Effect of Media Combination on in *vitro* Callus Induction in Sandalwood (*Santalum album* L.)

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Abstract

Present investigation aimed at standardizing media combinations for *in vitro* callus of *Santalum album* was conducted in Factorial Completely Randomized Design (FCRD) at Plant Biotechnology Centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Ratnagiri, Maharashtra, India, during 2014-2016. The different media combinations created variable response for callus establishment. Early callus induction observed in shoot explant (21 days) as compared to leaf explant (22 days). Callus establishment was maximum (67%) in leaf explant and performed better on M.S with 0.5 mg l^{-1} 2,4-D+ 0.5 mg l^{-1} NAA+ 0.5 mg l^{-1} BAP in 22 days. Whereas, in shoot explant, maximum callus induction frequency observed was 46.67%. Medium MS+ 0.5 mg l^{-1} 2,4-D+ 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} BAP observed to be the best media combination for establishment in leaf explant on which induction of callus observed in 23 days with maximum frequency of 57%. The current investigation showed efficient callus establishment capabilities of *Santalum album* from leaf and shoot explants.

Keywords : *Santalum album*, callus establishment, callus induction.

Introduction

Sandalwood (*Santalum album* L.) is a valuable tree associated with Indian culture. It is the second most expensive wood in the world. *Santalum album* L. belongs to the Santalaceae family. A medium-sized evergreen hemi-root parasitic tree is highly valued for its fragrant heartwood. The widely distributed and economically important genus *Santalum* consists of 29 species (Hewson and George 1984), which are xylem tapping root hemi-parasites belonging to the family Santalaceae.

Tissue culture or micro-propagation technique in forest trees is reported to be one of the most efficient method of micro-propagation and has got the advantage of producing several plantlets within a limited period to meet the ever increasing demand in reforestation activities (Sharma 1995). *In vitro* propagation of sandalwood was attempted as early as 1963. Induction of callus from mature endosperm on modified white's medium was reported but the callus did not proliferated further (Rangaswamy and Rao 1963). Induction of callus, differentiation of embryoids and subsequent development into plantlets from endosperm (immature seeds) has been reported by Lakshmi Sita *et al.* (1980). However, there is only one published report on shoot bud formation directly from *in vitro* cultured leaves for sandal (Mujib 2005). In woody species relatively little information is available on shoots formed directly on leaves without a callus phase (Preece *et al.* 1993).

Systematic study on the effects of combinations of plant growth regulators on morphogenesis is still insufficient which may overlook the potential combinations of certain plant growth regulators that are more suitable. Present study has thus been undertaken to develop a potential system of *In vitro* regeneration of sandalwood plant through callus culture and to understand the possible role of plant growth regulators in deciding morphogenic pathway.

Material and Methods

Plant material and surface sterilization: Leaves and axillary shoots of field-grown plants of *Santalum*

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album were collected from College of Forestry farm, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Ratnagiri, Maharashtra, India. Leaves were washed under running tap water for 30 minutes and placed into double distilled water containing Tween 20 (2%v/v), for 20 minutes to remove the adhering fine particles. The cleaned leaves were treated with 1% of Carbendazim® (BASF, Germany) for varied times in horizontal shaker at 30 rpm and treated with 70% (v/v) ethanol for different time spell, followed by a treatment of aqueous solution of 0.2% $HgCl_2$ for 10 minutes with initial vacuum of 100 psi. Finally, the leaves were subjected for 4-5 rinsing with sterile double distilled water (Table 1).

The shoots were washed thoroughly with a detergent solution and tap water. The washed material was then surface sterilized in 0.1% aqueous solution of mercuric chloride for 8 minutes and rinsed six times with sterile water. Following surface sterilization, all exposed ends of the explants were trimmed, and the remaining segment (~1–1.5 cm) was inoculated horizontally on the culture medium.

