Expression pattern of in vitro organogenesis-associated genes as transcriptional marker in Indian Sandalwood (Santalum album L.)

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Abstract

Indian sandalwood (*Santalum album* L.) is an expensive wood that requires reproducible method for mass propagation to ensure consistent production and sustainable use of sandalwood. For mass propagation of sandalwood, plant organogenesis requires different combinations of the tissue culture medium. The media is composed of exogenous phytohormones which decides the explant's morphological stages such as shooting or rooting induction. Early prediction of morphological stage from explant can potentially help in selecting the exogenous phytohormones combinations thereby saving time and resources for mass sandalwood propogation. An efficient protocol for the direct and indirect organogenesis (up to shooting development phase) of sandalwood were developed using Woody Plant Media (WPM). WPM supplemented with various concentrations of 6-Bezylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA) were tested for direct organogenesis, while different treatments consisting of various levels of 2,4-dichlorophenoxyacetic acid (2,4-D), NAA, BAP, Adenine sulphate (ADS), glycine and potassium nitrate were tested for indirect organogenesis.Three stages of leaf development were selected *viz*., the leaf just after inoculation in WPM media, initial stage of callus formation from leaf and shoot formation for expression pattern analysis. The targeted genes were *Alternative oxidase* (*ao*), *Late embryogenesis abundant* (*lea*), *Cytochrome P450* (*cyt-p450*), *ABC transporter* (*abct*), and *Serine-threonine phosphatase* (*stp*) which are associated with *in vitro* organogenesis. The expression patterns were evaluated to identify a transcription marker. During the initial stages of organogenesis, *ao*, *cyt-p450* and *abct* showed no/little change in expression in the direct pathway but up-regulation of *ao* and *abct* and downregulation of *cyt-p450* were observed in the indirect pathway. Expression of *lea* was increased up to 70-fold during direct and dropped to half during indirect organogenesis.

Key words: Absolute quantification, Micropropagation, Organogenesis, qPCR, Sandalwood

Introduction

Indian sandalwood (*Santalum album* L.) is one of the tree species popularly known as 'The Royal Tree' of the plant kingdom (Subasinghe 2013). *Santalum album* L., belongs to the family Santalaceae, is an approximately 12-15 meters tall, evergreen and hemi-root parasitic tree. In India, Indian sandalwood is more confined to the southern part, especially in Karnataka, Tamil Nadu, and Kerala (Kumar et al. 2012) and highly valued for its fragrant heartwood and medicinal purposes. Sandalwood has the capacity to grow under diverse conditions, viz. adaptability to low rainfall and wide variety of soil types and has innate survival capacity, short juvenile phase, and profuse coppicing ability (Singh et al. 2013). Though, the major constraints in growing sandalwood are long seed dormancy period (ranges from 2 months to 12 months and normally take 4-8 weeks for the germination) and flowering usually occurs after 3–4 years in only 60% plants (Srimathi et al. 1995). Due to limited regeneration ability and high demand of sandalwood products consequently increased sandalwood tree depletion rate. The over exploitation of sandalwood tree require an efficient solution for its conservation. For sandalwood conservation, since the conventional breeding methods is one of the techniques for creating consistency in production. However it is an expensive and difficult process because sandalwood has long generation time and heterozygous in nature (Rugkhla and Jones 1998).

Alternatively, *in vitro* organogenesis through micropropogation is the potential approach for rapid sandalwood propagation that fulfills the scarcity of sandalwood in the market (Mujib 2005). *In vitro* organogenesis consist of phytohormone application which initiates cell division from any plant parts to form specific organ primordia and meristems. As the technique rely on the application of exogenous phytohormones, in particular gradients in the concentration of auxin and cytokinin and also on the ability of the tissue to respond to these phytohormones (Thorpe 1990). Therefore, different combinations and gradients of pytohormones has been used for direct and indirect organogenesis pathways as an approach for sandalwood tree regeneration.

Further, to develop a better understanding of the physiological and molecular basis of regeneration potential in sandalwood, the gene expression analysis by reverse transcription (RT) followed by quantitative polymerase chain reaction (qPCR) was performed. At molecular level, switching to direct or indirect organogenesis at any developmental stage involves differential gene expression (Jain and Minocha 2013). It has been postulated that the different hormonal treatments induce different response in genes, and it is reflective in transcript number. RT- qPCR technique represents a powerful tool for the detection and quantification of mRNA. Since qPCR is the most sensitive method for the detection and quantification of gene expression levels in particular for low transcripts in tissues (Freeman et al. 1999; Steuerwald et al. 1999; Mackay et al. 2002). Therefore, with the consideration of the importance of organogenesis in sandalwood tissue culture and the genetic expression of genes involved in direct and indirect organogenesis,

the present investigation for standardization of tissue culture protocol and genetic expression was carried out.

Materials and Methods

Experimental material

Tender nodes were collected from approximately 20 years old sandalwood tree cultivated at Anand Agricultural University Campus, Anand, Gujarat, India. The 2-3 cm long stems containing nodes were selected as explants and surface sterilized using 1 mlL^{-1} Tween-20 (5 minutes), 1000 mgL^{-1} Carbendazim-50% (7 minutes), 200 mgL^{-1} Cefotaxime (5 minutes), 200 mgL^{-1} Kanamycin (5 minutes) and 1000 mgL^{-1} mercuric chloride (3 minutes). Surface sterilized explants were then inoculated on MS medium for the shoot induction response. Leaves emerged from the nodes were used as the experimental material in the present investigation.

Direct and Indirect Organogenesis

For both the organogenesis pathways, direct and indirect, *in vitro* leaves were placed on the shoot induction media. The shoot induction media for direct organogenesis was comprised of Woody Plant Medium (WPM) (Lloyd and McCown 1981) supplemented with various combinations of 6-Benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA) and 3% w/v sucrose (Table 1) while the shoot induction media for indirect organogenesis was comprised of WPM supplemented with various combinations of Glycine, Adenine Sulphate (ADS), Potassium nitrate KNO_3), NAA, BAP, 2,4-Dichlorophenoxyacetic acid (2,4-D) and 3% w/v sucrose (Table 2). The KH_2PO_4 concentrations for direct and indirect pathways were 170 mgL^{-1} and 85 mgL^{-1}, respectively while the K_2SO_4 concentrations for direct and indirect pathways were 990 mgL^{-1} and 495 mgL^{-1}, respectively. The media was jellified using 0.9% Agar and the pH of the medium was adjusted to 5.7 ± 0.01. All the cultures were incubated in a growth room maintained at 25 ± 1°C, 40–60% relative humidity and 16/8-hour light/dark regime was provided by a cool-white, fluorescent lights having 36 $\mu molm^{-2}s^{-1}$ intensity. Observation on bud frequency %, area covered by bud sprouting (%), number of shoots, length of shoots (cm), days to callus induction, callus induction (%), callus frequency (%), callus type, callus color and health range were recorded at the end of 40, 80 and 120 days.

