Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(6): 892-896
Received: 19-09-2017
Accepted: 20-10-2017

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Studies on *in vitro* regeneration of Sandalwood (*Santalum album* Linn) from Leaf disc explant

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Abstract
An Investigation has been conducted on *in vitro* regeneration of *Santalum album Linn* from Leaf disc explant at the Biotechnology-cum-Commercial Tissue Culture Center, OUAT, Bhubaneswar, during the year 2015-16.The results revealed that leaf discs treated with 0.1% $HgCl_2$ for 6 minutes was the best treatment for sterilization as it effectively reduced the percentage of fungal contamination (3.33), bacterial contamination (3.33), higher percentage of aseptic culture (93.33) and high survival percentage (86.67). It reduced the days callus initiation (8.33) with good callus spread. On subculture of the calli mass on MS medium fortified with different combinations of BAP (1.5 mg/l, 2.0 mg/l, 2.5 mg/l), NAA (0.5 mg/l), IAA (0.5 mg/l) no further development of has been recorded and the calli mass turned brown and died in the due course of time. Hence, it needs further research for obtaining quality plantlets in *Santalum album* Linn.

Keywords: Bacterial, contamination, fungal, sandalwood.

Introduction
The word, "sandalwood" in English, or "Chandan" in Hindi, evokes a world of ancient mystery, sanctity, and devotion. Sandalwood (*Santalum album*) has been part of Indian culture and heritage for thousands of years and was one of the first items traded with other countries. The heartwood yields fragrant oil, which is used mainly in the perfume industry but also has medicinal properties. The wood is used for carving and manufacturing incense. Generally, *S. album* is found in the dry deciduous forests of the deccan plateau, mostly in the states of Karnataka and Tamil Nadu, The evergreen tree regenerates naturally when conditions are favourable and have been spreading in its distribution.

Sandalwood is the name of a class of woods from trees in the genus *Santalum*. The woods are heavy, yellow, fine-grained, and unlike many other aromatic wood, they retain their fragrance for decades. Sandalwood oil is extracted from the woods for use. Both the wood and the oil produce a distinctive fragrance that has been highly valued for centuries. Consequently, species of these slow-growing trees have suffered over-harvesting in the past century. Sandalwoods are medium-sized hemiparasitic trees and part of the same botanical family as European mistletoe. Notable members of this group are Indian sandalwood (*Santalum album*) and Australian sandalwood (*Santalum spicatum*).

Santalum album is an important tree species belonging to family Santalaceae. The height of the evergreen tree is between 4 and 9 meters. The plant parasitizes the roots of other tree species, with a haustorium adaptation on its own roots, but without major detriment to its hosts. An individual will form a non-obligate relationship with a number of other plants. Up to 300 species (including its own) can host the tree's development supplying macronutrients phosphorus, nitrogen and potassium, and shade especially during early phases of development. It may propagate itself through wood suckering during its early development, establishing small stands. The reddish or brown bark can be almost black and is smooth in young trees, becoming cracked with a red reveal. The heartwood is pale green to white as the common name indicates. The leaves are thin, opposite and ovate to lanceolate in shape. This tree is native to semi-arid of the Indian subcontinent. It is now planted in India, China, Sri Lanka, Indonesia, Malaysia, the Philippines and Northern Australia.

International market volume of $ 1 billion, India accounts for 85% of this as the foremost producer and consumer Sandal is intricately linked to Indian culture, heritage and literature and it is depicted in epic Ramayana (2000 BC), Kautilya's Arthasastra (200 BC) and Kalidasa's (170 BC) works Sandalwood and its oil were among the first items traded along with spices and silk to the middle east and other countries.

The Indian sandalwood, one of the commercially important species produces essential oil in the heartwood which is used extensively in the incense and perfumery industries [1]. Sandalwood essential oil was popular in herbal medicine up to 1920–1930, mostly as a urogenital (internal) and skin (external) antiseptic. Its main component is santalol (about 75%). It is used in aromatherapy and to prepare soaps.

Producing commercially valuable sandalwood with high levels of fragrance oils requires *Santalum album* trees to be a minimum of 15 years old (*S. album*) the age at which they will be harvested Sandalwood is expensive compared to other types of woods, therefore to maximize profit sandalwood is harvested by removing the entire tree instead of sawing it down at the trunk close to ground level. This way wood from the stump and root, which possess high levels of sandalwood oil, can also be processed and sold.