Callus induction: Surface-sterilized leaf and shoot explants (1–1.5 cm) were placed with the adaxial surface in contact with the callus induction media. The callus induction media were composed of basal MS medium (Murashige and Skoog medium, 1962) containing myo-inositol (100 mgl^{-1}), K_2SO_4 (990 mgl^{-1}), and sucrose (3%, w/v). These media were supplemented with different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mgl^{-1}) of 2,4 dichlorophenoxy acetic acid (2,4-D), different concentrations (0.5 and 1.0 mgl^{-1}) of Naphthalene acetic acid (NAA) and different concentrations of Indole acetic acid (IAA) (0.5 mgl^{-1}). The pH of the medium was adjusted to 5.8 with 1 N NaOH before adding agar (0.8% w/v). The same medium without plant growth regulators was used as a control. All cultures were incubated in a culture room maintained at 25±2°C temperature, 40–60% relative humidity, and under uniform light (1600 Lux) provided by fluorescent tubes (7200 K) over a light and dark cycle of 16/8 hours except for cases where specific physiological conditions were required. The rate of callus formation was determined after 2 weeks.

Sub-culturing: After 21 days incubation, established cultures were transferred to the callus proliferation medium i.e. MS medium containing 0.5 mgl^{-1} 2,4-D+ 0.5 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP and grown for three weeks (same light and temperature condition). The sub culturing was done for two times on same media composition, light and temperature conditions.

Weight of callus (mg) after 30 days of inoculation: Weight of callus induced from leaf and shoot explant was recorded 30 days after inoculation i.e. after second subculturing. The cultured tissue grown on solid medium were carefully removed from the culture vessel and made free from specks of agar that had adhered at the point of contact. Accumulated water was blotted out gently without squeezing the tissue. The tissue was then transferred onto preweighed aluminum foil, and the weight was determined on a single pan digital balance.

Statistical analysis

The studies were carried out in laboratoryunder well-defined conditions of the medium, growth, temperature and light. Factorial Completely Randomized Design (FCRD) was employed for the experiment and the data were analysed in SAS (Statistical Analysis System V 9.1)

Result and Discussion

Disinfection of explants: In the present investigation, two explants viz. leaf and shoot were exposed to various sterilizing agents (Table 1 and 2). Results clearly indicated that in leaf explant, treatment involving Tween 20 (2%) for 20 minutes, Carbendazim (1%) for 10 minutes, HgCl2 (0.2%) for 10 minutes and Alcohol (70%) for 1 minutes duration, recorded maximum aseptic culture establishment (86.67). These results are in accordance with Bele *et al.,* (2012). Whereas in shoot explant, treatment involving Tween 20 (2%) for 10 minutes and HgCl2 (0.2%) for 8 minutes, duration recorded maximum aseptic culture establishment (82%). These results are in accordance with those reported earlier by Rao and Bapat (1984).

Table 1 : Effect of surface sterilizing agents on aseptic culture establishment in sandalwood leaf explants.

Tr. No.	Treatment details	Time(min)	Per cent aseptic culture
T1	Control (DDW washing)	20	0.00 (0.00)
T2	Tween 20 (2%)	20	26.67 (31.07)
T3	Carbendazim (1%)	10	16.67 (24.05)
T4	HgCl2 (0.2%)	10	23.33 (28.86)
T5	Alcohol (70%)	1	15.00 (22.79)
T6	Tween 20 (2%) + Carbendazim (1%) + HgCl2 (0.2%) + 70% alcohol	15 +10 +10 +1	63.33 (52.74)
T7	Tween 20 (2%) + Carbendazim (1%) + HgCl2 (0.2%) + Alcohol (70%)	20 +10 +10 +1	86.67 (68.66)
T8	Tween 20 (2%) + Carbendazim (1%) + HgCl2 (0.2%) + Alcohol (70%)	20 +15 +10 +1	68.33 (55.77)
T9	Tween 20 (2%) + Carbendazim (1%) + HgCl2 (0.2%) + Alcohol (70%)	20 +10 +15 +1	51.67 (45.96)
T10	Tween 20 (2%) + Carbendazim (1%) + HgCl2 (0.2%) + Alcohol (70%)	20 +10 +10 +2	78.33 (62.29)
	SE (m) ±		1.02
	CD at 1%		4.10

(*Figures in parenthesis are arcsine values.)