Table 1. Treatments for direct organogenesis

Media code	Basal	BAP(mgl^{-1})	NAA(mgl^{-1})
SD_1	WPM	0	0
SD_2	WPM	1	0
SD_3	WPM	1.5	0
SD_4	WPM	2	0
SD_5	WPM	2.5	0
SD_6	WPM	0	0.2
SD_7	WPM	1	0.2
SD_8	WPM	1.5	0.2
SD_9	WPM	2	0.2
SD_{10}	WPM	2.5	0.2
SD_{11}	WPM	0	0.4
SD_{12}	WPM	1	0.4
SD_{13}	WPM	1.5	0.4
SD_{14}	WPM	2	0.4
SD_{15}	WPM	2.5	0.4

All treatments have been provided 3% sucrose with 0.9% agar

BAP = 6-Benzylaminopurine NAA = 1-Naphthaleneacetic acid

WPM = Woody plant media

Table 2. Treatments for indirect organogenesis

Media code	Media composition	BAP (mgL^{-1})	2,4-D(mgL^{-1})	Glycin (mgL^{-1})	ADS (mgL^{-1})	KNO3 (mgL^{-1})	NAA (mgL^{-1})
SI_1	WPM	-	-	-	-	-	-
SI_2	Modified WPM	0	1	-	-	-	-
SI_3	Modified WPM	0.5	1	-	-	-	-
SI_4	Modified WPM	1	1	-	-	-	-
SI_5	Modified WPM	0	2	-	-	-	-
SI_6	Modified WPM	0.5	2	-	-	-	-
SI_7	Modified WPM	1	2	-	-	-	-
SI_8	Modified WPM	0.5	2.5	-	-	-	-
SI_9	Modified WPM	0	2.5	-	-	-	-
SI_{10}	Modified WPM	1	2.5	-	-	-	-
SI_{11}	Modified WPM	0	5	-	-	-	-
SI_{12}	Modified WPM	0.5	5	-	-	-	-
SI_{13}	Modified WPM	1	5	-	-	-	-
SI_{14}	WPM	-	-	1	-	-	-
SI_{15}	WPM	-	-	1	25	-	-
SI_{16}	WPM	-	-	1	25	-	-
SI_{17}	WPM	-	2	1	-	-	-
SI_{18}	WPM	-	3	1	25	-	-
SI_{19}	WPM	-	2	-	-	630	-
SI_{20}	WPM	-	3	-	-	-	-
SI_{21}	WPM	-	0.5	-	-	-	-
SI_{22}	WPM	-	1	-	-	-	-
SI_{23}	WPM	-	1.5	-	-	-	-
SI_{24}	WPM	-	2.5	-	-	-	-
SI_{25}	WPM	-	2	-	-	-	-
SI_{26}	WPM	-	3.5	-	-	-	-
SI_{27}	WPM	-	4	-	-	-	-
SI_{28}	WPM	-	4.5	-	-	-	-
SI_{29}	WPM	-	5.0	-	-	-	-
SI_{30}	WPM	-	3	-	-	-	-
SI_{31}	WPM	0	0.5	-	-	-	-
SI_{32}	WPM	1	0	-	-	-	-
SI_{33}	WPM	1	0.5	-	-	-	-
SI_{34}	WPM	2	0	-	-	-	-
SI_{35}	WPM	2	0.5	-	-	-	-
SI_{36}	WPM	4	0	-	-	-	0.4

All treatments have been provided 3% sucrose with 0.9% agar

Modified WPM = Half of macronutrients
ADS = Adenine sulphate
BAP = 6-Benzylaminopurine
WPM = Woody plant media
2, 4-D = 2, 4-Dichlorophenoxyacetic acid
NAA = 1-Naphthaleneacetic acid
KNO_3 = Potassium nitrate

Composition of WPM (Woody Plant Media)

Stock	**Chemical**	**Mgl^{-1}**	**Stock**		
			200 ml	**500 ml**	**1000 ml**
L & M – 18	$Ca(NO_3)_2.H_2O$	556	2224 mgs	5560 mgs	11120 mgs
A	NH_4NO_3	400	1600 mgs	4000 mgs	8000 mgs
20X	$CaCl_2.2H_2O$	96	384 mgs	960 mgs	1920 mgs
50mlL^{-1}	$MgSO_4.7H_2O$	370	1480 mgs	3700 mgs	7400 mgs
Fresh	KH_2PO_4	170			
	K_2SO_4	990			
			200 ml	**500 ml**	**1000 ml**
L & M – 18	H_3BO_3	6.2	124 mgs	310 mgs	620 mgs
B	$MnSO_4.4H_2O$	22.3	446 mgs	1115 mgs	2230 mgs
100X	$ZnSO_4.7H_2O$	8.6	172 mgs	430 mgs	860 mgs
10mlL^{-1}	$Na_2MoO_4.2H_2O$	0.25	5 mgs	12.5 mgs	25 mgs
	$CuSO_4.5H_2O$	0.25	5 mgs	12.5 mgs	25 mgs
L & M – 18			**200 ml**	**500 ml**	**1000 ml**
F	Na_2 EDTA	37.3	746 mgs	1865 mgs	3730 mgs

100X **10mlL^{-1}**	$FeSO_4.7H_2O$	27.8	556 mgs	1390 mgs	2780 mgs
Mullin et al., (1974) **B G** **100 X** **10 mlL^{-1}**			**200 ml**	**500 ml**	**1000 ml**
	Nicotinic Acid	0.5	10 mgs	25 mgs	50 mgs
	Pyridoxin HCL	0.5	10 mgs	25 mgs	50 mgs
	Thiamin HCL	1.0	20 mgs	50 mgs	50 mgs
	Glycin	2.0	40 mgs	100 mgs	100 mgs
	Inositol	100.0			

Lloyd and McCown (1981)

L & M-81 Macro + L & M-81 Micro + Mullin et al (1974) B (Vitamin) + Skoog (1944) A.A

Gene expression profiling

In silico selection of genes and primer designing

A total of thirty-one various gene sequences for callus and shoot development stage were selected from the findings of Che et al. (2006) on *Arabidopsis* and subjected to BLAST analysis. Sequences for the primer design were selected from the conserved region of selected genes using primer BLAST tool available at NCBI web site. Two pairs of primers were selected for each of the *in silico* identified genes. The primers were checked for dimer or hairpin formation using Oligo IDT analyzer tool. Along with the gene specific primers, a DNA specific primer has also been designed from the intron region of gene to check the cDNA sample for DNA contamination. Five set of primers, representing five different genes *viz*. *Alternative oxidase* (*ao*), *Late embryogenesis abundant proteins* (*lea*), *Cytochrome P-450* (*cytp450*), *ATP binding cassette transporter proteins* (*abct*), and *Serine threonine phosphatase* (*stp*) were designed using primer BLAST tool available at NCBI website (Table 3).