Lack of understanding of the dynamics of hemiparasitism by sandalwood has caused failure of pure plantations in the past; haustorial connections with its hosts supply sandalwood with nitrogen, phosphorus, and potassium [2]. In 1998, this species was recognized as "vulnerable" by the International Union for the Conservation of Nature's (IUCN) Red List, due to disease, fire (to which the sandalwood trees are extremely sensitive), and exploitation through illegal activity.

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 material, commercial plantations of this important aromatic and medicinal species have not been widely attempted and presently only wild population is exploited for the extraction process. Hence there is an urgent need for efficient plant regeneration protocol to be developed.

Conventionally this species is propagated either by means or by vegetative means. Sandalwood is cross pollinated plant and seeds show greater degree of heterozygosity for which naturally genetic uniformity is not maintained. Hence, there is an urgent need to develop alternative propagation techniques to enhance the reproduction of the quality planting materials of sandalwood trees. Few works have been reported in *in vitro* multiplication of *S. album* using nodal, hypocotyl, endosperm and *in vitro* leaves have [3]. However, a systematic study on the effects of combination of Plant growth regulators on morphogenesis is still insufficient which may overlook the potential the combination of certain plant growth regulators that are more suitable. *In vitro* techniques are widely accepted to produce virus free plants in a very large number for production of virus free plantlets was a challenge in this technique. Maintaining an aseptic or sterile condition has been identified as essential in successful tissue culture procedure [4]. The desire of every researcher in tissue culture studies is to eliminate or prevent contamination, but unfortunately contamination cannot be eliminated totally but can only be managed to reduce both the frequency and occurrence, this can be achieved by chemical sterilization [5]. Chemical sterilization is effective and cheap for getting contamination free culture, determination of appropriate exposure time in order to standardize the use of sterilants to minimize the contamination and to minimize the injury for better result so keeping in view the importance and high demand for these tree species the present, experiment was carried out to find out the best timing for surface sterilization with 0.1% $HgCl_2$ and to standardize the plant bioregulators for regeneration of plantlets.

Materials and Methods

The investigation was carried out at the Biotechnology-cum-Tissue Culture Center, OUAT, Bhubaneswar. The chemicals used for the present study were analytical reagents of excel R grade of Merck (India), Qualigen fine Chemicals, and Himedia Laboratories Ltd. (India). Auxins, Cytokinins, Myo-inositol and Fe-EDTA were from Sigma (USA) and Agar from Himedia Lab Ltd (India). For the preparation of MS culture [6] required quantities of macronutrients, micronutrients, Fe-EDTA, vitamins and plant bio regulators were taken from the stock solution and required quantity of sucrose dissolved in water was added fresh to the medium. The pH of the solution was adjusted to 5.7± 0.1 using 0.1N NaOH or 0.1 N HCl. Then volume was made up to 1 liter of distilled water. Agar (0.6% w/v) was added to the medium boiled and poured to the culture tubes and plugged with non-absorbent cotton. Plugged culture tubes containing culture medium were autoclaved for the 20 minutes at 121^0C and 15 Psi pressure. The autoclaved medium was kept in a laminar air flow bench for cooling. All the glassware were dipped in the detergent solution for overnight and washed under running tap water. They were rinsed with distilled water and then dried in an oven for 2hrs at 150^0C. Forceps, petridishes and scaples were thoroughly cleaned with isopropanol or rapped with paper and kept in a clean sterilized in the autoclave at 15 psi and 121^0C for 20 minutes. The working chamber of laminar air flow cabinet was wiped with isopropanol. Filtered air (80-100 cft/min) to ensure that particles do not settle in working area was blown for 5min. The sterilized materials to be used (except living tissue) were kept made the chamber and exposed to UV light for 30 minutes. While working, filtered air was continuously passed the laminar air flow cabinet. The explants of *Santalum album* Linn. were collected from the Arboretum of College of Forestry, Orissa University of Agriculture & Technology, Bhubaneswar. These were properly washed under running tap water for 30 minutes to remove all the dirt particles and impurities. After that, they were again washed with liquid detergent Tween 20 (Himedia Laboratories, India) for 10 minutes with vigorous shaking. After washing with detergent, the explants were again washed with running tap water to remove any traces of detergent for 30 minutes and kept at 1% and 0.5% w/v solution of Bavistin for 15 min. again they were washed in running tap water for 30 minutes. After that the explants were kept in a1 liter beaker covered with a sterilized lid. The explants (leaf discs) were surface sterilized with 0.1% $HgCl_2$ for the different time duration (2 min, 3 min, 4 min, 5 min 6 min, 7 min, 8 min, 9 min, 10 min and a control). The $HgCl_2$ treatment was always followed by washing the explants in sterile distilled water for 3-4 times to remove excess Hg^{++} ions and dried on autoclaved filter paper. All these were done in the laminar air flow chamber. Then the explants were inoculated into basal MS medium containing 2.0 mg/l, 2, 4-D and 1.0 mg/l BAP for initial culture establishment. Three replication per treatments and ten cultures per replication were used. Observation on the percentage of contamination, Percentage of aseptic culture, Percentage of explants death and Percentage of survival were recorded. After sterilization of explants with its best sterilant, they were further taken for callus regeneration and transferred to the MS medium containing different concentration of BAP (1.5, 2.0 and 2.5 mg/l) and 2, 4-D (1.0 and 1.5 mg/l) along with a control, there was three replication per treatments and ten cultures per replication were used. Observation on days to callus initiation, size of callus, callus colour and callus texture were recorded. After callus formation the calli mass was divided into 2-4 equal pieces with a sharp sterilized blade and

