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Accumulation patterns of phenylpropanoids and enzymes in East Indian sandalwood tree undergoing developmental progression *in vitro*

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Abstract

The East Indian Sandalwood tree, *Santalum album* L., is sought for its fragrant essential oil and heartwood. The prolonged harvestable economic phase, over-exploitation, and poaching have contributed to extensive micro propagation endeavors in this woody tropical species. However, till date there is no information available regarding the metabolic changes associated with its development *in vitro*. Established *in vitro* cultures (i.e. callus, somatic embryo, and somatic seedlings) were examined extensively for phenylpropanoid pathway enzymes and metabolite accumulation patterns. Two to twelve fold increases in critical enzymatic activities across the three stages suggested a progressive developmental organization. Phenylpropanoid analysis by reverse phase-high performance liquid chromatography (RP-HPLC) and liquid chromatography-electro spray ionization-mass spectrometry (LC-ESI-MS/MS) revealed changes and distribution patterns of 18 phenolics and 46 phenylpropanoids, respectively. Moreover, anatomical studies yielded insights into the vasculature's progressive organization and enhanced complexity. This study constitutes the first ever report on a comprehensive phenylpropanoid profiling analysis in East Indian sandalwood tree.

Keywords: anatomical; flavonoids; LC-ESI-MS; micropropagation; phenolics; RP-HPLC; *Santalum album* L.; somatic embryo.
Abbreviations: CAD_cinnamyl alcohol dehydrogenase; GAE_gallic acid equivalence; POX_peroxidase; PAL_ phenylalanine ammonia lyase; PPO_ polyphenol oxidase.

Introduction

The East Indian Sandalwood tree, *Santalum album* L., a woody tropical species belonging to the family *Santalaceae*, yields the sandalwood essential oil deposited in the fragrant heartwood. Bioactive principles like anti-cancer (Arasada et al., 2008), anti-tumor (Kim et al., 2006), anti-viral (Koch et al., 2008), anti-*Helicobacter pylori* (Ochi et al., 2005), and cytotoxic santalols as well as lignans (Matsuo and Mimaki, 2010) are obtained from the heartwood. Estimated global annual requirement of wood is about 10,000 tons, involving a trade of about $ 125 million, of which only 10% is met from natural resources. The expanded economic trade of this natural resource, poaching, and over-exploitation related to the lengthy harvestable economic phase have resulted in a decline among its natural populations. To counteract the severe loss and hence, to meet the global demands for this tree species, extensive efforts in large-scale micropropagation have been implemented. The worldwide natural population of this forest tree has been in decline due to (1) the mycoplasmal seed-born spike disease and (2) the illegal poaching by organized gangs. Hence, micropropagation of this plant was reported as early as 1963 (Rangaswamy and Rao, 1963), while somatic embryogenesis was documented to be a successful means for producing disease-free somatic seedlings as risk free planting stock (Pal et al., 2003). Perhaps, somatic embryogenesis represents *in vitro* differentiation events and provides an important model system to study the early development in higher plants. Till now no studies have been performed to delineate the development of this tree associated with phenylpropanoid pathway enzymes and metabolites, which govern lignification and hence wood formation that contribute to sandalwood oil deposition. Henceforth, in this study we intend to explore the phenylpropanoid metabolism in the sandalwood tree using established *in vitro* stages. This article reports for the first time the major phenylpropanoid compounds across various growth and developmental stages with regards to the enzymes and metabolites involved.

Results

Enzymes of the phenylpropanoid pathway

Based on the evaluation of preliminary experiments most of the phenylalanine ammonia lyase (PAL) activity was noted to be present in the supernatant fraction from 12,000 g centrifugation of filtered homogenates. The PAL accumulation levels are an indirect measure of the PAL enzymatic activity. In seedling the PAL activity was lower (0.25 pkat mg^{-1} protein). On the other hand, a 4-fold higher PAL activity was observed in callus (0.96 pkat mg^{-1} protein) and somatic embryo (1.13 pkat mg^{-1} protein) (Fig 1A). However, unlike the PAL activity, cinnamyl alcohol

dehydrogenase (CAD) activity showed a continual increase from callus (9.63 pkat mg^{-1} protein) as compared to seedling (Fig 1B). Anionic lignin peroxidase (POX) activity was estimated with the help of pyrogallol peroxidase (PPOX) and guaiacol peroxidase (GPOX) as substrate donors by two different colorimetric methods. However, both PPOX and GPOX activities, ranging from 0.83-10.17 nkat mg^{-1} protein, showed a consistent increase from callus to seedling (Fig 1C). Whereas, polyphenol oxidase (PPO) activity, ranging from 0.75-1.27 µkat mg^{-1} protein, showed an increase from callus to somatic embryo, followed by a significant ($p< 0.05$) decrease in the seedling (Fig 1D).

Distribution of phenylpropanoids

The total phenolic content, in the three developmental stages increased continually from callus [2.5 mg GAE (gallic acid equivalence) g^{-1}] to seedling (15 mg GAE g^{-1}) (Fig 2A). Besides, the first level of histo-differentiation caused a two-fold increase in the phenolic content from callus to somatic embryo stages. Moreover, the total anthocyanin content, expressed as color value (CV) g^{-1}, revealed a continual increase from the lowest in callus to the highest in seedling (Fig 2B). Thus, a 2.5-fold increase in anthocyanin content was observed at the first instance of differentiation from callus to somatic embryo. Total flavonoids content demonstrated a consistent increase from somatic embryo to seedling (Fig 2C). No flavonoids were detectable from callus. Compared to the two vascularized developmental stages, the total thioglycollate lignin content was lowest for callus (7.7) (Fig 2D, E). Levels of shikimic acid, a central primary metabolite was comparable across all stages (Fig 2E). The photosynthetic seedlings accumulated higher amounts of total UV-A absorbing compounds in comparisons to the non-photosynthetic stages of callus and somatic embryo (Fig 2F), whereas no such differences were observed for UV-B absorbing compounds amongst them (Fig 2G).