Table 3. List of genes in callus development and shoot development in Arabidopsis

Sr. No.	Gene	Primer Name	Sequence	*In silico* product length (bp)
1	Alternative oxidase (AO)	SW1 F	TGCCTGCACCGGCTATTG	90
		SW1 R	CTTCATCAGCACGGACCACC	
2	LEA family protein (LEA)	SW2 F	GAGAAGGGAAGCGAAGTGGG	145
		SW2 R	ACTCCGAAGCAAACTGAGCA	
3	Cytochrome P-450 (CYTP450)	SW3 F	AAGAGTCGGCTTACGAGCTG	110
		SW3 R	CTTGGGGCTGAAGAGATGGG	
4	ATP Binding Cassette Transporter proteins (ABCT)	SW4 F	GGATGAGCCAACTTCAGGCT	82
		SW4 R	TGCTAGGCTGGTGTATGGTG	
7	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	SW5 F	ATGCGGACATTAAGGCTGCT	113
		SW5 R	TGACCTGTTGTCGCCAATGA	
6	Serine Threonine Phosphatase (STP)	SW6 F	CACACCGTGGTTGATGGCT	150
		SW6 R	TGAACGTGACCGGCAAAAAC	

DNA/RNA extraction, cDNA synthesis

Total DNA was extracted from the sandalwood leaf tissue using the method described by Doyle and Doyle (1990). The quantity and purity (A260/A280 ratio) of DNA was measured in Nanodrop N.D.1000 (Thermo Scientific, U.S.A.). The DNA samples were diluted to 20 ng/µl with TE buffer and stored at 4°C. Three developmental stages of sandalwood were selected for direct organogenesis, namely, inoculated leaf (D1), proliferated leaf (D2) and shoot formation stage (D3). Similarly for indirect organogenesis, the samples were collected from inoculated leaf (ID1), proliferated leaf (ID2) and callus formation stage (ID3). The various direct and indirect stages are shown in figure 1. RNA extraction was carried out from the samples collected from the different stages of organogenesis using the method described by Ghavana et al. (2011) with minor modification. The quantity and quality (A260/A280 ratio) of RNA was determined in

Nanodrop N.D.1000 (Thermo Scientific, U.S.A.). *DNase* treatment was given to all the samples using *DNase I* (Thermo Scientific) to avoid any DNA contamination. Preparation of cDNA from total RNA was carried out using first strand cDNA synthesis kit (Takara, Japan) as per the manufacturer's instructions.

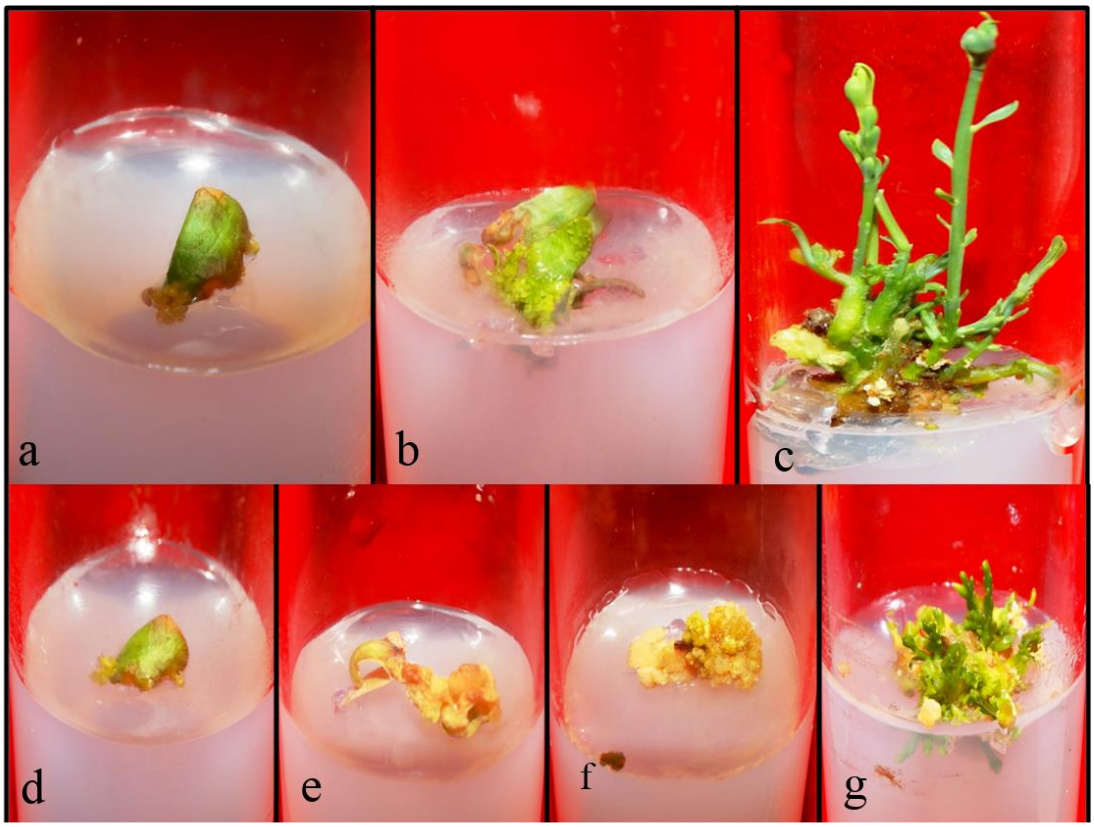

Fig. 1: Various developmental stages of direct (a, b, c) and indirect (d, e, f, g) organogenesis in sandalwood; a & d – Inoculated leaf; b & e – Proliferated leaf; c & g – Shoot formation; and f – Callus formation.

Primer screening through end-point PCR and validation

Primer screening was carried out with the DNA extracted from sandalwood tissues of different stages. RAPD amplification was performed in a 25 µl reaction system containing 100 ng DNA, 1x DreamTaq Green PCR buffer with 2 mM $MgCl_2$ (Thermo Fisher Scientific, U.S.A.), 0.2 mM each of dNTPs (Thermo Fisher Scientific, U.S.A.), 0.5 U *Taq* DNA polymerase (Kapa Biosystems, U.S.A.), 4 pmol of forward and reverse primers (SW1, SW2, SW3, SW4, SW5 and

SW6) and sterile distilled water. Amplification was automated using a PCR machine (Applied Biosystems, USA). The PCR cycles involved an initial denaturation step at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 30 seconds, extension at 72 °C for 1 minute and final extension at 72 °C for 5 minute. Agarose gel (1.5 %) was prepared in 1x TBE buffer to separate the amplified products. Gel documentation system (Alpha Innotech, USA) was used for visualization of amplified DNA fragments. Genes that did not amplify were eliminated and not considered for further downstream application. The screened primers were then validated with the cDNA of all samples to determine the genes expressed during organogenesis qualitatively. Each primer pair was amplified with all the samples along with an NTC (No template Control) to decipher the intensity of primer dimmers or DNA contamination. The primer pairs were selected on the basis of presence of specific and expected sized band in gel analysis. These primers were used for absolute quantification in Real Time PCR.

