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High Frequency Shoot Regeneration from Internodal Explants of *Santalum album* L.

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Abstract: An efficient plant regeneration protocol was developed for *Santalum album* L. (Santalaceae), an economically important species. Plant regeneration was achieved using internodal explants on Murashige and Skoog (MS) medium. Effect of Plant Growth Regulators (PGR) like 6-Benzyl Adenine (BA), Kinetin (KN) and 2-Isopentenyl adenine (2-iP) on shoot multiplication; 2-Isopentenyl adenine supplemented with varying concentrations of Coconut Milk (CoM) for shoot elongation and Indole-3-Butyric Acid (IBA) and α-Naphthalene Acetic Acid (NAA) on rooting was studied. Multiple shoots of *S. album* were significantly induced on internodal explants cultured on MS medium supplemented with 2-isopentenyl adenine (2iP) and Coconut Milk (CoM). The highest shoot multiplication was achieved on MS medium containing 1.0 mg L^{-1} (2iP) showed better growth response and produced 41.0±2.0 shootlets with an average length of 2.90±0.10 cm after 45 days of culture. The cluster of shoots were cultured on same media with 10% coconut Milk (CoM) showed shoot elongation up to 4.6 cm. Roots were induced after transfer to half strength MS medium supplemented with 0.5 mg L^{-1} IBA and 0.25 mg L^{-1} NAA produced 4.2±0.10 roots with an average height of 4.8±0.2 cm after six weeks in culture. The rooted plantlets were transferred for hardening, 70% of plantlets survived and resumed growth in the mixture of soil, vermiculite and farm yard manure (1:1:1).

Key words: *Santalum album,* coconut milk, 2-isopentenyladenine, intermodal explants, micro propagation

INTRODUCTION

Santalum album L. is an important tree species cultivated in a wide range of areas because of its wide applications. It belongs to the family Santalaceae. The Indian sandalwood (*S. album* L.), one of the commercially important species produces essential oil in the heartwood which is used extensively in the incense and perfumery industries (Rao and Bapat, 1995). Sandalwood harvesting, usually involves removal of the entire tree resulting in a critical loss of genetic diversity and valuable agronomic characters. Also due to non-availability of sufficient quality planting materials, commercial plantations of this important aromatic and medicinal species have not been widely attempted and presently only the wild population is exploited for extraction purposes. Hence, there is an urgent need for efficient plant regeneration protocol to be developed.

Conventionally this species is propagated either by vegetative means or by seeds. In vegetative propagation success rate is always very low and time consumption is an important factor in vegetative propagation. Since sandal is cross pollinated seeds show a greater degree of heterozygosity, naturally genetic uniformity is not maintained. Hence there is an urgent need to develop alternative propagation techniques to fulfil the current requirement and also to conserve this endangered species immediately (Rugkhla and Jones, 1998). *In vitro* multiplication of *S. album* using nodal, hypocotyl, endosperm and *in vitro* leaves has been reported (Rao and Bapat, 1992; Mujib, 2005; Surajit *et al.,* 1998). However, *in vitro* regeneration from internodal explant has not been reported so far. This is the first report to develop a simple, rapid, economical and high-frequency regeneration protocol from internodal explants of *Santalum album*, for large scale propagation.

MATERIALS AND METHODS

Plant material: *Santalum album* plants were collected during the middle of February 2010 from the field area of Vandalur Forest Research Institute (VFRI), Chennai, Tamil Nadu, India. The healthy seeds were collected from VFRI and seeds were cleaned in tap water, the cleaned seeds were stored at 4°C in Department of Biotechnology of our College.

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Explant preparation: The seeds collected from 8 year old mature plants of *S. album* were cleaned thoroughly under running tap water for 20 min washed with a solution of Tween 20 (2 drops in 100 mL of water) for 1 min and again washed with sterile distilled water. The cleaned explants were finally treated with 0.1% (w/v) $HgCl_2$ for 5 min under aseptic conditions and washed 4 times with sterile distilled water to remove traces of $HgCl_2$.

Germination and plant development: After surface sterilization, *S. album* seeds without pericarp were separated and transferred individually and inoculated on MS (Murashige and Skoog, 1962) basal medium supplemented with different concentrations of Benzyl adenine BA (0.5-2.5 mg L^{-1}) and Gibberellic acid GA_3 (0.5 mg L^{-1}) for germination of plants from the seeds.