Table 2 : Effect of surface sterilizing agents on aseptic culture establishment of sandalwood shoot explants.

Tr. No.	Treatment details	Time (min)	Per cent aseptic culture
T1	Control (DDW washing)	10	0.00 (0.00)
T2	Tween 20 (2%) + HgCl2 (0.1%)	20 + 8	38.33 (38.24)
T3	Tween 20 (2%) + HgCl2 (0.1%)	15 + 8	35.00 (36.27)
T4	Tween 20 (2%) + HgCl2 (0.1%)	10 + 8	81.66 (64.69)
T5	Tween 20 (2%) + HgCl2 (0.1%)	5 + 8	21.66 (27.71)
T6	Tween 20 (2%) + HgCl2 (0.1%)	10 + 10	43.33 (41.16)
T7	Tween 20 (2%) + HgCl2 (0.1%)	10 + 12	36.66 (37.22)
T8	Tween 20 (2%) + HgCl2 (0.1%)	10 + 5	73.33 (58.93)
T9	Tween 20 (2%) + HgCl2 (0.1%)	10 + 3	65.00 (53.73)
T10	Tween 20 (2%) + HgCl2 (0.1%)	10 + 1	11.67 (19.88)
	SE (m) ±		0.95
	CD at 1%		3.83

(*Figures in parenthesis are arcsine values.)

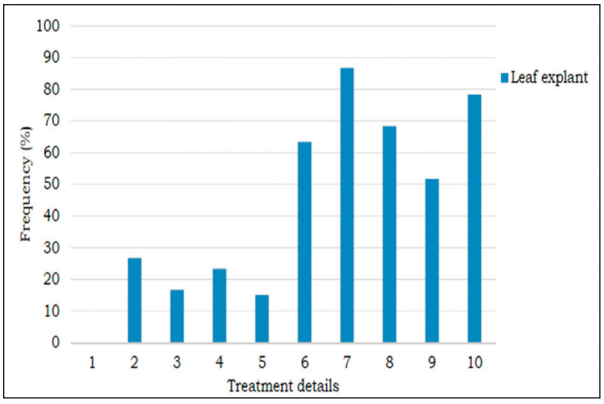

Fig. 1 : Effect of surface sterilizing agents on aseptic culture establishment of sandalwood leaf explants.

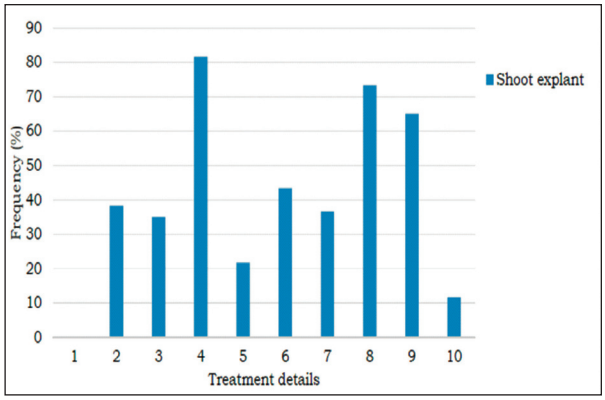

Fig. 2 : Effect of surface sterilizing agents on aspectic culture establishment of sandalwood shoot explants.

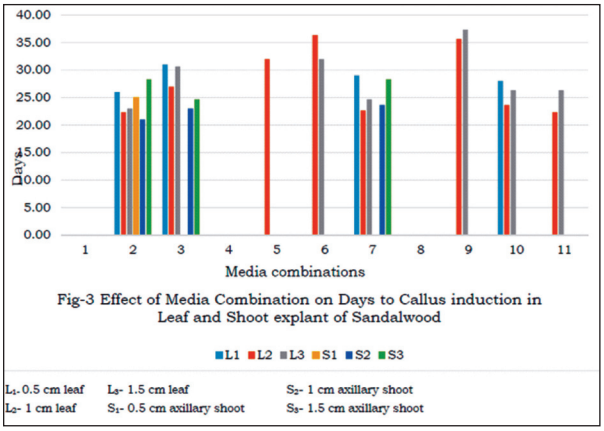

Fig. 3 : Effect of Media combination on days to Callus induction in Leaf and Shoot explant of Sandalwood.