Absolute quantification

The preparation of standard curve of each gene was carried out by purifying and quantitating the PCR product for each gene. Standards for all the genes were ranged from 10^9 to 10^1 copies per µl with each step differing by 10-fold. Using these standards and cDNA samples, absolute quantification was done in 7500 Fast Real Time PCR (Applied Biosystems, USA). SYBR Green chemistry was used for real time PCR reaction. Cycling conditions involved an initial denaturation at 94˚C for 5 minutes and 40 cycles of amplification at 94˚C for 10 seconds followed by 60˚C for 30 seconds. Standard curve was prepared for each gene using Cq values and initial copy number of the transcript. Concentrations of unknown samples were determined using the data generated from the plot. Sample transcript copy number was calculated using the equation obtained from the graph. Moreover, considering the inoculated stage leaf (both D1 and ID1) as control, their transcript copy numbers were designated as 1.0 and corresponding relative numbers were given to other treatments.

Results and discussion

The present study used *in vitro* leaf tissue for direct as well as indirect organogenesis. The detailed consequences of the direct and indirect organogenesis and *in vitro* expression profiling of the various genes have been described here and elucidated through tables and figures.

In vitro organogenesis

In attempts towards direct organogenesis, the relationship between gradient levels of BAP and NAA and the morphogenic potential of plantlets were found out which could give best establishment and multiplication rates. The percentage establishment and growth of cultures was highest in the establishment phase. After 40 days of incubation, treatment SD_{14} (2 mgL^{-1} BAP and 0.4 mgL^{-1} NAA) generated a sprouting frequency of 40%. Treatment, SD_{14} and SD_{15} indicated as the best health representative among all treatments with score (++++). Following SD_{14}, SD_{15} showed second highest sprouting frequency (30%). Remaining treatments demonstrated poor health with little necrotizing growth. In the proliferating phase, after 80 days, SD_{14} (2.0 mgL^{-1} BAP and 0.4 mgL^{-1} NAA) recurred as the best media in terms of the various observations recorded e.g. bud sprouting frequency, number of shoots and length of shoots were 60%, 3 and 2 cm, respectively, highest among the other treatments. Subsequently, SD_7 was listed as the subsequent efficient media with 52.50% bud sprouting frequency and had an excellent shoot health (++++). Even after 120 days, SD_{14} continued to give highest proliferation. The sprouting frequency has increased to 70% which was 40% earlier and number of shoots have increased to five. Treatments SD_{14} and SD_7 came up with potential numbers of shoots formation per explant (five) after 120 days but considering the overall efficient health SD_{14} dominated over SD_7. These observational analyses were in correlation with the report by Singh et al. (2013) where they used WPM supplemented with 2.5 mgL^{-1} BAP and 0.2 mgL^{-1} NAA and obtained a bud frequency of 79.16%, whereas incongruent with results of Mujib (2005) who reported the BAP concentration as low as 0.5 mgL^{-1} without incorporating NAA. Greenish shoot bud formation in all the treatments in the present study might have been observed due to low auxin:cytokinin ratio as explained by the work of Behbahani et al. (2011) and Singh et al. (2013). The detailed results are presented in Supplementary Tables. Among all treatments tested, SI_{24} (2.5 mgL^{-1} 2,4-D) followed by the treatment SI_{18} (3 mgL^{-1} 2,4-D and 1 mgL^{-1} glycine and 25 mgL^{-1} ADS) and SI_{33} (1 mgL^{-1} BAP and 0.5 mgL^{-1} 2,4-D) reflected as the best treatments for achieving early callus induction response after 40 days. This finding is in accordance with the results of Adesoye and Orkpeh (2009) and Behbahani et al. (2011) who also supported WPM over MS. Rashmi and Trivedi (2014) postulated that 2,4-D may have encouraged the synthesis of endogenous purines and cytokinins ultimately resulting in higher rates of cell division. The days to callus induction ranged from 35 to 37 days while the callus induction % ranged from 10% to 50%. After 80 days, the callusing reached maximum in treatment, SI_{24} (2.5 mgL^{-1} 2,4-D) which generated callus induction and frequency percent of 70% and 50%, respectively. The callus looked very healthy with green in colour and friable in nature. Tissue morphologies ranged from white, translucent and watery in appearance to green, opaque and compact. A few emerging propagules were observed as indicative of shoot regeneration in treatment SI_{33} (1 mgL^{-1} BAP and 0.5 mgL^{-1} 2,4-D) after 120 days. It generated callus induction percent of 87.50% and callus regeneration frequency percent of 75%. Any change in the concentrations of growth regulators resulted in change in morphology and growth of the callus developmental pattern. Apart from control, treatments SI_2, SI_3, SI_4, SI_{23}, SI_{26}, SI_{27}, SI_{28}, SI_{29}, SI_{30}, SI_{31}, SI_{32}, SI_{34} and SI_{36} were

incompetent for leaf proliferation and shoot initiation even after 120 days. The detailed results are presented in Supplementary Table 1-6.

Gene expression studies

The screening of gene specific primers with various stages of sandalwood organogenesis cDNA samples was performed. The primers of genes which amplified the cDNA samples were *Alternative oxidases*, *Cytochrome P-450*, *Late Embryogenesis Abundant*, *ABC transporter*, and *serine-threonine phosphatase* and their product lengths recorded were 90 bp, 82 bp, 149 bp, 150 bp, and 113 bp, respectively. The absolute quantification was performed for all mentioned primers in the real time PCR. Absolute quantification of each gene was carried out using standard curve as prepared from the graph of C_q values versus copy number through which absolute copy number was calculated and converted into relative quantity value taking leaf inoculated sample (for both direct and indirect organogenesis) as control (Table 4, Figure 2, 3).