Shoot multiplication and elongation: Young and immature internodal explants obtained from aseptically germinated seeds (two months old) were used for primary inoculation. Internodal explants were trimmed to 0.8-1.0 cm and inoculated on MS basal medium supplemented with individual concentration of 2iP (0.25-2.5 mg L^{-1}), BA (0.25-2.5 mg L^{-1}) and KN (0.25-2.5 mg L^{-1}) for shoot induction. Additionally coconut milk was added to the medium in different concentrations (5.0, 10.0 and 15.0%) to induce shoot elongation.

Rooting and acclimatization: The proliferated shootlets were transferred to half strength MS medium supplemented with IBA and NAA (0.1-1 mg L^{-1}) for root development. Shootlets of 4.0-4.5 cm length were used for rooting experiments. Root number and root length were recorded after five weeks in culture. For *ex vitro* establishment, well rooted plantlets were rinsed thoroughly with sterile water to remove residual nutrient from the plant body and transplanted to paper cups containing a mixture of red soil, vermiculite and farm yard manure in a 1:1:1 ratio. The rooted plantlets were transferred to green house under 60-70% humidity. The plantlets were fertigated with dilute MS basal media. After 15 days, the fully acclimatized plantlets were transferred to pots (6 cm dia) containing red soil, vermiculite and farmyard manure in 1:1:1 ratio. Established plantlets were then transferred to bigger pots (14 cm dia).

Culture medium and conditions: MS basal medium containing 3% sucrose was used for all *in vitro* culture studies. The pH of the medium was adjusted to 5.6±0.2 prior to adding 0.9% agar, and autoclaved at 121°C at 15 psi for 15 min. The explants were implanted in to 15 mL aliquots of agar MS medium in 25×150 mm culture tubes, in such a way that the internodes were in contact with the surface of the medium. All the cultures were maintained at 25±1°C under 16/8 h light and dark cycle. Lighting was provided using white cool fluorescent tubes of 40 μmol/m^2/s light intensity with 55-60% relative humidity. The plant growth regulators were filter-sterilized using 0.2 μm filter (Minisart ®, Sartorius) prior to addition to culture media.

Statistical analysis: Each experiment was repeated three times and each treatment had six replicates. The data were analyzed using analysis of variance (ANOVA) and the means were compared using the Duncan's Multiple range Test (DMRT) using SPSS (SPSS version 16.0) at 5% level of significance ($p<0.05$).

RESULTS AND DISCUSSION

The *in vitro* seedlings were developed from seed explants inoculated on MS medium containing BA (0.5-2.0 mg L^{-1}) in combination with GA_3 (0.5 mg L^{-1}). Seed germination in most of the treatment was recorded within 3 weeks of culture (Fig. 1a,b). The entire seedling developed on MS basal medium supplemented with 1.0 mg L^{-1} BA+0.5 mg L^{-1} GA_3 showed significant growth response of 71.6±2.8% germination with an average shoot length of 6.43±0.05 cm and an average root length of 3.76±0.25 cm (Table 1) and healthy seedlings were developed after 40 days of culture (Fig. 1c, d). The *in vitro* seedlings were used for further experimental studies.

Multiple shoots developed from internodal explants cultured on MS medium supplemented with 2iP, BA and KN (0.25-2.5 mg L^{-1}) and fortified with various concentrations of coconut milk (5.0, 10.0 and 15.0%) showed differential response according to the hormonal concentration used. Initiation of multiple shoots in most of the treatments was observed within 3 weeks of culture in application of 2iP (Fig. 2a,b,c). High number of multiple shoot proliferation from internodal explants was observed in MS medium containing 2iP (1.0 mg L^{-1}) produced

Table 1: *In vitro* seedlings developed from seeds of *Santalum album*

PGR(s) (mg L^{-1})				
BA	GA_3	Germination response (%)	Mean shoot length (cm)±SD	Mean root length (cm)±SD
0.5	0.5	$43.3±5.7^{ab}$	$3.86±0.20^{bc}$	$2.03±0.15^{a}$
1.0	0.5	$71.6±2.8^{c}$	$6.43±0.05^{d}$	$3.76±0.25^{b}$
1.5	0.5	$53.3±2.8^{b}$	$4.36±0.50^{c}$	$2.43±0.11^{a}$
2.0	0.5	$48.3±5.7^{ab}$	$3.33±0.15^{ab}$	$2.10±0.52^{a}$
2.5	0.5	$40.0±5.0^{a}$	$2.90±0.10^{a}$	$1.86±0.11^{a}$

