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RESEARCH ARTICLE

In vitro propagation of *Pterocarpus santalinus* L. (Red Sandalwood) through tissue culture

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Abstract: Studies have been conducted to develop an *in vitro* propagation protocol for *Pterocarpus santalinus* L., an endangered medicinal plant. Stem cuttings excised from one year old plants were surface sterilized successfully using 15 % clorox (5.25 % NaOCl) for 10 minutes followed by 70 % ethanol for two minutes and established on Mc Cowns woody plant medium (WPM) with 0.1 % activated charcoal. Different explant types were tested including cotyledonary nodal segments, mesocotyl segments, *in vitro* derived shoot tips, immature and semi-hard wood cuttings detached from one year old plants and *in vitro* germinated seedlings. Maximum number of shoots and shoot branches were obtained with the Gamberg medium (B5) with 8.0 µM 6-benzyle amino purine (BAP) and 2.0 µM napthalene acetic acid (NAA). Adventitious roots were formed on micro shoots on half-strength solid Murashige and Skoog (MS) medium containing 0.5 µM indole butric acid (IBA) after exposing to pulse treatment with 250 µM IBA for 12 hours. *In vitro* rooted plantlets were successfully acclimatized in pots containing sand:coir dust (1: 1) in a humid chamber during the first four weeks followed by keeping in a plant-house for another four weeks before repotting or field establishment.

Keywords: Explant, *in vitro, in vivo*, proliferation, *Pterocarpus santalinus* L., surface sterilization.

INTRODUCTION

Red sandalwood, *Pterocarpus santalinus* L. (Fabaceae), is a highly valued woody plant with dark claret-red heartwood, which contains 16 % Santalin, a red colouring agent. Santal, pterocarpin and homopterocarpin are three other crystalline colouring agents present in the heartwood that are used in the pharmaceutical, paper, pulp, soap, food and textile industries to obtain a dark maroon to purple colour. In ayurvedic medicine, red sandalwood is used as an antiseptic, wound-healing agent, and in anti-acne treatments. Because of its hard texture, the red sandalwood is used in making furniture and in wood carvings. The natural habitat of *P. santalinus* is in the southern Ghats states in India, which is a hilly region with a hot dry climate. It is now considered as an endangered species and placed in the red list of endangered species under IUCN guidelines (International Union for Conservation of Nature, 2011) compelling the Indian government to ban exportation of this commodity. This decision has negatively affected Sri Lanka, as we have been importing red sandalwood from India to fulfill the national demand. In Sri Lanka, only a limited number of red sandalwood trees, which had been brought from India, are present, mainly in the southern region of the country (Arunakumara *et al*., 2005). Red sandalwood is conventionally propagated through seeds, which is somewhat difficult due to the hard seed coat coupled with low viability and seed dormancy due to the presence of phenolic compounds (Kumarasinghe *et al*., 2003).

The conventional vegetative propagation of red sandalwood has proven to be ineffective (Dayathilake *et al*., 2001) as the cuttings were found to be hard to root even with the application of growth regulators (Subasinghe *et al*., 2004). Therefore the conservation and large-scale propagation of this endangered species has some inherent limitations. In this context tissue culture seems to be a promising technique for conservation and large scale multiplication of several woody species (Razdan, 1994). Studies on tissue culture of red sandalwood began in 1980s with more recent attempts to improve shoot sprouting using seedling explants in India (Anuradha & Pullaiah, 2001). However, an efficient protocol for micropropagation of elite and quality trees of red sandalwood has not been reported to date in Sri Lanka.

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Therefore, an attempt was made to develop a feasible *in vitro* propagation protocol for red sandalwood through tissue culture techniques, to enable mass propagation of this plant for biodiversity conservation and to meet the increasing demand.

METHODS AND MATERIALS

Pods were picked directly from mature red sandalwood trees (over 25 years old) at the green to brownish stage. Seeds were surface sterilized using 10 % clorox for 20 min followed by 70 % ethanol for 2 min and cultured on Gamberg (B5) medium (Gamborg *et al*., 1968). Six different explant types were used for the study. *In vitro* germinated seedlings were excised after 20 d to derive mesocotyl segments, cotyledonatory nodal segments and shoot tips. Mother plants were maintained under plant-house conditions to excise stem cuttings. Fresh and immature terminal shoot cuttings and subsequent nodal shoot segments (semi-hard wood, 3- 5 cm in length with dormant auxiliary buds) were collected during the early hours of the day at 2 wk intervals. *In vitro* germinated 20 day old seedlings were used as the control.

