was also included. Two homologues of cytochrome P450 genes involved in terpenoid oxidation from subfamilies 72 and 716B in other plants (*SaCYP72* and *SaCYP716B*: Supplement S1) were selected (Fukushima et al. 2011; Vetter et al. 1992) as they also had unusually high transcript abundance in oil producing ray parenchyma of *S. album*. Finally, *Sawrky1* and *Sawrky2* are homologs of GaWRKY1, a transcription factor which was found to regulate δ-cadinene synthase in *Gossypium arborium* (Xu et al. 2004). Given the significant role WRKY transcription factors played in cotton terpenoid regulation, changes in the expression of these sandalwood homologues could provide useful leads for future work.

Quantitative RT-PCR was done at the Biomolecular Resource Centre at the Australian National University using the BioMark Fluidigm system. Leaf, stem and root cDNA was probed on three 48×48 microfluidic chips, respectively. Three technical replicates of each primer pair were used. A pre-amplification step employing 12 cycles of amplification with a mixture of all primer pairs was performed for each cDNA sample and this pre-amplified template mixture was loaded into the chip. Thermocycling was carried out according to the manufacturers' instructions; denaturing at 96°C for 5 min, then 36 cycles of 96°C for 5 s, 60°C for 10 s. All gene expression was analysed relative to *actin* as reference gene. Melt curves for each amplicon were determined for quality control, while the products were sequenced for verification of primer specificity. Only *actin, histone3*, *wrky1*, *hmgr*, *fpps*, *SaSQBS*, *SaSTPS*, *SaCYP716B* and *SaCYP76F38v2* were successfully amplified in any tissues, and generated single amplicons of the expected size with acceptable melt curves. All primer pairs used in this study are listed in Table 1.

Total sesquiterpene concentrations in the stems of MeJ treated seedlings appeared to decrease relative to the control seedlings (Figure 2) whereas sesquiterpene concentrations in the roots were unchanged and an order of magnitude higher than in stems (Figure 3). A gradual increase in stem sesquiterpene concentrations over time for both MeJ treated and control seedlings is observed. Total root sesquiterpene concentrations were about 10-fold higher than stems, and consisted predominantly of santalenes and santalols (based on matching retention times). No other sesquiterpenes known to be present in sandalwood oil were detected in any of the extracts.

Of the 16 genes used to probe expression patterns, only 10 were successfully amplified and yielded reliable data for cDNA derived from leaf tissue, and 8 from stem cDNA (Table 1). Root cDNA was not successfully amplified with any probes due to degradation of the cDNA template in storage. In both leaves and stems, the two housekeeping genes (*actin* and *histone3*) remained unchanged throughout the time course and did not vary with MeJ treatment (data not shown), we therefore selected one of them (*actin*) as a reference. An average of the expression value of both housekeeping genes

Table 1. List of the genes and primers used for quantitative RT-PCR.

Gene name	Abbreviation	Forward primer (5′-3′)	Reverse primer (5′-3′)	amplification
Actin	N/A	GGAGATGATGCTCCACGGGC	GCCCCAGAAGAGCATCCTGTC	yes
Histone3	N/A	GACCGCTCGTAAGTCGACGG	CGGATCTGCGTTTCCAGAGCC	yes
WRKY transcription factor	*wrky1*	GGGTTCGATGTCTAGCCCGGAC	CAGGCCATGGGCCAGACG	yes
WRKY transcription factor	*wrky2*	CCACTGTCCATCGACACTCCGG	CGACCACCGGAACAGGCC	no
WRKY transcription factor	*wrky3*	TTCATGGTTCCCCCTGGGATGAG	CCAGCTTCTTCAGGATCATTGACCC	no
3-Hydroxy-3-methylglutary-CoA reductase	*hmgr*	GTCCGATAAAGCCGTCGGACG	CGGCTTCTTCGGCGTCGAT	yes
1-Deoxyxylulose-5-phosphate synthase 1	*dxs1*	GGGGCACTGGGTTAAATCTCTTC	CCTGGTCATAAGCTCTCTGCAAG	no
Isopentyl diphosphate isomerase	*ippi2*	GTATGAGCTACTGCTTCAGCAACG	CAAGTTCATGCTCTCCCCACTTCC	no
Farnesyl diphosphate synthase	*fpps*	CATACACGCCGAGGTCAGCC	GGTCGAGTTCCAAACAACTTCAGGA	yes
Santalene synthase	*SaSSy*	CCAATGAGGTTGGCCTTCGAGTC	TCCCTCTCTTGTTAATCACGGGC	partly
Bisabolene synthase	*SaBS*	TAGGCTATGACCTCCTGAGAGACC	GCAGACGGAAATCCTGGAGAATT	partly
Sesquiterpene synthase	*SaSTPS*	GGAAGAGATGGACAATCAAGGAAGCC	CACGACTATATCATACCTTGGAATGGGC	partly
Sesquisabinene synthase	SQBS	ACTTCTAACGATATCGGCTACTGGGC	CCAAACCTTCTTGTCACCAGCTACCTA	yes
Cytochrome P450 subfam 716B	*SaCYP716B*	CATGAGGCTGACGCCACCAG	GAAGAAGTAGGAGCAACCCCGTTT	yes
Cytochrome P450 subfam 72	*SaCYP72*	GAACGTAGGAGGGTCCGCG	CCATCAGAATTGGCAGTCGCGC	no
Cytochrome P450 subfam 76 (F38v2)	*SaCYP76F38v2*	CGAGCTGATTCCGTTCGGCG	GACGATGGAATGGGGTCGCC	yes