RP-HPLC and LC-ESI-MS/ MS based profiling of phenylpropanoids

The phenolics profiles derived from the three sandalwood developmental stages, i.e., *in vitro* grown callus, somatic embryo, and seedling are represented in the RP-HPLC chromatograms (Fig 3). All compounds were identified from their diode-array detector (DAD)-spectra and co-elution experiments with appropriate standards and matching their retention times. Phenolics such as, 4-hydroxybenzylalcohol, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, p-coumaric, ferulic and chlorogenic acids were largely present in all developmental stages. Hydroxycinnamates, such as protocatechuic, p-coumaric, ferulic, sinapic and caffeic acids displayed a consistent increase alongside development. At least one common hydroxycinnamic acid, i.e., p-coumaric, caffeic, ferulic or sinapic acid is present in all three stages of development. Protocatechuic acid, coniferaldehyde, sinapic acid, vanillic acid, benzyl alcohol were detected only in callus (absent in somatic embryo). Coniferaldehyde was only present in callus, while 3, 4-dimethoxybenzaldehyde and t-cinnamaldehyde were detected only in somatic embryos. Similarly, gentisic acid was found exclusively in seedlings. Exclusive presence of vanillic acid in callus was noteworthy. Contrary to syringaldehyde and syringic acid, vanillic acid and benzyl alcohol were absent in the photosynthetic developmental stage, i.e. seedlings. However, the types of phenolics identified in each developmental stage were as follows: maximum in callus (13 no's) and minimum in seedling (11 no's). Phenylpropanoids were identified based on comparisons with spectra from known standards, available library and the existing literature on LC-ESI-MS/ MS (Tian et al., 2005). Detailed explanation for the interpretation of LC-MS/ MS spectra is beyond the scope of this manuscript. Analysis by LC-MS/ MS revealed the presence of 46 phenylpropanoids belonging to phenolic acids, benzoates, monolignols, anthocyanins, catechins, flavonoids and phenolic-conjugates (Table 2). All phenylpropanoic acids detected with ESI^+ gave characteristic fragment ions $(A\text{-}OH)^+$ that correspond to the detachment of the hydroxyl group from their carboxyl group. This was confirmed by the fragmentation of the authentic standards, available library and documented literature (Fang et al., 2002). In ESI-MS/MS analysis, anthocyanins lost the sugar moiety and the pseudomolecular ion $(M)^+$ occurred together with an aglycone ion $(M\text{-sugar moiety})^+$. MS/ MS experiments performed on the $(M\text{-sugar})^+$ ions of all anthocyanins, produced daughter ions at m/z 149 providing confirmation of their anthocyanic nature. The ethyl acetate and n-butanol fractions contained the most phenolic acids and their derivatives. Diethyl ether fractions contained several esters of cinnamoylquinic acids such as caffeoyl and quinic acid derivatives. Somatic embryos represented the majority of phenylpropanoids identified, i.e., 5 phenolic acids, 2 benzoates, 4 monolignols, 9 flavonoid (glycosides) and 7 phenolic conjugates, whereas no anthocyanins were detected. Similarly in callus, 2 each phenolics, monolignols and catechins could be identified, while no flavonoids were detected. Highest numbers of anthocyanins were present in seedlings, while none could be identified in somatic embryos. Apart from protocatechuic acid, none of the phenolics were represented across all stages of development.

Histochemical evidences of differentiation and maturation events

Autofluorescence studies based on fluorescent microscopy analysis discriminated the metabolic complement of the tissues, i.e., green (lignin), yellow to green (phenolics and flavonoid) and red (chlorophyll) autofluorescence. This provided a robust tool to visualize the progression of lignification and related vascularization events. The fresh callus could not be visualized in dark field florescent microscopy during the log phase of growth, probably, due to absence of any autofluorescence. The process of histodifferentiation begins *in vitro* with pro-embryogenic mass (PEM) formation. The wall-bound phenylpropanoids accumulated around the PEMs portrayed the phenomena of first level of cellular differentiation (Fig 4 A). The somatic embryos in cross section showed the development of two concentric phenolic-rich zones, one representing the central stele that eventually forms the vasculature and the one outer to epidermis, i.e. cuticular layer (Fig 4 B). The yellow tinges were attributed to accumulation of phenylpropanoids (monolignols, phenolics, and flavonoids). In a cross section across the stem of the seedling revealed scattered chlorophylls (red autofluorescence) throughout the ground parenchymatous tissue, implicating the attainment of autotrophy by virtue of photosynthesis. The nascent developing procambial precursor elements could be observed in the stellar regions in the seedling (Fig 4 C). For comparison, a matured stem of field grown tree was examined. The stem vasculature was characterized by fully developed xylem and phloem elements, phenolic deposition rich scattered clusters of collenchymatous strands in the ground tissue and phenylpropanoid rich cuticular layers.

Table 1. Chromatographic and spectral characteristics of phenolics detected in three different sandalwood developmental stages by RP-HPLC analysis.