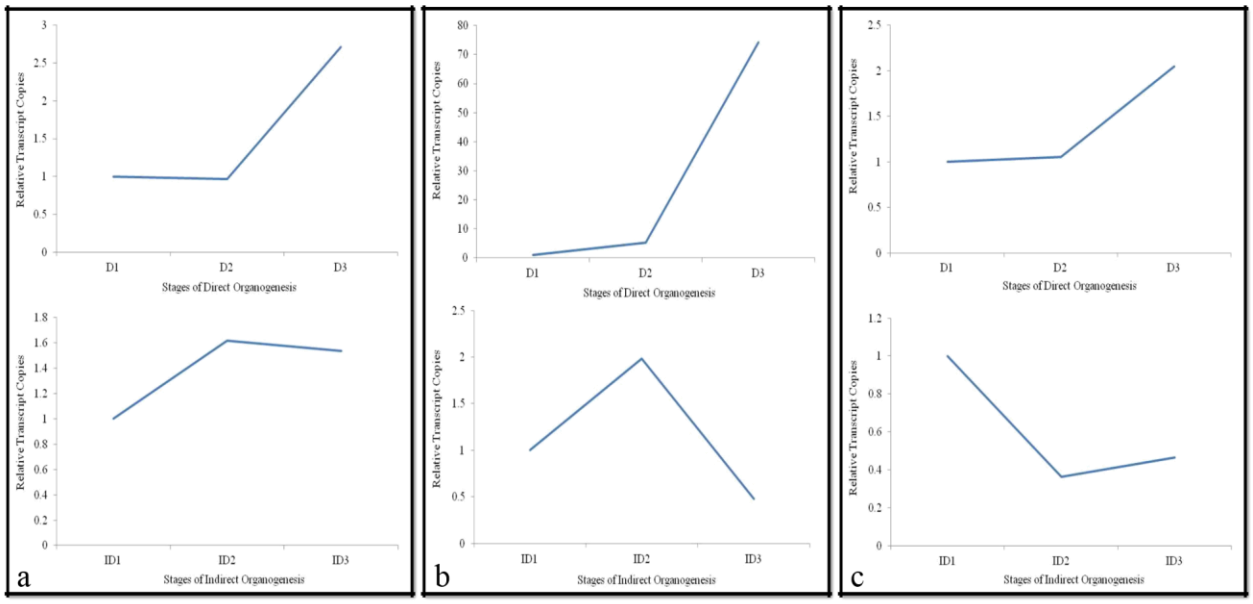

Fig. 2: Relative gene expression pattern of *ao* (a); *lea* (b); and *cyt-P450* (c) within three stages of direct and indirect organogenesis. D1 – inoculated leaf, D2 – proliferated leaf and D3 – shoot formation stage, ID1 – inoculated leaf, ID2 – proliferated leaf and ID3 – callus formation.

Table 4. List of genes in callus development and shoot development (from Arabidopsis database) used for the gene expression profiling in micropropagation of *S. album.*

Sr. No.	Gene	Primer name	Sequence (5' - 3')	*In silico* product length (bp)	Stage	Absolute quantity (copy no./µg of total RNA)	Relative quantity
1	Alternative oxidase (*ao*)	SW1 F	TGCCTGCACCGGCTATTG	90	D1	15276.1	1.0
					D2	14721.9	0.9
					D3	41355.4	2.7
		SW1 R	CTTCATCAGCACGGACCACC		ID1	10470.2	1.0
					ID2	16953.3	1.6
					ID3	16082.7	1.5
2	LEA family protein (*lea*)	SW2 F	GAGAAGGGAAGCGAAGTGGG	145	D1	1067.8	1.0
					D2	5635.8	5.2
					D3	79284.9	74.2
		SW2 R	ACTCCGAAGCAAACTGAGCA		ID1	1994.9	1.0
					ID2	3952.5	1.9
					ID3	951.8	0.4
3	Cytochrome P-450 (*cytp450*)	SW3 F	AAGAGTCGGCTTACGAGCTG	110	D1	39.6	1.0
					D2	41.8	1.0
					D3	81.1	2.0
		SW3 R	CTTGGGGCTGAAGAGATGGG		ID1	73.9	1.0
					ID2	26.9	0.3
					ID3	34.5	0.4
4	ATP	SW4 F	GGATGAGCCAACTTCAGGCT	82	D1	407.7	1.0

	Binding Cassette Transporter proteins (*abct*)				D2	327.2	0.8
					D3	2348.2	5.7
		SW4 R	TGCTAGGCTGGTGTATGGTG		ID1	304.1	1.0
					ID2	674.1	2.2
					ID3	337.0	1.1
5	Serine Threonine Phosphatase (*stp*)	SW6 F	CACACCGTGGTTGATGGCT	150	D1	15749091	1.0
					D2	14435999	0.9
					D3	5599633	0.3
		SW6 R	TGAACGTGACCGGCAAAAAC		ID1	13914516	1.0
					ID2	15238483	1.0
					ID3	ND	ND

ND – not detected

Alternative oxidase (ao)

Alternative oxidase (ao) is ubiquitous protein in all plants which play role in stress response, in addition, major contribution in regulating cellular thermodynamic responses by alteration of radicals (Van Aken et al. 2009). Relative gene expression pattern of *ao* observed during direct and indirect organogenesis as depicted in Figure 2a. During direct organogenesis, gene expression remained nearly consistent in terms of transcript copy number from inoculated leaf to proliferated leaf. This can be amenable with the report by Millenaar and Lambers (2003) where the excess production of reactive oxygen species free radicals was controlled by *ao* by continuing the citric acid cycle and regulating mitochondrial ubiquitin pool.

Indirect organogenesis was induced by different doses of 2,4-D phytohormone which act as a stress growth regulator that forms the unorganized mass called callus (Ikeuchi 2013 and Tahir et al. 2011). During indirect organogenesis, increase in gene expression was visualized from inoculated leaf to leaf proliferation stage. This indicate that *alternative oxidase* capacity for the storage of carbohydrates may have increased as mentioned by Steingrover 1981; Lambers et al. (1981). Linus et al. (1983) stated functions of *ao* as a reserve electron transport capacity in callus in which cytochrome pathway activity was impaired. Further, expression remained consistent in callus induction phase. Noteworthy point was that the next stages of shoot

formation through indirect organogenesis showed reduction in transcript level as *ao* as illustrated by Lambers (1980). The *ao* disproportionate curve indicates that *ao* set the defense equilibrium or threshold in plant cells, in the absence of which plants showed irregularity in growth pattern and responded abruptly to stress as noticed in indirect organogenesis under stress condition induced by 2, 4-D (Fiorani. 2005).

Late embryogenesis abundant (lea)

Late embryogenesis abundant (lea) are low molecular weight (10–30 kDa) proteins which mainly involved in protecting higher plants from damage caused by environmental stresses, especially drought (dehydration). The expression of *lea* gene had increased from 1.0 (inoculated leaf stage) to 5.2-fold (proliferated one) and sudden up-regulation (74.2-fold) was observed at shoot formation stage during direct organogenesis.

However, in case of indirect organogenesis *lea* expression reached its peak from 1.0 (leaf inoculation) to 1.9 (leaf proliferation stage) followed by sharp decline (0.4) at callus formation stage (Figure 2b). Looking at the fold change value of *lea* during direct and indirect organogenesis, the level of expression increased to 2-3 fold, from inoculated leaf to proliferated leaf stage but at the time of shoot formation/callusing, the value increased to 70 fold higher and to approximately half during direct and indirect organogenesis, respectively. Such expression pattern of *lea* during indirect organogenesis indicated the vital role in differentiated tissue rather than undifferentiated one. For survival, higher plants acclimatize themselves to changing environment through various mechanisms involved at different levels. The present report also deduced coherent results which stated that due to stress in shooting stage of direct organogenesis, the expression of *lea* had been significantly higher due to high signal transduction. However during indirect organogenesis, the stress response was negligible and therefore the low transcript profiling was detected. Gao and Lan (2016) reported late embryogenesis abundant (*lea*) proteins as a large and highly diverse gene family in pines that played a vital role not only in various stress tolerance responses but also crucial during embryonic development. This was evident during direct organogenesis where the transcript copy number was significantly high from inoculated leaf to leaf proliferation stage whereas during indirect organogenesis the expression declined as compared to normal developmental levels.