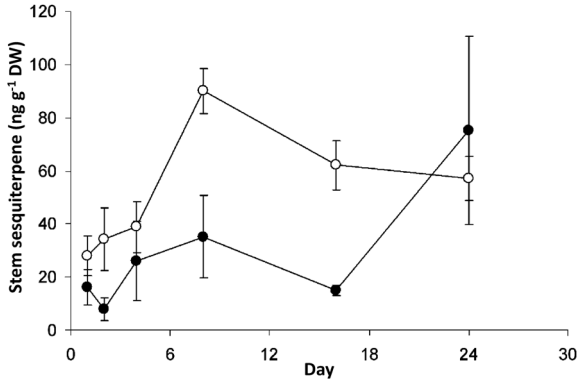

Figure 2. Total stem sesquiterpene concentrations for treated (●) and untreated (○) plants over a 24-d time course (± SE, N=4 for each time point).

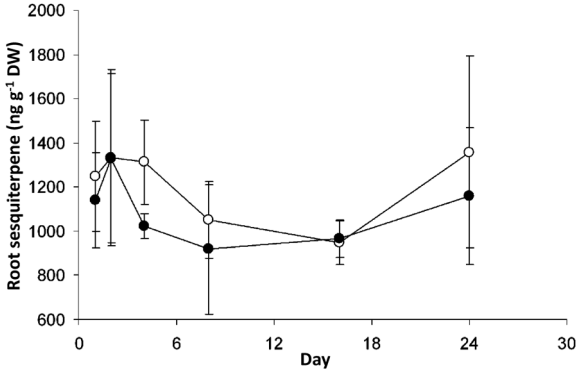

Figure 3. Total root sesquiterpene concentrations for treated (●) and untreated (○) plants over a 24-d time course (± SE, N=4 for each time point).

was used for normalisation of gene expression values. Several genes showed an initial increase in expression levels, both in treated and untreated samples. Expression of *wrky1* was higher in early time points (up to 8 d) of MeJ treated stem samples, but lower at late time points in untreated leaf samples (Figure 4A and B). At the earliest time point (4 h after treatment) *hmgr* expression in leaves of treated plants was roughly 6 fold higher than that of untreated leaves (Figure 4C). At all later time points, expression levels were similar between treated and untreated samples. A similar trend was observed in stems with treated samples showing 5 fold higher expression levels at the 4 h time point and 4 fold higher