Sl. No.	Phenolic Name	Code	Molecular Formula	λ_{max}	Ret. Time (mins.)	Content (mg 100 g^{-1} DW) [a]		
						Callus	Somatic Embryo	Seedling
1	Protocatechuic acid	PCA	$C_7H_6O_4$	260, 295	6.3	0.26	ND	0.48
2	4- hydroxybenzylalcohol	4-HBAlc	$C_7H_8O_2$	274	6.67	1.15	2.74	3.21
3	Gentisic acid	GA	$C_7H_6O_4$	333	8.5	ND	ND	2.41
4	Caffeic acid	CfA	$C_9H_8O_4$	325	8.25	3.52	4.76	ND
5	4- hydroxybenzoic acid	4- HBA	$C_7H_6O_3$	255	9.31	0.96	0.85	1.23
6	4- hydroxy benzaldehyde	4-HBAld	$C_7H_6O_2$	329	10.5	1.23	2.33	1.64
7	Vanillic acid	VA	$C_8H_8O_4$	261, 293	11.28	0.81	ND	ND
8	Vanillin	V	$C_8H_8O_3$	269	12.54	*ND*	*ND*	*ND*
9	Benzyl alcohol	BA		255	14.5	0.89	ND	ND
10	Ferulic acid	FA	$C_{10}H_{10}O_4$	310	19.8	0.66	1.54	0.77
11	p- coumaric acid	pCA	$C_9H_8O_3$	309	23.45	0.79	0.94	1.31
12	Coniferaldehyde	CA	$C_{10}H_{10}O_3$	340	10.6	0.55	ND	ND
13	Sinapic acid	SiA	$C_{11}H_{12}O_5$	322	23.7	0.23	ND	ND
14	Chlorogenic acid	ClA	$C_{16}H_{18}O_9$	235, 325	7	1.65	2.31	3.3
15	p- hydroxy benzoic acid	pHBA	$C_7H_6O_3$	255	10.4	0.18	0.34	0.21
16	t- cinnamaldehyde	T- CAld	C_9H_8O	225, 280	27.25	ND	0.22	ND
17	Syringic acid	SA	$C_9H_{10}O_5$	275	17	ND	1.19	0.95
18	Syringaldehyde	SAld	$C_9H_{10}O_4$	306	21	ND	0.23	0.15
19	Veratryl alcohol	VAlc	$C_9H_{12}O_3$	278	11.6	ND	ND	ND
20	3,4- dimethoxy benzaldehyde	3,4-DMBAld	$C_9H_{10}O_3$	258, 290	27.9	ND	0.65	ND
	Types of Phenolics (Numbers)					13	12	11
	Total Phenolics[c] Identified by RP-HPLC (mg 100 g^{-1} DW)					12.9	18.1	15.7
	Total Phenolics[d] Quantified by Folin- Ciocalteau (mg 100 g^{-1} DW)					260	550	790
	Total Phenolics Identified (expressed as % of Total Phenolics Content)					4.9	3.2	2

[a]: Phenolics content in three developmental stages of sandalwood; [b] Each value is the mean (mg GAE 100 g^{-1} DW) of three independent runs; [c] Sum of the individual phenolic compounds; [d] Determined following the Folin-Ciocalteau method (mg GAE100 g^{-1} DW), ND: Not Detected.

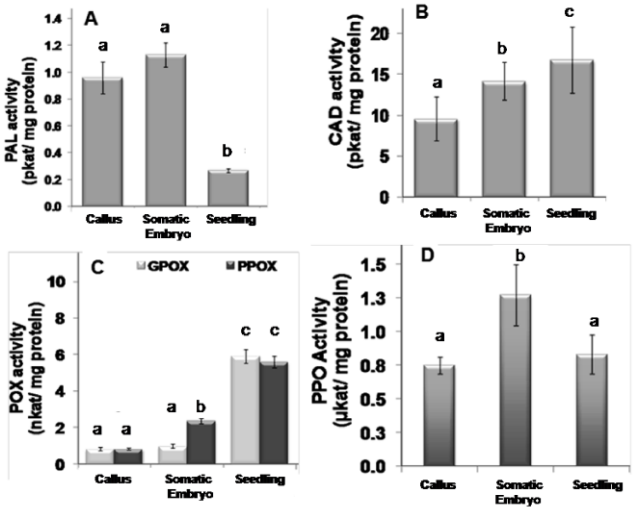

Fig 1. Phenylpropanoid enzyme activities in sandalwood developmental stages. A. PAL activities expressed as pkat mg^{-1} protein. B. CAD activities expressed as pkat mg^{-1} protein. C. POX activities (GPOX and PPOX) expressed as nkat mg^{-1} protein. D. PPO activities expressed as µkat mg^{-1} protein. The data, representative of six interday assays (n= 6) are provided as mean ± SD (error bars), and the values followed by the same letters are not statistically different ($p< 0.05$).

Besides, lignin was localized to the xylem of vascular bundles and collenchyma but never in the phloem tissues or parenchyma. Moreover, three to five vascular bundles of endarch and open nature were observed forming an arc by joining each other (Fig 4 D). Thus, the *in vitro* seedlings and field grown stem were observed to be anatomically similar, except secondary growth and vast xylem elements