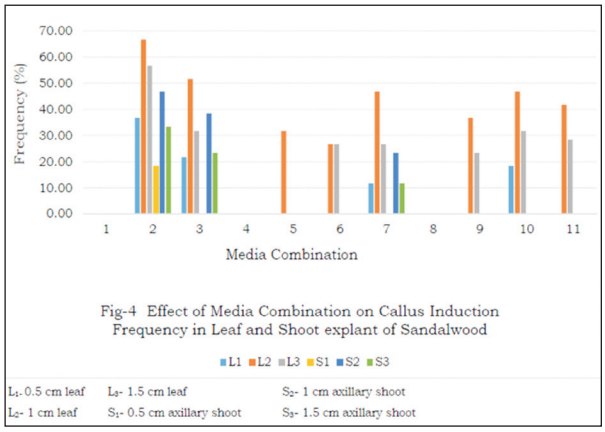

Fig. 4 : Effect of Media combination on Callus Induction Frequency in Leaf and Shoot explant of Sandalwood.

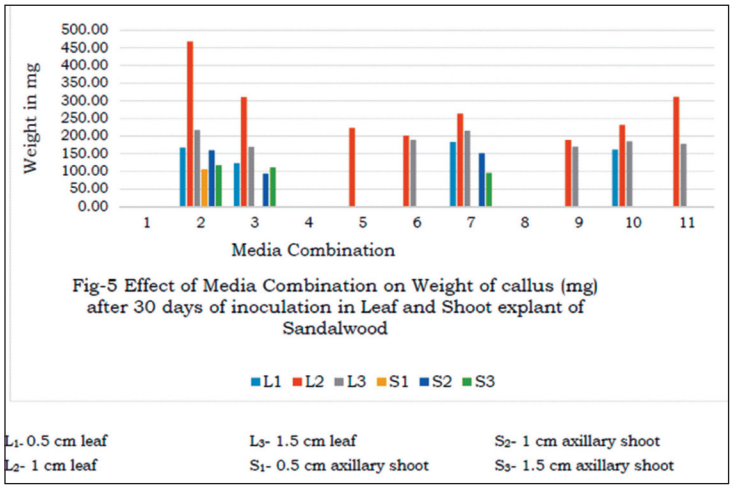

Fig. 5 : Effect of Media Combination on Weight of callus (mg) after 30 days of inoculation in Leaf and Shoot explant of Sandalwood.

Callus induction: Callus was initiated from the cut ends of leaf sections on Woody Plant Medium (WPM) media supplemented with different concentrations of 2,4-D NAA and IAA. After 2 weeks, the entire surface of the explants was covered with callus. In the present investigation, leaf explant showed highest (53.33 %) callus establishment on media combination M.S +0.5 mgl^{-1} 2,4-D+ 0.5 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP. Among leaf explants, explant L2 showed highest (67%) callus establishment on media combination M.S +0.5 mgl^{-1} 2,4-D+ 0.5 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP. The calli obtained were friable and translucent at the beginning. After 12 weeks of incubation the calli turned into brownish colour and lost the fragile texture, since the regenerative capacity of the cells became reduced or was lost totally after a certain period of time. Similar findings were also reported by Singh *et al.* (2012).

Similarly, shoot explant showed highest (33%) callus establishment on media combination M.S +0.5 mgl^{-1}

Table 3: Callus induction frequency in sandalwood.