was carefully transferred to the shoot initiation media containing different concentration of cytokinins (BAP) and Auxins (IAA, NAA) and in combination with Adenine sulphate (100 mg/l) along with a control (MS alone). The data recorded for the experiments were analyzed following the method of Gomez and Gomez (1984)[7] using one way ANOVA in Completely Randomized Design (CRD). The significance and non- significance of the treatment effect were judged with the help of 'F' variance ratio test. Calculated 'F' value was compared with the table value of 'F' at 5% level of significance. The data were transferred from where ever required before suitability of Analysis of Variance (ANOVA) analyzed in statistical package SAS version 7.0.

Results and Discussion

Impact of timing of sterilant in leaf disc explants

The results of the experiment on timing of sterilant in leaf disc explants (Table.1) revealed that the leaf disc explants surface sterilised by 0.1% $HgCl_2$ for 6 minutes significantly reduced the percentage of fungal infection (3.33%) and the data stood at par with the treatment T_5 (0.1% $HgCl_2$ for 5 minutes), T_7 (0.1% $HgCl_2$ for 7 minutes), T_8 (0.1% $HgCl_2$ for 8 minutes) and T_9 (0.1% $HgCl_2$ for 9 minutes). Significantly maximum fungal infection 100% was recorded in control i.e. T_1. The result of bacterial infection showed non-significant results. In treatment T_1 (control) condition there is no bacterial infection as all the explants have died due to fungal infection. However lower percentage of bacterial infection (3.33) was recorded in treatment T_4 (0.1% $HgCl_2$ for 4 minutes), T_6 (0.1% $HgCl_2$ for 6 minutes). Aseptic culture was significantly higher (93.33%) with T_6 (0.1% $HgCl_2$ for 6 minutes) and the data stood par with T_7 (0.1% $HgCl_2$ for 7minutes), T_9 (0.1% $HgCl_2$ for 9 minutes), T_{10} (0.1% $HgCl_2$ for 10 minutes). There was no aseptic culture as all the explants have died due to fungal infection in treatment T_1 (control). Percentage of the death of the explants was significantly low (6.67) in T_6 (0.1% $HgCl_2$ for 6 minutes) and no results were at par with this value. Significantly highest number of dead explants were reported inT_9 (0.1% $HgCl_2$ for 9 minutes) and T_{10} (0.1% $HgCl_2$ for 10 minutes) (40%). There is no death of the explants naturally as all the explants have died due to fungal infection in treatment T_1 (control). The explants surface sterilised with by 0.1% $HgCl_2$ for 6 minutes recorded significantly higher percentage of survival (86.67%). Sterilization is a procedure for elimination of microorganisms[4]. Leaf disc were used in the present investigation as explants. Due to the presence of the tree in wild forest habitat hence, possibilities of presence of contaminants were higher. To make explants free from contaminants, surface sterilization with 0.1% $HgCl_2$ for 2,3,4,5,6,7,8,9,10 minutes along with a control were undertaken. There is a need of different time exposure. The results are in agreement with Rezadost *et al*., (2012), [8] who reported that surface sterility chosen for the an explant typically depends on explants type and plant species and time was depends on the type of tissue. Previously $HgCl_2$ has been used for surface sterilization in sandalwood by many workers Bele *et al.*, 2012 [9], Sanjay *et al.*, 2006 [10], Janarthanam *et al.*, 2011 [11], and Singh *et al.*, 2013 [12].