Discussion

Lignification and xylogenesis (also known as wood formation) are well characterized phenomena in land plants (Raes et al., 2003). However, limited information is currently available regarding the enzymes, metabolites and vasculature that participate in developmental processes. The first enzyme in the phenylpropanoid pathway PAL is suggested to play distinct and overlapping roles in growth and development, which is evident from the involvement of different isoforms of PAL implicated in biosynthesis of phenolics, anthocyanins, and flavonoids, as well as UV-B protection in a differential manner (Huang et al., 2010). In the *Zinnia* mesophyll model, the PAL and POX have been recommended as marker proteins for tracheary element differentiation (Fukuda and Komamine, 1982), which represents the process of xylogenesis *in vitro*. The higher PAL activities in the callus and somatic embryo of *S. album* suggest that PAL plays a different role in these stages compared to the photosynthetic seedlings (Fig 1A). Recently, PAL, CAD, and POX have been proposed as major enzymes which should be investigated in fruit lignification models. CAD and POX gene expression levels were temporally associated with lignification in the ripening fruits of two loquat (*Eriobotrya japonica*) cultivars (Shan et al., 2008), in agreement with our results (Fig 1 B, C). Increased CAD activity in *S. album* stages (Fig 1C) was concomitant with growth and development. Studies conducted in maize revealed that POX activity and the phenolic concentration increased in parallel with root and leaf age, reflecting increased lignin production during maturation (Maksimović et al., 2008). The peaking of PPO activity only in somatic embryos was notable during sandalwood development (Fig 1D). Similarly, the specific PPO activity in apricots (*Prunus armeniaca*) during fruit development started from 1.75 µkat mg^{-1} at the immature-green stage to reach a peak (4.5 µkat mg^{-1}), while the half-ripe stages showed value of 0.95 µkat mg^{-1} (Chevalier et al., 1999). In fact, during wheat seed germination it quickly passes from a state of metabolic arrest to the highest growth rate in its life history (Williams, 1972), which reflects the developmental transition from predominantly thus reflecting major adjustments in the cellular biochemical processes. Whereas it is well established that lignin synthetic pathway genes regulate multiple aspects of plant growth and development (Shadle et al., 2007), transcriptome profiling of secondary xylem development indicates that lignin biosynthetic pathway genes are up regulated during secondary cell wall formation (Hertzberg et al., 2001). Thus, we propose that monitoring these enzymatic activities during developmental stages across various forms can provide information on the developmental status in sandalwood. The total phenolics content in sandalwood (2.5-15 mg GAE g^{-1} DW) is higher than that found in dietary cereals (0.2-1.3 mg g^{-1}), which is comparable to vegetables (0.4-6.6 mg g^{-1}) and is lower than phenolic rich berries (12.4-50.8 mg g^{-1}) (Kähkönen et al., 2001). Moreover, the RP-HPLC studies (Table 1) revealed that the accumulated phenolics acids, i.e., protocatechuic, ferulic, p-coumaric, chlorogenic, syringic, p-hydroxybenzoic, and caffeic acids fall within of 0.13-5.56 mg g^{-1}, a range well augmented in many important plants (Mattila and Hellstrom, 2007; Zhao and Moghadasian, 2008). Accumulated amounts of thioglycollate lignin remained similar in all developmental stages, except callus (Fig 2. D, E). Shikimic acid is a central metabolite in the biosynthesis of the amino acid phenylalanine, and thus provides the precursor for first step of lignin biosynthesis. However, quantity of shikimic acid did not differ significantly across the three developmental stages thus reflecting a steady state of primary metabolism. In addition to this, it was observed that the anthocyanin contents followed similar trends with that of flavonoids, but differed in quantities from total UV-absorbing pigments across development. Levels of anthocyanins varied independently of the concentrations of flavonols, flavones, hydroxycinnamic acids and total UV-absorbing pigments, where anthocyanin accumulation served to compensate for deficiencies in levels of other protective pigments (Gould et al., 1995). Developmental studies conducted in blueberries (*Vaccinium corymbosum* L.) through maturation and ripening using HPLC-DAD revealed an increase in anthocyanins while decreased flavonols and hydroxycinnamic acids (Castrejón et al., 2008). Absence of detectable levels of flavonoids in callus is striking. In fact, in flavonoid rich *Genista* flavonoids such as apigenin, luteolin were not detectable in callus cultures (Łuczkiewicz and Głód, 2003). The hydroxycinnamic acids are associated with plant cell walls i.e., ferulic and coumaric acid form ester linkages with pectins in dicots (Faulds and Williamson, 1999). In angiosperms, the lignin is composed of guaiacyl (G-units), p-hydroxyphenyl (H-units), and syringyl units (S-units) which are formed from the monolignols. Our results are indicative of lignin composition across three developmental stages in terms of the monolignols, i.e., caffeic, ferulic, p-coumaric and sinapic acids and coniferaldehyde. Similar studies conducted in *Araucaria angustifolia* across various stages of development suggest that the adult tissues accumulated increased amounts of benzaldehydes, vanillin, p-hydroxybenzaldehyde and coniferaldehyde as compared to callus and seedling stems (Fonseca et al., 2000). Development and growth is accompanied with a phenolic complexity and enrichment, not only in quantity but also in terms of diversity. Of the total 20 phenolics measured across the three developmental stages indicate that, only 4.9 % of the total phenolics detected in callus could be identified and quantified, though their diversity was limited (Table 1). On the contrary, the somatic embryos reflect a metabolically enriched stage in the developmental transition, which is reflected by the presence of 28 phenylpropanoids as compared to 15-20 in the preceding and following stages (Table 2). In fact it was shown that, besides lignin biosynthesis, ester-linked phenolic acids (ferulic, protocatechuic, vanillic, p-hydroxy benzoic acids and coniferyl alcohol) are involved in alterations of the cell wall composition during differentiation and morphogenesis (Lozovaya et al., 1996). Nevertheless, the authors are aware of the fact that sampling, handling, and biases associated with data acquisition using instrumental analysis could not be ruled out while doing such comparative studies. Differentiation of plant cells is accompanied by characteristic changes in the cell walls that are easily discernible by optical microscopy. Localized accumulation of wall-bound phenolics in cross sections using UV-fluorescence microscopy has been documented (Chapple et al., 1992). Xylem elements emit green florescence because of the existence of ferulic acid (Hartley and Harris, 1976). In a wide variety of dicots, quercetin and kaempferol derivatives are known to be mainly

Table 2. LC-ESI-MS/MS-based profiling of sandalwood phenylpropanoids. [a] Results presented were steady over the course of six inter-day assays.