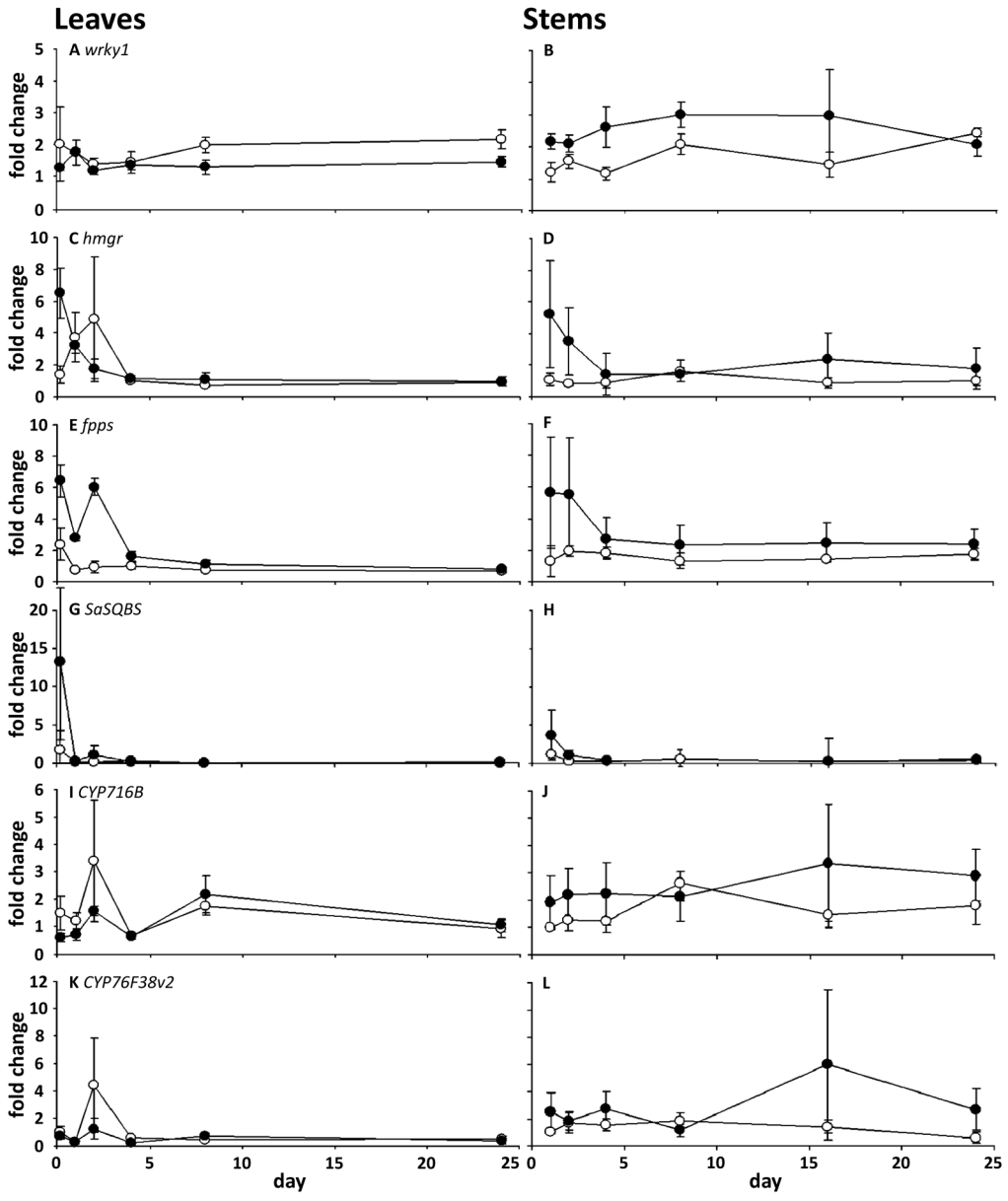

Figure 4. Transcript levels of six genes in leaves and stems, represented as fold change relative to *actin*, over a 24-d time course (± SE, N=4 for each time point) for MeJ treated (●) and untreated seedlings (○). 4A and B show relative expression levels of transcription factor *wrky1*, 4C and D show *hmgr*, 4E and F show *fpps*, 4G and H show SQBS, 4I and J show CYP716B and 4K and L show CYP76F38v2.

expression levels at the two day time point compared to control samples (Figure 4D). Expression of *fpps* peaked in leaves of MeJ treated seedling, and was almost 6-fold higher than that of leaves from untreated plants at just 4 h, but returned to control levels by day 4 (Figure 4E). This trend was also reflected in stems (Figure 4F). Some of the terpene synthases investigated appeared to have higher expression levels after MeJ treatment in both stems and leaves, but high levels of sample-to-sample variation meant the results were inconclusive (data not shown). Sesquisabinene B synthase (*sqbs*) transcripts showed early increases in treated samples, however there were high levels of variation between biological replicates (Figure 4G). Cytochrome P450 expression was largely unchanged with MeJ treatment, in both leaves and stems (Figure 4 I–L).