BA-6: Benzyl adenine, GA_3: Gibberellic acid. Results represent Mean±SD of three replicated experiments and data were recorded after 40 days of culture. Values denoted by different letters differ significantly at $p<0.05$ level using Duncan's Multiple Range Test (DMRT)

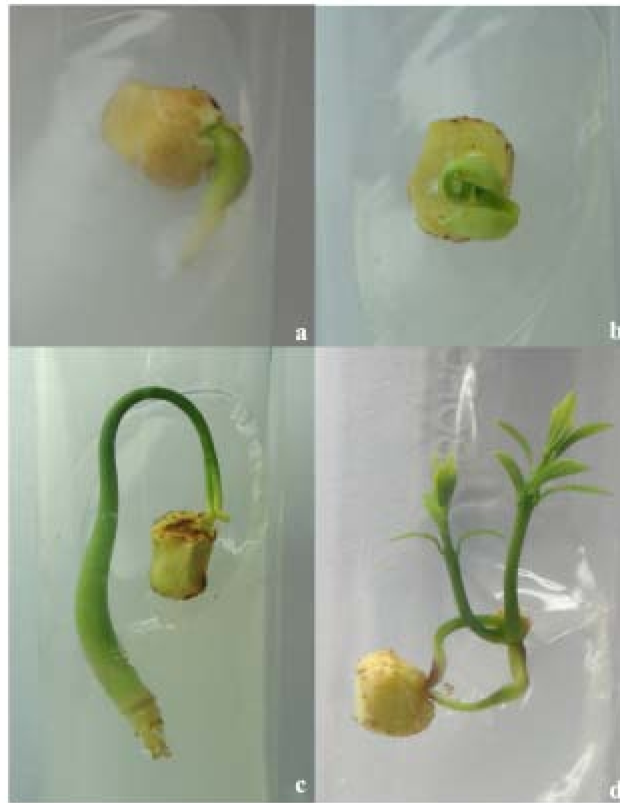

Fig. 1 (a,d): *In vitro* seedling developed from *Santalum album* seeds. A, B) Seeds without pericarp inoculated on MS medium supplemented with 4.44 μM BA+1.44μM GA3. (c, d) *In vitro* germinated seedling developed from *Santalum album* seeds

71.6±2.8% response and 41.0±2.0 shoots per explant with an average shoot length of 2.9±0.10 cm (Table 2) after 45 days of culture. Higher concentration of 2iP (2.0 mg L^{-1}) resulted in gradual decrease in the number of shoots per explants. The cluster of shootlets cultured on same media supplemented with 10% coconut milk showed significant increase in shoot length (Fig. 2d). The average shoot length with 10% coconut milk (CoM) was recorded to be 4.63±0.40 cm (Table 3).

Individual shoots from a multiple shoot complex were separated after 45 days of culture on shoot developed medium and transferred to half strength MS medium supplemented with IBA and NAA (0.1-1 mg L^{-1}). In all media, the first roots appeared after 4 weeks of culture and after 6 weeks the root system was well developed (Fig. 2 e, f). The significant increase in the rooting response (75.0±5.0%) was recorded on medium supplemented with 0.5 mg L^{-1} IBA and 0.25 mg L^{-1} NAA with an average number of 8.0±1.0 rootlets per shootlet, with an average root length of 4.8±0.2 cm with 45 days of culture (Table 4). This could be attributed to the nature of *in vitro* shootlets from internodal explants grown on 2iP.