Serial No.	Major ions (*m/z*)[a]	*Phenylpropanoid Type*	Mode	Fragment Ions		Callus	Somatic Embryo	Seedling
Phenolic Acids						*2*	*5*	*2*
1	136	Gentisic acid	Negative	$[M- H]^-$		√	√	√
2	154	Gallic acid	Negative	$[M- OH]^-$		-	-	-
3	155	Protocatechuic acid	Positive	$[M+H]^+$		√	√	√
4	167	Vanillic acid	Negative	$[M- H]^-$		-	√	-
5	173	*Quinic acid*	Negative	$[M- H_2O]^-$		-	√	-
6	181	Syringic acid	Negative	$[M- OH]^-$		-	√	-
7	210	*Quinic acid*	Positive	$[M+NH_4]^+$		-	√	-
Benzoates						-	*2*	-
8	141	4- hydroxybenzoic acid	Negative	$[M- H]^-$		-	√	-
9	401	Benzyl alcohol pentose. hexose	Negative	$[M- H]^-$		-	√	-
Flavonoid (glycosides)						-	*9*	*2*
10	196	Rhamnetin	Negative	$[M- H]^-$		-	√	-
11	271	Naringenin	Negative	$[M- H]^-$		-	√	-
12	285	Luteolin	Negative	$[M- H]^-$		-	√	-
13	296	Quercetin	Negative	$[M- H]^-$		-	√	-
14	319	Myricetin	Positive	$[M+H]^+$		-		√
15	435	Phloridzin/ phloretin- 2- glucoside	Negative	$[M- H]^-$		-	√	-
16	445	Apigenin- glucoside	Negative	$[M- H]^-$		-	√	-
17	447	Kaempferol- 3- glycoside	Negative	$[M- H]^-$		-	√	-
18	449	Luteolin- glucoside	Negative	$[M- H]^-$		-	√	-
19	471	Kaempferol- 3- hexoside	Positive	$[M+Na]^+$		-	√	-
20	475	Apigenin- aglycone	Negative	$[M- H]^-$		-	√	-
21	477	Quercetin- 3- β- d- glucuropyranoside	Negative	$[M- H]^-$		-	√	-
22	625	Myricetin rhamno- hexoside	Negative	$[M- H]^-$		-	√	-
23	649	Quercetin- 3- hexosyl- caffeoyl ester	Positive	$[M+Na]^+$		-	-	√
Monolignols						*2*	*4*	*4*
24	147	p- coumaric acid	Positive	$[M+OH]^+$		-	√	-
25	147	t- cinnamic acid	Negative	$[M- H]^-$		√	√	√
26	173	Caffeic acid	Negative	$[M- H_2O]^-$		-	√	√
27	179	Caffeic acid	Negative	$[M- OH]^-$		-	√	√
28	195	Ferulic acid	Positive	$[M+H]^+$		√	√	√
29	341	Caffeoyl glucose	Negative	$[M- H]^-$		-	-	√
Anthocyanins (anthocyanidin glycosides)						*4*	-	*9*
30	301	Cyanidin	Positive	$[M+H]^+$		-	-	√
31	419	Cyanidin- 3- arabinoside	Positive	$[M+H]^+$		-	-	√
32	433	Pelargonidin- 3- glucoside	Positive	$[M+H]^+$		√	-	-
33	463	Peonidin- 3- glucoside	Positive	$[M+H]^+$		-	-	√
34	465	Delphinidin- 3- galactoside	Positive	$[M+H]^+$		√	-	-
35	493	Malvidin- 3- glucoside	Positive	$[M+H]^+$		-	-	√
36	625	Peonidin 3, 5- diglucoside	Positive	$[M+H]^+$		√	-	√
37	627	Delphinidin 3, 5- diglucoside	Positive	$[M+H]^+$		√	-	-
38	771	Petunidin- 3- p- coumaroylrutinoside	Positive	$[M+H]^+$		-	-	√
39	809	Malvidin- 3- glucoside, catechin linked	Positive	$[M+H]^+$		-	-	√
40	919	Delphinidin- 3- p- coumaroylrutinoside- glucoside	Positive	$[M+H]^+$		-	-	√
41	949	Petunidin- 3- caffeoylrutinoside- 5- glucoside	Positive	$[M+H]^+$		-	-	√
Phenolic conjugates						*5*	*7*	*1*
42	367	Feruloyl quinic acid	Negative	$[M- H]^-$		-	-	-
43	487	Coumaric acid derivative	Negative	$[M- H]^-$		√	√	-
44	497	Dicaffeoylferuloylquinic acid	Negative	$[M- H]^-$		√	√	-
45	497	Trihydroxycinnamoyl, gallic alcohol	Positive	$[M+H]^+$		-	√	-
46	539	Caffeoylquinic acid- hexoside	Positive	$[M+Na]^+$		√	√	-
47	677	Tricaffeoylquinic acid	Negative	$[M- H]^-$		-	√	-
48	707	Caffeoylquinic acid dimer	Negative	$[M- H]^-$		-	√	-
49	195	Hydroxytyrosol acetate *(O- diphenol)*	Negative	$[M- H]^-$		√	√	-
50	438	Dicoumaroyl spermidine *(Polyamine conjugate)*	Positive	$[M+H]^+$		√	-	√
Catechins						*2*	*1*	*2*
51	289	Catechin/ epicatechin	Negative	$[M- H]^-$		-	-	-
52	441	Epicatechin gallate	Negative	$[M- H]^-$		√	√	√
53	457	Epigallocatechin gallate	Negative	$[M- H]^-$		√	-	-
54	471	3- methyl epigallocatechin gallate	Negative	$[M- H]^-$		-	-	√
		Total Identified Phenylpropanoids				*15*	*28*	*20*

(√): presence and (-): absence.