The results described in this paper indicate that transcripts of genes for the terpene biosynthetic pathway can be induced in stems of sandalwood seedlings. However, this response was not accompanied by a detectable increase in terpene concentrations of the stems or root. The stem samples of the sandalwood seedlings (ca. 9 months of growth) investigated contained between 5 and 90 ng g^{-1} DW total sesquiterpenes. Ten year old sandalwood trees contain up to 90 mg g^{-1} DW (Jones et al. 2006), a difference of 10^6-fold, indicating that sesquiterpene accumulation increases with age. Young sandalwood trees may not have the capacity to produce

or accumulate terpene in their xylem or parenchyma cells. Our results show the potential of induction of terpene production through the application of MeJ to the leaf surface, but MeJ had no effect on the up-regulation of sesquiterpene biosynthesis genes in the leaf of juvenile seedlings. While terpene induction/accumulation is a common response in plants to MeJ treatment, not all perennials show this response. Henery and co-workers (2008) found no effect of MeJ treatment in *Eucalyptus nitens* as indicated by a lack of change in foliar monoterpene and formylated phloroglucinol compound concentrations after MeJ treatment. Webb, Foley and Külheim (unpublished) found no changes in either foliar monoterpene concentration or gene expression of biosynthetic pathway genes, involved in biosynthesis of terpenes, upon MeJ treatment in *Melaleuca alternifolia*.

Several studies have found correlations between gene expression levels for genes upstream of terpene biosynthesis and terpene concentrations in leaves (Phillips et al. 2007; Webb et al. 2013). Most of these studies have concentrated on the synthesis of monoterpenes and gene expression in methylerythritol phosphate (MEP) pathway genes, which is located in the chloroplast. The product of both the MEP pathway and the cytosolic mevalonate (MVA) pathway is isopentyl diphosphate IPP. In several plant species IPP is exported from the plastids to the cytosol where it can be used in sesquiterpene biosynthesis (Dudareva et al. 2005; Laule et al. 2003). Two early pathway genes involved in the biosynthesis of sesquiterpenes, *hmgr* and *fpps* were investigated. Both showed transient induction upon MeJ treatment suggestive of short-term induction of sesquiterpene biosynthesis. The results indicate that there may have been a signal transduction within the plant for this response. This is indicated by the stem gene expression results for some genes, but this needs further clarification using clonal plant material to reduce the variation within the experimental system.

Despite the negative results of this present study with seedlings, these results do not exclude the possibility that sandalwood oil yields can be enhanced by MeJ treatment of trees that are older than those trees used in our work. If sandalwood trees were found to respond to MeJ when they reached a commercial wood volume, the harvest time could be potentially reduced by multiple years (currently 15 years or more). Many plants respond to multiple applications of MeJ with enhanced production of defence compounds (Ku et al. 2014), therefore several rounds of application of MeJ could further enhance sandalwood oil yield. A field experiment where groups of plants of different age (e.g. 2, 6 and 10 years) are divided into control/treatment groups, where the treatment groups are sprayed with MeJ solution, could provide better information on the effects of MeJ on older plants under field conditions. Treatment groups could further be divided into single or multiple treatment groups. Such an experiment would however take many years to completion. An alternative to foliar application of MeJ would be intra vascular application of MeJ. Such an application would have the advantage of not having to rely on signal transduction within the plant, but also contains the risk of secondary fungal infections. From a practical perspective, this method would be much more labor intensive than foliar spraying.

High levels of variability were seen between biological replicates, despite technical replicates of these individuals showing quite consistent results. This indicates that there are other factors that need to be considered when exploring the relationship between MeJ and sesquiterpene synthesis. Between sample variation was particularly common with the terpene synthases *SaSSy* and *SaBS*. It cannot be ruled out that some individual seedlings may be more responsive to MeJ treatment than others, and the expression of certain TPS genes over others may be consistent with the variation in oil profiles seen in samples of diseased wood (Barbour et al. 2010). This study shows that seedlings of sandalwood react to treatment with MeJ by inducing genes involved in the biosynthesis of sesquiterpenes. While this is not reflected by an increase in stored terpenes in wood or root, it could nevertheless enhance sandalwood oil yield and/or reduce rotation times.

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