Tr. No.	Treatment details	Explant							
		Leaf explant				Shoot explant			
		L1	L2	L3	Average	S1	S2	S3	Average
T1	MS medium (control)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T2	MS + 0.5 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	36.67 (37.26)	66.67 (54.74)	56.67 (48.83)	53.33 (46.90)	18.33 (25.30)	46.67 (43.09)	33.33 (35.25)	32.77 (34.92)
T3	MS + 1 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	21.67 (27.71)	51.67 (45.96)	31.67 (34.23)	35.00 (36.27)	0.00 (0.00)	38.33 (38.24)	23.33 (28.85)	20.55 (26.95)
T4	MS +1.5 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T5	MS + 2 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	0.00 (0.00)	31.67 (34.23)	0.00 (0.00)	10.55 (18.95)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T6	MS + 2.5 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	0.00 (0.00)	35.00 (36.27)	26.67 (31.07)	20.55 (26.95)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T7	MS + 0.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	11.67 (19.88)	46.67 (43.09)	26.67 (31.07)	28.33 (32.15)	0.00 (0.00)	23.33 (28.85)	11.67 (19.88)	11.66 (19.96)
T8	MS +1 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T9	MS + 1.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	0.00 (0.00)	36.67 (37.26)	23.33 (28.85)	20.00 (26.56)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T10	MS + 2 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	18.33 (25.30)	46.67 (43.09)	31.67 (34.23)	32.22 (34.58)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
T11	MS + 2.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	0.00 (0.00)	41.67 (40.20)	28.33 (32.14)	23.33 (28.88)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	SE (m) ±	0.77	0.76	0.81	8.16	0.40	0.57	0.67	4.87
	CD at 1%	3.11	3.06	3,27	32.82	1.60	2.28	2.69	19.58
	Range	0-36.67	0.66.67	0.56.67	0-53.33	0-18.33	0-46.67	0-33.33	0-32.77

(*Figures in parenthesis are arcsine values.)

2,4-D+ 0.5 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP, (Table 3). Among shoot explants, explant S2 showed highest (47%) callus establishment on media combination M.S +0.5 mgl^{-1} 2,4-D+ 0.5 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP. These results are in accordance with Muralidharan E. M. (1997). These could be attributed to the fact that many stimuli are communicated across the plant body by plant growth regulators which consequently play an important role in diverse aspects of plant growth and development. At a cellular level, auxin affects division, expansion and differentiation. Cytokinins are necessary in concerts with auxin in many cases for cell division at G1-S and G2-M transitions in a variety of cultured plant cells as well as in plants. (Deb and Arenmongla (2011).

Days to callus induction: Early induction of callus contributes significantly in deciding the success of micro-propagation in any species. In the present investigation number of days for callus induction ranged from 10 to 26 days in both explants. In leaf explant minimum number of days (10) to callus induction were recorded in treatment T2 M.S + 2 mgl^{-1} 2, 4-D + 0.5 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP. Among leaf explants, explant L2 showed minimum days (22) to callus induction in treatment T2 M.S +2 mgl^{-1} 2,4-D+ 0.5 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP, T7 MS + 0.5 mgl^{-1} 2,4-D + 1 mgl^{-1} NAA + 0.5 mgl^{-1} BAP and T11 MS + 2.5 mgl^{-1} 2,4-D + 1 mgl^{-1} NAA + 0.5 mgl^{-1}BAP. In shoot explant minimum number of days (17) to callus induction were recorded in treatment T2 M.S +0.5 mgl^{-1} 2,4-D+ 1 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP (Table 4). Among Shoot explants, explant S2 showed minimum days (21) to callus induction in treatment T2 M.S +0.5mg/l 2,4-D+ 1mg/l NAA+ 0.5mg/ l BAP. Muralidharan (1997) had also observed the difference in callus induction in Teak and other species.

Table 4 : Days required for callus induction in sandalwood.

Tr. No.	Treatment details	Explant							
		Leaf explant				Shoot explant			
		L1	L2	L3	Average	S1	S2	S3	Average
T1	MS medium (control)	0	0	0	0	0	0	0	0
T2	MS + 0.5 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	26	22	23	23	25	21	28	26
T3	MS + 1 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	31	27	30	29	0	23	24	15
T4	MS + 1.5 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	0	0	0	0	0	0	0	0
T5	MS + 2 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	0	32	0	10	0	0	0	0
T6	MS + 2.5 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	0	36	32	22	0	0	0	0
T7	MS + 0.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	29	22	24	25	0	23	28	17
T8	MS + 1 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	0	0	0	0	0	0	0	0
T9	MS + 1.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	0	35	37	24	0	0	0	0
T10	MS + 2 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	28	23	26	25	0	0	0	0
T11	MS + 2.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	0	22	26	16	0	0	0	0
	SE (m) ±	0.45	0.47	0.46	6.30	0.18	0.28	0.32	3.74
	CD at 1%	1.80	1.90	1.85	25.36	0.73	1.12	1.27	15.05
	Range	26-31	22-36	23.37	10-29	25	21-23	24-28	15-26