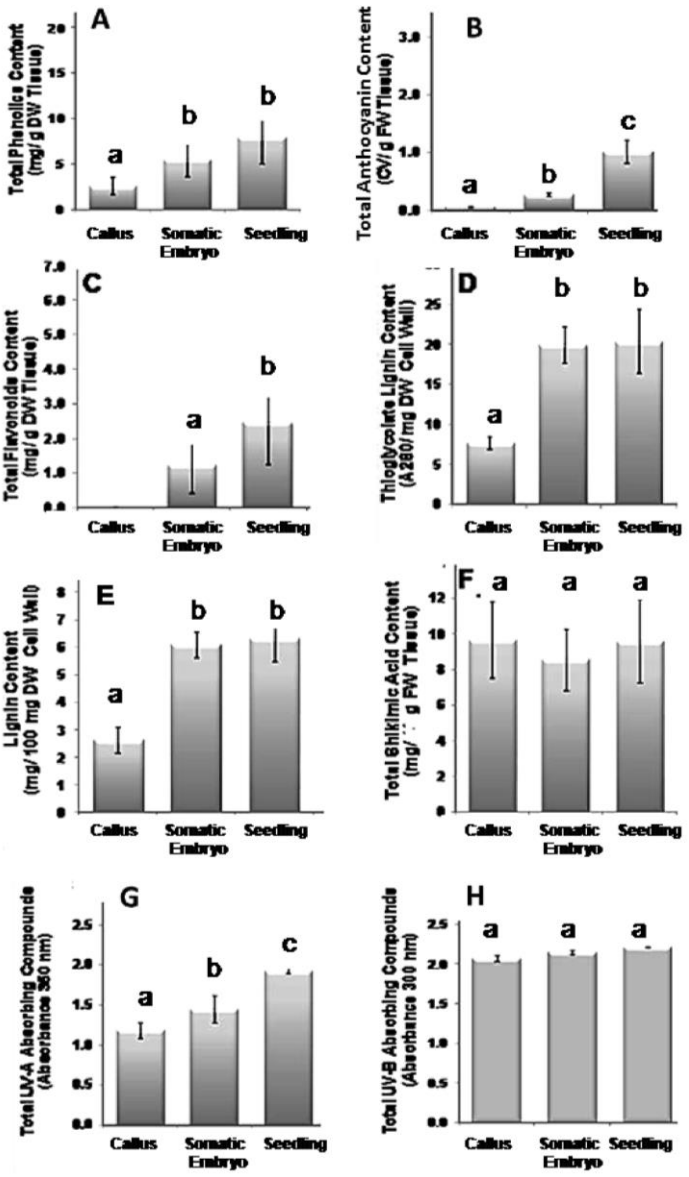

Fig 2. Quantification of phenylpropanoid metabolites in sandalwood developmental stages. A. Total phenolics content of expressed as mg g^{-1} DW. B. Total anthocyanin content expressed as CV g^{-1} FW. C. Total flavonoids content expressed as mg g^{-1} DW. D. Thioglycolate lignin content expressed as A_{280} mg^{-1} DW cell wall. E. Total lignin content expressed as mg 100 mg^{-1} DW of cell wall. F. Total shikimic acid content expressed as mg 10 g^{-1} FW. G. Total UV-A absorbing compounds expressed as a function of absorbance at 350 nm. H. Total UV-B absorbing compounds expressed as a function of absorbance at 300 nm. The data, representative of six interday assays (n= 6) are provided as mean ± SD (error bars), and the values followed by the same letters are not statistically different ($p< 0.05$).

glycosylated and located in epidermal vacuoles and they absorb UV light (Cerovic et al., 2002). In fact, autofluorescence of the cell walls has been previously used to characterize the content and nature of lignin in gymnosperms and dicotyledons (Yoshizawa et al., 1993). Similar to the somatic embryos of dicotyledonous, the phenylpropanoid accumulation in the cell walls continue through the globular and oblong-heart shaped stages (Schiavone and Cooke, 1985). In *Arabidopsis*, prominent absence of matured and differentiating vascular elements and well discernible procambium precursor cell network are observed during the transition from the globular to the heart stage of embryogenesis (Busse and Evert, 1999), as vascular differentiation culminates in the formation of phloem and xylem. Recently, the anionic and cationic peroxidases were proposed as biochemical markers to define the rooting status (Hatzilazarou et al., 2006) in gardenia (*Gardenia jasminoides*), an ornamental pot plant. Additionally, tea leaf polyphenols were proposed as phenetic markers and as tools for chemosystematics (Li et al., 2010). In fact, phenolic compounds are implicated to be involved indirectly in developmental patterning (Vassiliki et al., 2007). Thus, in this study, we propose that characterization of the patterns of progression of enzymes (i.e., PAL, CAD, POX) and stage-specific accumulation of characteristic phenylpropanoids [i.e., phenolic acids, flavonoids] can be used as potential biomarkers of development, possibly in cell and tissue-specific manner to assess the metabolic status and processes underway in the developmental cycle of *S. album*.

Materials and methods

Plant materials

The three *in vitro* cultured developmental stages used in this study were; callus, somatic embryos and seedlings. Callus: A highly proliferating cell line (IITKGP/ 91, from a elite Kerala, India clone), yielding a friable mass of single cells were grown either on solid MS (Murashige and Skoog, 1962) or WPM media (Lloyd and McCown, 1981) supplemented with 2,4-D (1 mg L^{-1}), 4 % sucrose, 0.35 % Phytagel (Sigma Aldrich, USA). Cultures were maintained in dark at 25±2°C with 50-60% relative humidity. Callus cultures were harvested 14 weeks post subculture for analyses or to further sub cultures. Somatic embryo: Somatic embryos were grown on solid MS media, supplemented with IAA (0.5 mg L^{-1}), BAP (0.5 mg L^{-1}), 3 % sucrose, 0.35 % Phytagel (Sigma Aldrich, USA) and were harvested 2 weeks after induction into embryogenic media from callus line mentioned above. Cultures were maintained in dark at 25±2°C with 50-60% relative humidity.

Seedlings: Seedlings were cultured on MS media supplemented with IAA (1 mg L^{-1}), IBA (1 mg L^{-1}), GA (0.5 mg L^{-1}), 4 % sucrose, 0.35 % Phytagel (Sigma Aldrich, USA) and were harvested after 6 weeks from induction of organogenesis. Seedlings were maintained under white fluorescent light of 1000-1500 lux (16 h light/ 8 h dark cycle) at 25±2°C with 50-60 % relative humidity.