Table 1: Effect of sterilant on sterilization of leaf disc explants Duration- 30 days Basal media- MS +2,4-D (2.0 mg/l) + BAP (1.0 mg/l)

	Treatments	Fungal infection (%)	Bacterial infection (%)	Aseptic culture (%)	Death (%)	Survival (%)
T_1	Distilled water (controlled)	100(90.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)
T_2	0.1% $HgCl_2$ (2 min.)	56.67(48.84)	16.67(23.85)	36.67(37.22)	3.33(6.14)	30.00(33.21)
T_3	0.1% $HgCl_2$ (3 min.)	23.33(28.78)	6.67(12.29)	63.33(52.78)	16.67(23.85)	46.67(43.07)
T_4	0.1% $HgCl_2$ (4 min.)	20.00(26.56)	3.33(6.14)	73.33(59.00)	33.33(35.22)	36.67(37.22)
T_5	0.1% $HgCl_2$ (5 min.)	6.67(12.29)	3.33(6.14)	86.67(72.29)	26.67(30.99)	60.00(50.85)
T_6	0.1% $HgCl_2$ (6 min.)	3.33(6.14)	3.33(6.14)	93.33(77.71)	6.67(12.29)	86.67(68.66)
T_7	0.1% $HgCl_2$ (7 min.)	6.67(12.29)	10.00(18.43)	86.67(68.86)	23.33(28.78)	40.00(39.23)
T_8	0.1% $HgCl_2$ (8 min.)	10(18.43)	10.00(18.43)	80.00(63.93)	36.67(37.22)	43.33(43.25)
T_9	0.1% $HgCl_2$ (9 min.)	13.33(21.14)	6.67(12.29)	80.00(63.93)	40.00(39.23)	40.00(39.23)
T_{10}	0.1% $HgCl_2$ (10 min.)	16.67(23.85)	6.67(12.29)	83.33(66.14)	40.00(39.23)	36.67(37.22)
	SE(m)±	3.70	4.84	4.25	3.17	2.47
	CD (5%)	10.90	14.26	12.53	9.36	7.29

*values in the parentheses are the arc sine values

Impact of timing of sterilant on callus initiation and development of the leaf disc explants

The data presented in table.2 revealed that explants treated with 0.1% $HgCl_2$ for 6 minutes significantly reduced the days for callus initiation (8.33) remaining at par with T_5 (0.1% $HgCl_2$ for 5 minutes),T_7 (0.1% $HgCl_2$ for 7 minutes), T_9 (0.1% $HgCl_2$ for 9 minutes) and T_{10} (0.1% $HgCl_2$ for 10) minutes, and days to callus development were significantly higher (21.67) in T_6 (0.1% $HgCl_2$ for 6 minutes) remaining at par with T_5 (0.1% $HgCl_2$ for 5 minutes), T_7 (0.1% $HgCl_2$ for 7 minutes) and T_{10}(0.1% $HgCl_2$ for 10 minutes). The callus spread was higher (1.63 × 1.33) in T_6 (0.1% $HgCl_2$ for 6 minutes).

Considering all the characters of the impact of sterilant, leaf disc surface sterilized with 0.1% $HgCl_2$ for 6 minutes was found to be best for *Santalum album Linn.* Leaf disc surface sterilized with 0.1% $HgCl_2$ for 6 minutes significantly reduced the fungal contamination and days to callus initiation. A higher percentage of aseptic culture was recorded in the leaf disc explants when surface sterilized with 0.1% $HgCl_2$ for 6 minutes and the callus spread was also good. However none of explants survived when no sterilant was used. Better callusing were obtained from leaf discs, the callusing started from injured sides cuts. Similar results were obtained by Mujib, 2005 [13] and Bele *et al.*, 2012 [9].