Cytochrome p-450 (cyt-p450)

To better understand the organogenesis events, another gene expression pattern of *Cytochrome P-450* was included. Like *ao, cyt-p450* showed insignificant change in expression

during first two stages of direct organogenesis and then doubled at shoot formation stage while in indirect organogenesis continuous down-regulation pattern was observed (Figure 2c). The double number of transcripts at shoots formation stage in direct organogenesis pathway have been obtained due to regulation of auxin biosynthesis by *cytochrome p450* family proteins which ultimately affected the growth and development of the plant as explained by Bak and Feyereisen (2001) and Vadassery et al. (2008) in *Arabidopsis* plant. Further, the *cytochrome p-450* and *alternative oxidases* pathways were interlinked and differed reciprocally highlighting the simultaneous decrease in *cytochrome p-450* with ascending *alternative oxidases* expression during indirect organogenesis which was in account of studies by Bahr and Bonner (1973) and Theologis and Laties (1978).

ATP Binding Cassette Transporter Proteins (abct)

The *abct* gene revealed the down regulation during direct organogenesis from inoculated leaf to leaf proliferation. However, almost six fold up regulation was observed at shoot formation stage. This finding clearly indicated that as the plant attained maturity, the transcript level has increased. Gaedeke et al. (2001) also postulated that *abct* is involved in stomata formation and expressed mainly in vascular bundles, epidermis especially in guard cells. It also works as auxin conjugate transporters that regulate auxin as well as ion channels. However, in case of callus mediated organogenesis two fold increase was observed during leaf proliferation stage and the level reached to its ground state, near to control, afterwards (Figure 3a). Kang et al. (2011), while working on Arabidopsis, also obtained similar expression patterns due to cellular detoxification processes that are necessary for organ growth, plant nutrition, plant development.

In agreement with the supporting literatures (Rea 2007; Schulz and Kolukisaoglu 2006; Theodoulou 2000), our analysis revealed a sharp up regulation at shoot formation stage during direct organogenesis contributed to the fact that plant at this stage require more ion transport, has high detoxification rate, and performs frequent stomata activity resulting in intense increase in *abct* transcript level. Furthermore, the decline in *abct* level at callus induction stages in indirect pathway revealed that the rate of stomata activity might have declined and as a result cell growth rate was affected as compared to normal. In conclusion, our curiosity led to prediction of *abct* proteins regulation successfully which clearly differentiated the direct and indirect organogenesis event, visually contradict expression pattern at different pathway.

Serine Threonine Phosphatase (stp)

Serine/threonine protein phosphatases plays critical role in the regulation of cell cycle. In the present study constant down regulation of *stp* was observed during both direct and indirect pathways (Figure 3b).

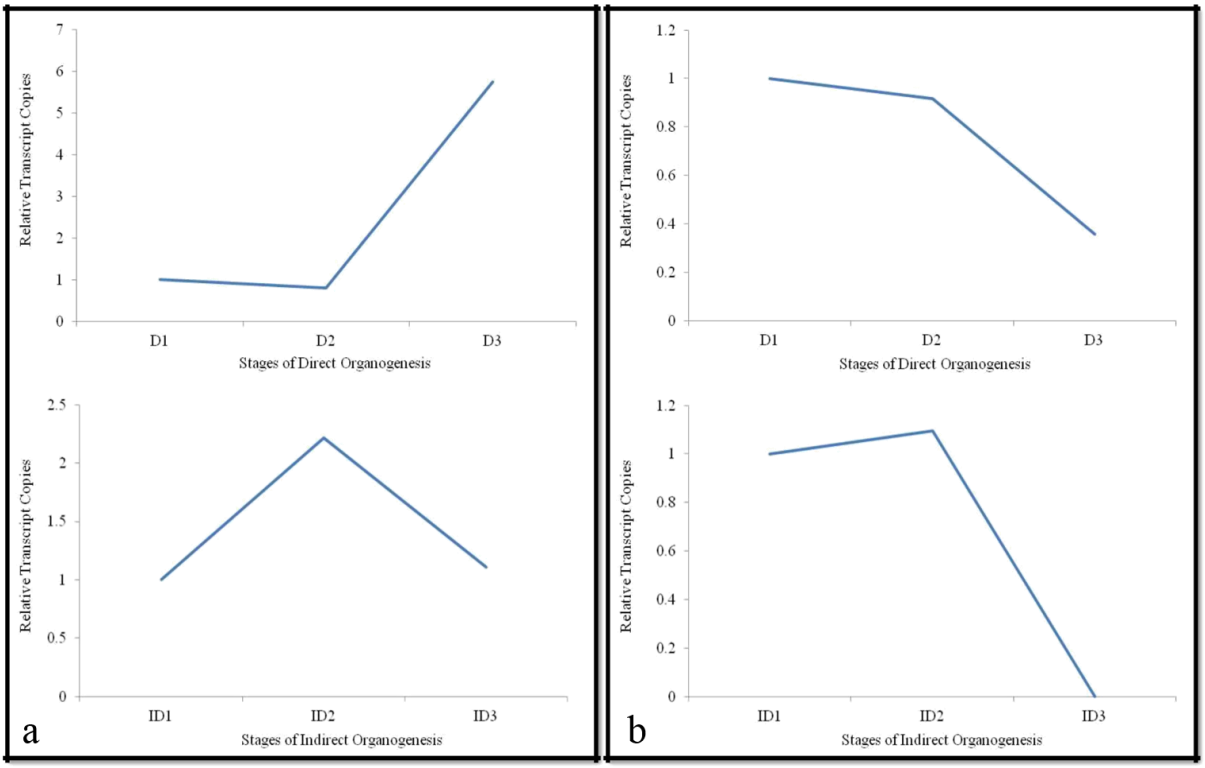

Fig. 3: Relative gene expression pattern of *abct* (a); and *spt* (b) within three stages of direct and indirect organogenesis. D1 – inoculated leaf, D2 – proliferated leaf and D3 – shoot formation stage, ID1 – inoculated leaf, ID2 – proliferated leaf and ID3 – callus formation.