Preparation of enzymes

Fresh tissues (~10 g) were homogenized in 10 mL of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.1 mM EDTA, 20 mM β-mercaptoethanol and 5 % (w/v) polyvinylpolypyrrolidone (PVPP). After filtration through four layers of cheesecloth, the homogenate was centrifuged at 12, 000 g for 20 min and the supernatant was used as source of crude enzyme. All steps to obtain enzyme preparation were carried out at 4 ºC. Total soluble protein content was determined following Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) assay

The PAL assays were conducted following the methods described (Cahill and McComb, 1992). The enzyme activity

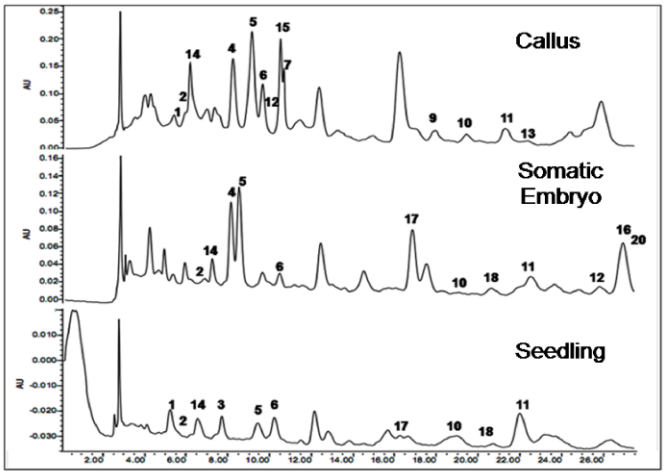

Fig 3. RP-HPLC chromatograms of the combined phenolic fractions of three developmental stages. Numbers denote identified phenolics, i.e., 1: protocatechuic acid, 2: 4-hydroxybenzylalcohol, 3: gentisic acid, 4: caffeic acid, 5: 4-hydroxybenzoic acid, 6: 4-hydroxy benzaldehyde, 7: vanillic acid, 8: vanillin (*not detected*), 9: benzyl alcohol, 10: ferulic acid, 11: p-coumaric acid, 12: coniferaldehyde, 13: sinapic acid, 14: chlorogenic acid, 15: p-hydroxy benzoic acid, 16: t-cinnamaldehyde, 17: syringic acid, 18: syringaldehyde, 19: veratryl alcohol (*not detected*), 20: 3, 4-dimethoxy benzaldehyde.

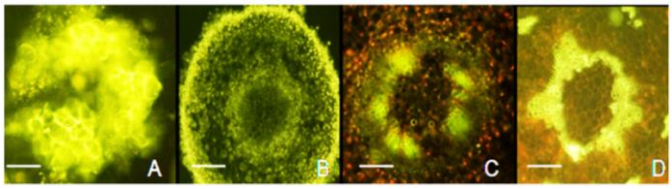

Fig 4. Autofluorescence of sandalwood tissues across various developmental stages showing progressive lignification and vascularization events. A. Pro-embryogenic masses showing accumulation of auto florescent phenolic matter (yellow) (scale bar: 100 µm). B. Transverse section of a somatic embryo showing development of two concentric phenolic-rich zones (yellow autofluorescence) (scale bar: 1 mm). C. Transverse section of a seedling showing development of insipid lignified elements i.e., protoxylem (green), scattered throughout the pith are chloroplasts (red) and phenolics (yellow) (scale bar: 1 mm). D. Transverse section of a field grown tree stem indicating full-fledged development of vasculature (green and yellow), regionated collenchymatous strands (yellow-green) and chlorenchymatous tissues (red) (scale bar: 2.5 mm).

was calculated from *t*-cinnamic acid formed (ε, 104 mM^{-1} cm^{-1}). The PAL activity was expressed in nkatals [(nM t-cinnamic acid formed s^{-1}) mg^{-1} protein].

Cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) assay

CAD assays were performed by following the oxidation of coniferyl alcohol to coniferaldehyde spectrophotometrically, as described (Mitchell et al., 1994). The CAD activity was expressed in nkatals [(nM coniferaldehyde formed s^{-1}) mg^{-1} protein].

Peroxidase (POX, EC 1.11.1.7) assays

Standard soluble anionic peroxidase (POX) assays were performed in the presence of following substrates: guaiacol [(GPOX, λ_{max}= 470 nm, conc. 18 mM, ε= 26.6 mM^{-1} cm^{-1}, i.e., formation of tetraguaiacol, pH 4.5, (Chance and Maehly, 1955)] and pyrogallol [PPOX, λ_{max}= 420 nm, conc. 20 mM, ε= 2.6 mM^{-1} cm^{-1}, pH 6 (Shannon et al., 1966)]. The reaction rate for each substrate at its pH optimum was converted to enzyme activity (EA) units (defined as the consumption/ production of 1 µmol of substrate/ product per min at 25 °C and the pH of the assay) and expressed as nkatals mg^{-1} protein.

Polyphenol oxidase (PPO, EC 1.14.18.1) enzyme preparation and assay

PPO enzyme assays in crude fractions were performed as described (Colak et al., 2005). PPO activity was determined by measuring the increase in absorbance at 500 nm using catechol as the diphenolic substrate (Espin et al., 1997) at pH 7 at 25 °C. One unit of PPO activity was defined as 1 µM of product formed per min in 1 mL of reaction mixture and expressed as µkatals mg^{-1} protein.

Extraction of free and wall-bound phenolics

Free and wall-bound phenolics were extracted as reported (Campbell and Ellis, 1992), and were used for Folin-Ciocalteau assay and RP-HPLC based identification of phenylpropanoids.

Quantification of phenolics

The concentration of total free phenolics, measured as gallic acid (GA) equivalents, was determined using the Folin-Ciocalteau's phenol reagent. To determine the phenolic content of the combined wall bound and soluble free phenolics the quantification was done as described (Julkunen-Tiitto et al., 2005). Gallic acid equivalence (GAE) (mg g^{-1} extract) for samples were determined from a gallic acid (GA) standard curve.

Quantification of anthocyanins

To analyze anthocyanin content, extraction and measurement methods followed, were as described (Qu et al., 2005). A color value (CV) of the pigment extract, which is a commercial indicator of anthocyanin content, was calculated using the following formula; CV= 0.1 x absorbance x dilution factor (CV. g-FCW^{-1}). In the above-described procedure, the dilution factor was 80 and FCW is fresh cell weight.