Table 2: Impact of timing of sterilant on callus development of leaf disc explants. Duration- 30 days

Treat. No.	Treatment	Days to callus initiation	Days to callus development	Size of the callus (cm)		Nature of the callus	Colour of the callus
T_1	Distilled water (controlled)	11.67	18.33	0.57	0.43	Friable	Whitish
T_2	0.1% $HgCl_2$ (2 min.)	11.00	19.00	0.57	0.33	Friable	Whitish
T_3	0.1% $HgCl_2$ (3 min.)	10.00	20.00	0.73	0.43	Friable	Whitish
T_4	0.1% $HgCl_2$ (4 min.)	9.67	20.33	0.70	0.50	Compact	Greenish
T_5	0.1% $HgCl_2$ (5 min.)	9.00	21.00	0.80	0.53	Compact	Whitish
T_6	0.1% $HgCl_2$ (6 min.)	8.33	21.67	1.63	1.33	Compact	Greenish
T_7	0.1% $HgCl_2$ (7 min.)	8.67	21.33	1.53	0.57	Compact	Greenish
T_8	0.1% $HgCl_2$ (8 min.)	9.67	20.33	0.87	0.37	Compact	Greenish
T_9	0.1% $HgCl_2$ (9 min.)	9.00	21.00	0.77	0.27	Compact	Greenish
T_{10}	0.1% $HgCl_2$ (10 min.)	8.67	21.33	0.63	0.30	Compact	Greenish
SE (m)±		0.32	0.32				
CD (5%)		0.93	0.95				

Impact of PBR's on callus formation on leaf disc explants

The data presented in table.3 revealed that MS medium fortified with BAP 2.5 mg/l and 2,4-D 1.5 mg/l and BAP 2.0 mg/l and 2,4-D 1.5 mg/l significantly reduced the days for callus initiation (7.33) remaining at par with(T_8) BAP 1.5 mg/l and 2,4-D 1.5 mg/l, (T_6) BAP 2.0 mg/l and 2,4-D 1.1 mg/l and (T_5) BAP 1.5 mg/l and 2,4-D 1.0 mg/l. The same trend was recorded for days to callus development where BAP 1.5 mg/l and 2, 4-D 1.5 mg/l significantly had maximum number of days for callus development. MS medium supplemented with BAP 2.5 mg/l and 2,4-D 1.5 mg/l gave greenish white callus with a good spread(1.67×1.55) followed by (T_9) BAP 2.0 mg/l and 2,4-D 1.5 mg/l (1.63×1.23) with a compact whitish callus. Hence, considering all the characters for callus formation on leaf disc explants of *Santalum album* Linn. BAP 2.5 mg/l and 2, 4-D 1.5 mg/l was found to be the best treatment.

Table 3: Effect of PBR's on Callus development of *Santalum album Linn*. Leaf disc Duration- 30 days Basal media- MS

Treat. No.	Treatments			Days to callus initiation	Days to callus development	Size of the callus (cm)		Nature of the callus	Colour of the callus
	BAP		2,4-D			Length	Breadth		
T_1		-	-	9.67	20.33	0.50	0.27	Compact	Greenish
T_2		1.5	-	10.00	20.00	0.78	0.42	Compact	Greenish
T_3		2.0	-	9.33	20.67	1.10	0.44	Compact	Greenish
T_4		2.5	-	8.67	21.33	0.82	0.33	Compact	Greenish
T_5		1.5	1.0	8.00	22.00	0.57	0.38	Compact	Greenish
T_6		2.0	1.0	8.67	21.33	0.82	0.53	Compact	Greenish
T_7		2.5	1.0	8.00	22.00	0.67	0.55	Compact	Greenish
T_8		1.5	1.5	8.67	21.33	1.29	0.44	Compact	Whitish
T_9		2.0	1.5	7.33	22.67	1.63	1.23	Compact	Whitish
T_{10}		2.5	1.5	7.33	22.67	1.67	1.55	Compact	Greenish
SE (m)±				0.33	0.33				
CD (5%)				0.98	0.98				

Standardizing the media supplements for shoot initiation and root formation under *in vitro* condition

The calli mass developed by the leaf disc explants were inoculated in MS medium alone or in combination with BAP (1.5, 2.0, 2.5 mg/l), NAA (0.5 mg/l), IAA (0.5 mg/l). The experiment was carried out for 1 month period. However, the calli mass and shoots started browning and eventually died in the due course of time.

Conclusion

It was concluded that the best surface sterilization treatment for leaf disc explants was 0.1% $HgCl_2$ for 6 minutes, where the treatment recorded minimum per cent of fungal contamination. MS medium fortified with BAP 2.5 mg/l and 2, 4-D 1.5 mg/l was the best treatment for early initiation (7.33), development in leaf disc explants with maximum spread of callus. No further shooting was obtained from any of the combination of MS with BAP (2.5, 2.0, 1.5 mg/l), NAA (0.5 mg/l), IAA (0.5 mg/l) and Ads (100 mg/l) from the calli mass and callus started browning and died in course of time. Further studies should be carried out in this direction.

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