The continuous decline in expression during direct organogenesis might be due to the ability of okadaic acid to activate other transcription factors which caused alteration in protein serine/threonine resulting in dephosphorylation as explained by Mumby and G. Walter (1993). Whilst, during indirect organogenesis, the transcript copy number did not change initially but sharply delineated to zero at callusing stage which marked the central role of protein *serine/threonine phosphatase* in the re-entry of quiescent cells into the cell cycle. This re-entry suggested the dedifferentiated phase of callus that may trigger the cell for redifferentiation resulting in increase in the mRNA level initially which further did not show any expression pattern as described by Villafranca (1996). The available information on the specific functions of different forms of protein serine/threonine phosphatases is still severely limited as mentioned by Mumby and Walter (1993).

From the above results and discussions, we anticipate the gene expression pattern can be used as a diagnostic tool for identification of different developmental stages in sandalwood. The combinations of gene expression patterns provides a potential information required for developmental stage identification. To widen our understanding about molecular aspects, the

futher understanding of plant quantitative traits that are controlled by many genes and intricate pathways is essential.

Conclusion

In conclusion, out of five genes used to obtain gene expression patterns, three of them, namely; *ao*, *cyt-p450* and *stp* showed little/no change in transcript copy number during initial two stages while two of these genes *ao* and *cyt-p450* showed up-regulation and *stp* showed down-regulation in direct pathway. Expression patterns of *lea* and *abct* showed strong molecular signature to predict the route of the organogenesis in sandalwood micropropagation as they were constantly up regulated during direct organogenesis and down regulated during indirect organogenesis. Thus the objective of the present investigation had come to the fulfillment with the help of dynamic expression patterns of *lea* and *abct* during initial stages of organogenesis and it is now clear that one can predict the fate of explant.

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Supplementary Table 1 Direct organogenesis - Morphology of the shoot buds derived from the defoliated leaf explant at the end of 40, 80 and 120 days

Media code	PGRs		After 40 days		After 80 days				After 120 days			
	BAP (mgl^{-1})	NAA (mgl^{-1})	Bud Frequency (%)	Health range	Area covered by bud sprouting (%)	Number of shoots	Length of shoots (cm)	Health range	Area covered by bud sprouting (%)	Number of shoots	Length of shoots (%)	Health range
SD_1	0.0	0.0	0.00^{f}	+	0.00^{t}	0.00^{a}	0.00^{g}	+	0.00^{h}	0.00^{f}	0.00^{t}	+
SD_2	1.0	0.0	0.00^{f}	+	5.00^{h}	1.00^{a}	0.30^{f}	+	10.00^{g}	2.00^{d}	0.70^{t}	+
SD_3	1.5	0.0	0.00^{f}	++	10.00^{fg}	1.00^{a}	0.40^{f}	++	20.00^{f}	2.25^{d}	1.00^{h}	++
SD_4	2.0	0.0	0.00^{f}	++	8.75^{g}	1.00^{a}	0.50^{f}	++	20.00^{f}	2.75^{c}	1.50^{g}	++
SD_5	2.5	0.0	0.00^{f}	++	12.50^{f}	2.00^{a}	1.00^{d}	++	20.00^{f}	4.00^{b}	2.00^{fe}	++
SD_6	0.0	0.2	0.00^{f}	++	0.00^{t}	0.00^{a}	0.00^{g}	++	0.00^{h}	0.00^{f}	0.00^{j}	++
SD_7	1.0	0.2	7.50^{f}	+++	52.50^{b}	3.00^{a}	1.75^{b}	+++	67.50^{b}	5.00^{a}	2.75^{bc}	+++
SD_8	1.5	0.2	20.00^{c}	+++	30.00^{d}	1.00^{a}	1.00^{d}	+++	40.00^{d}	2.00^{d}	2.25^{df}	+++
SD_9	2.0	0.2	10.00^{d}	+++	40.00^{c}	2.00^{a}	1.50^{c}	+++	47.50^{c}	3.75^{b}	3.00^{b}	+++
SD_{10}	2.5	0.2	0.00^{f}	+	0.00^{t}	0.00^{a}	0.00^{g}	+	0.00^{h}	0.00^{f}	0.00^{t}	+
SD_{11}	0.0	0.4	0.00^{f}	+	0.00^{t}	0.00^{a}	0.00^{g}	+	0.00^{h}	0.00^{f}	0.00^{t}	+
SD_{12}	1.0	0.4	0.00^{f}	+	20.00^{f}	1.00^{a}	0.50^{f}	+	30.00^{f}	2.00^{d}	1.75^{fg}	+
SD_{13}	1.5	0.4	20.00^{c}	+++	30.00^{d}	1.25^{a}	1.00^{d}	+++	40.00^{d}	3.00^{c}	2.00^{fe}	+++
SD_{14}	2.0	0.4	40.00^{a}	++++	60.00^{a}	3.00^{a}	2.00^{a}	++++	70.00^{a}	5.00^{a}	4.00^{a}	++++
SD_{15}	2.5	0.4	30.00^{b}	++++	40.00^{c}	2.00^{a}	1.50^{c}	++++	50.00^{c}	4.00^{b}	2.50^{cd}	++++
S.Em.			0.373		0.968	1.92	0.037		0.645	0.112	0.083	
C.D. at 5%			1.065		2.767	0.00	0.107		1.845	0.320	0.238	
C. V. %			8.77		9.41	0.00	9.36		4.64	8.89	9.82	

All the treatments were analyzed by DNMRT and treatment means with same letters were at par at 5% level of significance.

PGRs – Plant Growth Regulators. + Poor, ++ Moderate, +++ Healthy, ++++ Very healthy.

Supplementary Table 2 Indirect organogenesis - Morphology of the callus derived from the defoliated leaf explant at the end of 40, 80 and 120 days