Quantification of flavonoids

Total flavonoids were determined as described (Zhishen et al., 1999). Rutin was used to make the calibration curve, expressing the flavonoids content as mg rutin equivalents (RE) g^{-1} plant extract. The production of total flavonoids was calculated by the formula: production of total flavonoids (mg L^{-1}) = content of total flavonoids (%) × cell dry weight (g L^{-1}) × 1000.

Concentrations of total UV-absorbing compounds

Methanolic extracts of fresh tissues were prepared by macerating 1 g of tissue in 10 mL of methanol, followed by filtration with Whatman No. 1 filter paper, and centrifugation at 5, 000 g. Concentrations of 'total UV-absorbing compounds' were estimated from 50-fold dilutions of the methanolic extracts as A_{300} (UV-B) and A_{350} (UV-A) (Day, 1993).

Quantification of lignin

Lignin was extracted and measured by the thioglycollate method (Bruce and West, 1989). Lignin concentration was calculated from a linear calibration curve (0-20 mg) prepared with commercial alkali lignin, Indulin AT (Sigma-Aldrich, USA) (Musel et al., 1997).

Quantification of shikimic acid

The amount of shikimic acid present in sandalwood developmental stages were quantified as described (Singh and Shaner, 1998). Samples were quantified spectrophotometrically (λ_{max} for shikimic acid, 380 nm).

Reverse Phase-High Pressure Liquid Chromatography (RP-HPLC) analysis of phenolics

The combined soluble and bound phenolics fractions of plant materials from three developmental stages were prepared for HPLC by dissolving in 50 % (v/v) methanol. Ferulic acid was used as an internal standard during extraction process. RP-HPLC analyses were performed on Waters HPLC system (Milford, USA) using a Phenomenex^{TM} C_{18} (RP-Hydro, 250 x 4.6 mm, 4 µm particles; Torrance, USA) column, equipped with a C_{18} guard column, dual absorbance UV and diode array (DAD) detectors. Chromatograms were monitored simultaneously at 254, 280, 310 and 365 nm and analyzed with a BREEZE^{TM} software ver. 3.2 (Waters, Milford, USA). Separation of phenolics was achieved as previously described by Sachan et al. (2004). Identification of the phenolic acids was achieved by comparing their retention times (from co-elution as well as spiking experiments with individual standard compounds), UV-Vis absorbance spectra and DAD spectra with those from standards. The calibration curves were obtained by linear regression of peak areas versus concentrations of each phenolic standard for quantification purposes.

Liquid Chromatography –Electron Spray Ionization- Mass Spectroscopy (LC-ESI-MS/MS) analyses of phenylpropanoids

To extract phenylpropanoids, fresh plant materials representing the specific developmental stages were extracted sequentially with ethyl acetate, diethyl ether, n-butanol, methanol and distilled water. The obtained extracts were freeze dried in isolation, pooled and again reconstituted in methanol. Ferulic acid was used as an internal standard during extraction. Using LH-20 column and various elutants, fractions were collected for identification and characterization. Fractions were analyzed by 'on-line' LC-ESI-MS/ MS using a Waters, 2695 separation module and QuattroMicro^{TM} API HPLC coupled with a Micromass Ion-Trap model. The solvent flow rate was 0.250 mL min^{-1} and the injection volume was 10 µL. The temperature was set to 25 °C and the column backpressure was 490 bars. Sample solutions were injected into a reversed phase column XTerra MS C_{18}, 5 µm (particle size) and 2.1 (i.d.) x 100 mm (length) (Waters, Milford, USA), that was maintained at 20°C. Chromatographic separations were achieved as previously described (Gruz et al., 2008). The pressure ranged from 410-500 psi during the chromatographic run. The effluent was introduced into a PDA detector (scanning range of 190-370 nm, resolution 1.2 nm) and subsequently into an electrospray source (source block temperature 130 °C, desolvation temperature 300°C, capillary voltage 3 kV, cone voltage 28 V). Argon was used as both collision (collision energy 2 eV) and desolvation gas (650 L h^{-1}). The flow generated by chromatographic separation was directly injected into the ESI source. The spectrometer was operated in both, negative and positive mode and the detected mass range was set to 50-900 m/z. For fragmentation pattern study, two scan events were prescribed to run simultaneously in the LCQ mass spectrometer. The first event was a full scan MS to acquire data on ions in the range (scan mode) 50-900 m/z and the second event was a MS/ MS product scan event on selected mass ions molecule specific m/z, respectively. The data were processed using the MassLynx^{TM} 4.1 software. Identification of compounds was based on the spectra generated from an in house established library as well as inference from previous studies.

Fluorescent microscopy

Fresh tissues (i.e., callus, PEM, somatic embryos, seedlings and field grown stem) were hand-cut with a razor blade or wholly mounted and observed directly in a drop of water for autofluorescence. All the images were taken with an inverted phase-contrast microscope (Model PM-10AD5, Olympus) attached to a Nikon camera. Images were processed for size and resolution using Adobe Photoshop CS2 version 9.0.2 (Adobe systems, Mountain View, CA). Autofluorescence was monitored for visualization of whole-mounts or cross-sections for visualization of lignin, phenolics, chlorophyll and flavonoids.

Statistical analyses

Statistical analysis was performed with SPSS software package (version 17) (SPSS Inc., Chicago, IL, USA). To understand the difference in values of parameters obtained from assays, one-way analysis of variance (ANOVA) was performed; where significant differences were identified using Fisher's protected least significant difference at 5 % confidence interval. Results were expressed as mean ± SD (error bars), obtained from six inter-day assays performed in triplicates, unless stated otherwise.

Conclusion

To our knowledge, this is the first ever study in *S. album* to explore phenylpropanoid metabolism, metabolites and lignification across representative developmental stages. Pending further investigation the 18 characteristic phenolic constituents and 46 phenylpropanoids may correlate with lignification pathway and its relevance with oil accumulation.

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