Table 2: Effect of different concentration of cytokinins (BA, KN and 2iP) on *in vitro* shoot multiplication from internodal explants of *Santalum album*

PGR(s) (mg L^{-1})	Percentage response	Mean number of shoots/explant±SD	Mean shoot length (cm)±SD
BA			
0.25	28.3±2.9^{abc}	1.67±0.5^{a}	1.40±0.10^{a}
0.5	40.0±5.0^{defgh}	3.00±0.0^{ab}	2.10±0.20^{de}
1.0	43.3±5.7^{fgh}	4.00±1.0^{c}	2.33±0.15^{ef}
1.5	23.3±5.8^{ab}	2.33±1.2^{ab}	2.23±0.05^{ef}
2.0	-	-	-
KN			
0.25	21.7±2.8^{a}	1.67±0.6^{a}	1.50±0.10^{ab}
0.5	33.3±5.7^{cde}	3.00±1.0^{ab}	2.50±0.20^{f}
1.0	46.6±2.8^{gh}	3.33±1.1^{ab}	1.90±0.10^{cd}
1.5	45.0±8.6^{fgh}	2.67±1.1^{ab}	1.73±0.12^{bc}
2.0	31.7±2.8^{bcd}	2.00±0.0^{a}	1.40±0.10^{a}
2iP			
0.25	45.0±8.6^{fgh}	2.67±1.2^{ab}	2.16±0.11^{de}
0.5	50.0±5.0^{h}	8.00±1.0^{c}	2.30±0.20^{ef}
1.0	71.6±2.8^{i}	41.0±2.0^{d}	2.90±0.10^{g}
1.5	38.3±7.6^{cdefg}	3.00±1.0^{ab}	1.57±0.21^{ab}
2.0	35.0±5.0^{cdef}	2.33±0.5^{ab}	1.40±0.26^{a}

BA-6: Benzyl Adenine, KN: Kinetin, 2iP: 2-Isopentenyl adenine. Results represent Mean±SD of three replicated experiments and data were recorded after 45 days of culture. Values denoted by different letters differ significantly at $p<0.05$ level using Duncan's multiple range test (DMRT)

Fig. 2 (a,i): Multiple shoots regeneration from internodal explants of *Santalum album*. (a) Internodal explants inoculated on MS medium supplemented with 2ip 1.0 mg L^{-1}. (b) Initiation of shoot from internodal explants after two weeks of culture. (c, d) Proliferation of multiple shoots from internodal explants at 45 days of cultured on MS medium containing 2ip 1.0 mg L^{-1} with 10% coconut milk. (e) Healthy *in vitro* shootlets inoculated on half strength MS medium containing IBA 0.5 mg L^{-1} and NAA 0.25 mg L^{-1}. (f) A well established plant. (g) Well established and hardened in *vitro* plants successfully transferred to the paper cups. (h,i) Hardened plants transferred to external environment condition showing luxurious growth

Table 3: Effect of Coconut Milk (CoM) on elongation of *in vitro* microshoots of *Santalum album*

Concentration of coconut milk (%)	Mean No. of shoots/explan±SD	Mean shoot length (cm)±SD
Control	41.0±2.0	2.09 ± 0.05^a
5	41.0±2.0	3.06 ± 0.25^a
10	41.0±2.0	4.63 ± 0.40^b
15	41.0±2.0	3.43 ± 0.57^a

Control- MS medium with 2iP 1.0 mg L^{-1}. Results represent Mean±SD of three replicated experiments Data were recorded after 45 days of culture Values denoted by different letters differ significantly at $p<0.05$ level using Duncan's Multiple Range Test (DMRT)

Eighty percent plantlet survival was seen after hardening of the regenerated *S. album* in red soil, vermiculite and farmyard manure (1:1:1) for 3 weeks. However, the rate decreased as some plants died over the next 4- 5 weeks after transfer to soil. It was observed that very gradual acclimatization of *in vitro* grown plants to the external environment is most essential to *S. album.* Seventy percent of the plants transferred to pots survived and resumed growth (Fig. 2g, h, i).

2iP has been considered to be one of the most active cytokinins in organogenic differentiation in plant tissue culture (Butiuc-keul and Deliu, 2001; Pereira, 2006; Ondruskova *et al*., 2006). Even in *S. album* the role of TDZ and BAP has been reported earlier but the present investigation clearly demonstrated the supremacy of 2iP which is in agreement with the report of *Leucaena leucocephala* (Maity *et al*., 2005). The overall number of shoots in this study from internodal explants recorded 41.0±2.0 shoots on MS medium containing 2iP (1.0 mg L^{-1}) produced was significantly higher than the other concentrations. An earlier study reported on the production of shoots from nodal explants (Sita *et al*.,