Media Code	PGRs						After 40 days						After 80 days					After 120 days				
	BAP (mgl^{-1})	2,4-D (mgl^{-1})	Glycine (mgl^{-1})	ADS (mgl^{-1})	KNO_3 (mgl^{-1})	NAA (mgl^{-1})	Days to callus induction	Callus induction (%)	Callus frequency (%)	Callus type	Callus colour	Health range	Callus induction (%)	Callus frequency (%)	Callus type	Callus colour	Health range	Callus Induction (%)	Callus regeneration frequency (%)	Callus type	Callus colour	Health range
SI_1	0.0	0.0	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_2	0.0	1.0	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_3	0.5	1.0	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_4	1.0	1.0	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_5	0.0	2.0	-	-	-	-	37.00^{a}	20.00^{f}	5.00^{f}	F	G	+++	10.00^{g}	40.00^{d}	F	G	+++	20.00^{g}	50.00^{f}	F	G	+++
SI_6	0.5	2.0	-	-	-	-	37.00^{a}	10.00^{f}	10.00^{f}	F	G	+++	20.00^{f}	30.00^{f}	F	G	+++	30.00^{ef}	40.00^{f}	F	G	+++
SI_7	1.0	2.0	-	-	-	-	37.00^{a}	10.00^{f}	10.00^{f}	F	Y	+++	30.00^{d}	20.00^{g}	F	Y	+++	40.00^{d}	30.00^{h}	F	Y	+++
SI_8	0.5	2.5	-	-	-	-	35.00^{g}	20.00^{f}	20.00^{d}	F	G	+++	20.00^{f}	20.00^{g}	F	G	+++	30.00^{ef}	32.50^{gh}	F	G	+++
SI_9	0.0	2.5	-	-	-	-	36.00^{df}	10.00^{f}	20.00^{d}	C	G	+++	30.00^{d}	20.00^{g}	C	G	+++	50.00^{c}	30.00^{h}	C	G	+++
SI_{10}	1.0	2.5	-	-	-	-	36.25^{cd}	20.00^{f}	20.00^{d}	C	Y	+++	20.00^{f}	30.00^{f}	C	Y	+++	40.00^{d}	40.00^{f}	C	Y	+++
SI_{11}	0.0	5.0	-	-	-	-	37.00^{a}	30.00^{c}	5.00^{f}	C	G	+++	10.00^{g}	30.00^{f}	C	G	+++	22.50^{g}	40.00^{f}	C	G	+++
SI_{12}	0.5	5.0	-	-	-	-	36.75^{ab}	20.00^{f}	5.00^{f}	F	G	+++	10.00^{g}	32.50^{f}	F	G	+++	27.50^{f}	42.50^{f}	F	G	+++
SI_{13}	1.0	5.0	-	-	-	-	36.50^{bc}	10.00^{f}	10.00^{f}	F	Y	+++	20.00^{f}	20.00^{g}	F	Y	+++	30.00^{ef}	30.00^{h}	F	Y	+++
SI_{14}	-	-	1.0	-	-	-	35.50^{f}	10.00^{f}	5.00^{f}	F	G	+++	10.00^{g}	32.50^{f}	F	G	+++	27.50^{f}	40.00^{f}	F	G +Y	+++
SI_{15}	-	-	1.0	25	-	-	36.75^{ab}	27.50^{d}	5.00^{f}	F	G	+++	20.00^{f}	42.50^{d}	F	G	+++	30.00^{ef}	50.00^{f}	F	G + Y	+++
SI_{16}	-	-	1.0	25	-	-	36.50^{bc}	10.00^{f}	10.00^{f}	F	G	++++	20.00^{f}	30.00^{f}	F	G	++++	32.50^{f}	42.50^{f}	C	G	++++
SI_{17}	-	2.0	1.0	-	-	-	37.00^{a}	20.00^{f}	20.00^{d}	F	G	++++	27.50^{f}	25.00^{f}	F	G	++++	42.50^{d}	35.00^{g}	F	G	++++
SI_{18}	-	3.0	1.0	25	-	-	35.00^{a}	50.00^{a}	20.00^{d}	F	G	++++	30.00^{d}	50.00^{c}	F	G	++++	42.50^{d}	62.50^{cd}	F	G	++++
SI_{19}	-	2.0	-	-	630.0	-	37.00^{a}	30.00^{c}	20.00^{d}	F	G	++++	30.00^{d}	50.00^{c}	F	G	++++	50.00^{c}	52.50^{f}	F	G	++++
SI_{20}	-	3.0	-	-	-	-	36.00^{g}	40.00^{b}	30.00^{c}	F	G	+++	40.00^{c}	67.50^{a}	F	G	+++	52.50^{c}	65.00^{c}	F	G	+++
SI_{21}	-	0.5	-	-	-	-	37.00^{a}	10.00^{f}	5.00^{f}	F	G	+	10.00^{g}	20.00^{g}	F	G	+	20.00^{g}	30.00^{h}	F	G	+
SI_{22}	-	1.0	-	-	-	-	36.00^{df}	10.00^{f}	10.00^{f}	F	G	++	20.00^{f}	20.00^{g}	F	G	++	20.00^{g}	30.00^{h}	F	G	++
SI_{23}	-	1.5	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	++	0.00^{h}	0.00^{h}	NR	ND	++	0.00^{h}	0.00^{t}	NR	ND	++
SI_{24}	-	2.5	-	-	-	-	36.50^{df}	50.00^{a}	50.00^{a}	F	G	++++	70.00^{a}	50.00^{c}	F	G	++++	80.00^{b}	70.00^{b}	F	G + Y	++++
SI_{25}	-	2.0	-	-	-	-	35.75^{a}	30.00^{c}	20.00^{d}	F	G	+++	20.00^{f}	40.00^{d}	F	G	+++	40.00^{d}	60.00^{d}	F	G	+++
SI_{26}	-	3.5	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_{27}	-	4.0	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_{28}	-	4.5	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_{29}	-	5.0	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_{30}	-	3.0	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_{31}	0.0	0.5	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_{32}	1.0	0.0	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_{33}	1.0	0.5	-	-	-	-	37.00^{a}	40.00^{b}	37.50^{b}	F	G	++++	60.00^{b}	62.50^{b}	F	G	++++	87.50^{a}	75.00^{a}	F	G	++++

Media Code	PGRs						After 40 days						After 80 days					After 120 days				
	BAP (mgl^{-1})	2,4-D (mgl^{-1})	Glycine (mgl^{-1})	ADS (mgl^{-1})	KNO_3 (mgl^{-1})	NAA (mgl^{-1})	Days to callus induction	Callus induction (%)	Callus frequency (%)	Callus type	Callus colour	Health range	Callus induction (%)	Callus frequency (%)	Callus type	Callus colour	Health range	Callus Induction (%)	Callus regeneration frequency (%)	Callus type	Callus colour	Health range
SI_{34}	2.0	0.0	-	-	-	-	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
SI_{35}	2.0	0.5	-	-	-	-	36.75^{ab}	10.00^{f}	20.00^{d}	F	G	++++	30.00^{d}	40.00^{d}	F	G	++++	80.00^{b}	72.50^{ab}	F	G	++++
SI_{36}	4.0	0.0	-	-	-	0.4	0.00^{h}	0.00^{g}	0.00^{g}	NR	ND	+	0.00^{h}	0.00^{h}	NR	ND	+	0.00^{h}	0.00^{t}	NR	ND	+
S.Em.							0.134	0.417	0.417				0.417	1.049				1.179	1.318			
C.D. at 5%							0.375	1.167	1.167				1.167	2.936				3.300	3.689			
C. V. %							6.21	6.15	8.39				5.38	9.77				9.48	9.30			

All the treatments were analyzed by DNMRT and treatment means with same letters were at par at 5% level of significance.

SI1 and SI14 to SI36 (Full WPM). SI2 to SI13 (Modified WPM - Half of the suggested concentrations of the macronutrients).

PGRs – Plant Growth Regulators. G – Green. Y – Yellow. G + Y - Greenish yellow. C – Compact callus texture, F - Friable callus texture. ND- Not detected. NR - No response. + Poor, ++ Moderate, +++ Healthy, ++++ Very healthy