Footnote: L1- 0.5 cm leaf L2- 1 cm leaf L3- 1.5 cm leaf S1- 0.5 cm axillary shoot S2- 1 cm axillary shoot S3- 1.5 cm axillary shoot

Weight of callus after 30 days of inoculation: The weight of callus produced ranged from 68 mg to 284 mg. Better results of callus weight was recorded in leaf explant i.e. 283.89 mg on media combination of M.S +0.5mg/l 2,4-D+ 0.5mg/l NAA+ 0.5mg/l BAP, as compared to shoot explant in which maximum weight of callus was observed i.e. 118 mg (Table 5). Among leaf explants, explant L2 showed maximum callus weight i.e. 467.67 mg on media combination of M.S +0.5 mgl^{-1} 2,4-D+ 0.5 mgl^{-1}NAA+ 0.5 mgl^{-1} BAP and in shoot explants, explant S2 showed maximum callus weight i.e. 159.67 mg on media combination of M.S +0.5 mgl^{-1} 2,4-D+ 0.5 mgl^{-1} NAA+ 0.5 mgl^{-1} BAP. These results are in accordance with Haque *et al.* (2009)**.** These could be attributed to the fact that optimum concentration of growth regulators induces better callus growth which ultimately leads to increase in the weight of callus.

Table 5 : Weight of callus (mg) after 30 days of inoculation in sandalwood.

Tr. No.	Treatment details	Explant							
		Leaf explant				Shoot explant			
		L1	L2	L3	Average	S1	S2	S3	Average
T1	MS medium (control)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T2	MS + 0.5 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	167.00	467.67	217.00	283.89	104.33	159.67	118.00	127.33
T3	MS + 1 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	124.00	311.00	169.00	201.33	0.00	93.00	111.00	68.00
T4	MS + 1.5 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T5	MS + 2 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	0.00	222.67	0.00	74.22	0.00	0.00	0.00	0.00
T6	MS + 2.5 mg/l 2,4-D + 0.5 mg/l NAA + 0.5 mg/ l BAP	0.00	200.33	189.67	130	0.00	0.00	0.00	0.00
T7	MS + 0.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	183.33	262.67	214.67	220.22	0.00	150.67	95.33	82.00
T8	MS + 1 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
T9	MS +1.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	0.00	188.67	170.67	119.78	0.00	0.00	0.00	0.00
T10	MS + 2 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	161.67	231.67	185.33	192.89	0.00	0.00	0.00	0.00
T11	MS + 2.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/ l BAP	0.00	311.67	178.67	163.44	0.00	0.00	0.00	0.00
	SE (m) ±	0.57	0.93	0.76	52.13	0.28	0.54	0.57	18.43
	CD at 1%	2.28	3.75	3.06	205.73	1.12	2.16	2.28	74.16
	Range	0-183.33	0-467.67	0-217.00	0-283.89	0-104.33	0-159.67	0-118.00	0-127.33

Conclusion

Among different media combinations tried in sandalwood, M.S. with 0.5mg/l 2, 4-D+ 0.5mg/l NAA+ 0.5mg/ l BAP, proved to be the best media combination for callus induction. Weight of callus recorded maximum after second subculture in leaf explant than shoot explant. Shoot explant after second subculture did not show better callus growth. The results obtained from the present investigations clearly indicate that the standardized callus culture technique will provide a successful and rapid technique that can be successfully used for mass *in vitro* propagation of elite species like sandalwood. Furthermore, these techniques may be used for production of secondary metabolites and may also be used in creation of variability among sandalwood plant. In future, there is a need to standardize regeneration protocol from this developed callus culture technique.

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