Table 4: Effect of different concentration of auxins (IBA, NAA) on rooting response of *Santalum album*

PGR (s) (mg L^{-1})		Percentage	Mean number of roots/	Mean root length
IBA	NAA	rooting	shoot±SD	(cm)±SD
0.1		30.00 ± 5.00^{bc}	$2.67\pm0.58a^{bcde}$	2.10 ± 0.53^{bc}
0.25		31.67 ± 2.89^{bcd}	2.67 ± 1.15^{abcde}	1.93 ± 0.12^{b}
0.5		60.00 ± 5.00^{g}	4.00 ± 1.00^{e}	3.40 ± 0.10^{f}
1.0		20.00 ± 5.00^{a}	1.67 ± 0.58^{ab}	1.40 ± 0.10^{a}
	0.1	-	-	-
	0.25	45.00 ± 5.00^{ef}	2.67 ± 0.58^{abcde}	2.43 ± 0.12^{cd}
	0.5	36.67 ± 2.89^{cd}	2.00 ± 1.00^{abc}	2.40 ± 0.10^{cd}
	1.0	25.00 ± 0.00^{ab}	1.67 ± 0.58^{ab}	1.40 ± 0.17^{a}
0.5	0.25	30.00 ± 5.00^{b}	3.67 ± 0.58^{de}	2.43 ± 0.12^{cd}
	0.5	33.33 ± 5.77^{cd}	3.00 ± 1.00^{bcde}	3.77 ± 0.25^{g}
	1.0	46.67 ± 2.89^{f}	3.33 ± 1.15^{cde}	3.43 ± 0.06^{f}
0.5	0.25	75.00 ± 5.00^{h}	8.00 ± 1.00^{f}	5.77 ± 0.21^{h}
	0.5	35.00 ± 0.00^{cd}	3.67 ± 0.58^{de}	3.97 ± 0.06^{g}
	1.0	25.00 ± 5.00^{ab}	3.00 ± 0.00^{bcde}	2.87 ± 0.15^{e}
1.0	0.25	38.33 ± 2.89^{ed}	3.00 ± 1.00^{bcde}	2.97 ± 0.21^{e}
	0.5	30.00 ± 0.00^{bc}	2.33 ± 0.58^{abcd}	2.67 ± 0.12^{de}
	1.0	25.00 ± 5.00^{ab}	1.33 ± 0.58^{a}	1.87 ± 0.12^{b}

IBA: Indole-3-Butyric Acid, NAA-α: Naphthalene Acetic Acid. Explants were cultured on half-strength MS media supplemented with IBA and/or NAA. Data were recorded after 45 days of culture. Results represent Mean±SD of six replicated experiments. Values denoted by different letters differ significantly at $p<0.05$ level using Duncan's Multiple Range Test (DMRT)

1979), hypocotyl (Bapat and Rao, 1979) and *in vitro* leaves (Mujib, 2005) of S. album. The development of micro shootlets and the leafy shoot emergence failed when the internodal explants were cultured on MS medium supplemented with individual concentrations (0.25 - 2.0 mg L^{-1}) of BA and KN but the application of 2iP at lower concentration proved to be effective. In *S. album* herewith reported supremacy of 2iP than other growth regulators like BAP and KN. Similar results have been reported that the role of 2iP in leaf explants of *Garcinia mangosta* and *Pinus strobus* (Goh *et al.,* 1990; Flinn *et al.*, 1986).

Coconut milk is an undefined complex mixture of organic substances that has been successfully employed for culturing different plant species (Arditti and Ernst, 1993; Suttle, 1996; Janarthanam and Sumathi, 2010). It is evident in their report that coconut milk stimulated the development of shoot buds. In general, CoM is known to promote callus formation and also plays a suppressive role in the formation of shoots buds. However, in our investigation the results were contrary to the general trends and coincide with the findings of Maity *et al.* (2005). Although, exogenous 2iP is regarded as the most crucial factor for the direct induction of shoot buds and the response is enhanced with addition of coconut milk.

CONCLUSION

The results showed the ability of the internodal explants to produce higher number of shootlets without any intervening callus phase, where all the plantlets were uniform in height and growth. Hence, we propose this protocol a simple, economical, rapid and highly reproducible to obtain more plantlets within a short period of time.

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