As a rejuvenation procedure, decapitation and spraying of 10.0 ppm 6-benzyle amino purine (BAP) solution was done for the mother plants, at 2 wk intervals. Plants were maintained under plant-house conditions free from pests and diseases and watered when necessary. Overhead watering was avoided. A 100.0 ppm solution of thiophanate methyl 70 % (topsin) was sprayed 24 h before collecting the buds for culturing to prevent fungal infections. Plants were treated with 200.0 ppm Albert's solution (complete fertilizer mixture) at 2 wk intervals.Cultures were kept aseptically in a growth room (23 ± 2 ^{0}C temperature, 60 % RH) and cool white fluorescent lights were used to generate a 16 h photoperiod under 1220 lx of light intensity. All media contained 3.0 % sucrose and 0.01 % myo-inositol. The pH of the medium was adjusted to 5.8. Prepared media were autoclaved at 121 ^{0}C and 1.05 kg cm^{-2} pressure for 20 min. Experiments were designed according to complete randomized design with 20 replicates in each treatment and all parametric data were analyzed using SAS statistical software. Mean separations were carried out using least significant difference test (LSD). Non-parametric data were analyzed using the Chi Square test.

Surface sterilization of shoots

Immature and semi-hard wood shoots excised from mother plants were immediately dipped in a beaker containing distilled water. In the laboratory, shoot segments were treated with liquid detergent (Teepol 5 % v/v) for 5 min and placed under running tap water for half an hour. Shoots were transferred to 0.3 % topsin solution and kept for 1 h, thoroughly washed twice using sterile distilled water and transferred to different concentrations of clorox separately (10 %, 15 % and 20 % v/v) for three different time durations (10, 15 and 20 min). To enhance the activity of the detergent, two drops of Tween 20 (poly oxyethelene sorbitanmonolaurate) was mixed with each of the clorox solutions**,**introduced to 70 % ethanol for 2 min and washed twice using sterile distilled water. This procedure was repeated twice. Finally, cut ends of the explants were trimmed and implanted vertically on a basal culture medium [MS (Murashige & Skoog, 1962) or WPM (Lloyd & McCown, 1980)]. Percentages of non contamination, browning and survivals as well as the number of shoot buds initiated and the new leaves formed were recorded over a period of 4 wks.

Establishment of shoots

Surface sterilized immature and semi-hard wood shoots were cultured on MS (Murashige & Skoog, 1962) and WPM (Lloyd & McCown, 1980) media with and without 0.1 % activated charcoal. Percentages of browning and survivals as well as the number of shoot buds initiated and the new leaves formed were recorded over a period of 4 wks.

Effect of explant type, culture medium and combination of plant growth regulators on *in vitro* shoot proliferation

Separate experiments were conducted using six different types of explants. Explants were cultured on MS and B5 (Gamborg *et al*., 1968) media with different concentrations of BAP (4.0, 8.0, and 12.0 µM) and napthalene acetic acid (NAA) (0.5 and 2.0 µM). Number of shoots, mean shoot height, number of fully opened leaves and number of branches were measured at four week intervals up to the sixth sub-culture.

Effect of pulse treatment, IBA concentration and potting media on root formation of micro cuttings

Micro shoots were dipped in three indole butric acid (IBA) concentrations (25.0, 250.0 and 2500.0 ppm) for 12 and 24 hours for pulse treatment. They were transferred to liquid and solid (by adding 0.8 % agar) half strength MS media with two different IBA concentrations (0.5 and 5.0 µM). the number of roots, root length and percentage of browning were measured over a period of 4 wks. *Ex vitro* rooting was practiced with three IBA concentrations (1,000.0, 3,000.0 and 5,000.0 ppm) and 12 and 24 hour exposure durations. Sand and sand: coir dust (1: 1) were used as two potting

media. Root initiation rate was measured after 4 wks by uprooting the plants.

Acclimatization of plantlets

In vitro rooted plantlets were transferred to pots containing sand, coir dust, cow dung and top soil in different ratios and placed under high humid conditions in the laboratory for 2 wks. Potted plantlets were transferred to plant-house and maintained under high humidity for another 4 wks (Figure 1a).

Plantlets were transferred to poly bags containing cow dung: top soil: sand (1: 1: 2) and placed under plant-house conditions for another 4 wks (Figure 1b). Survival rates, time taken to produce new branches, number of new leaves formed and height increment of the plants were observed over a period of 10 wks. After 4 wks, *ex vitro* rooted plants were transferred to poly bags with a mixture of cow dung: top soil: sand (1: 1: 2) and placed under planthouse conditions for another 4 wks. Well acclimatized plantlets were finally transferred to the field.

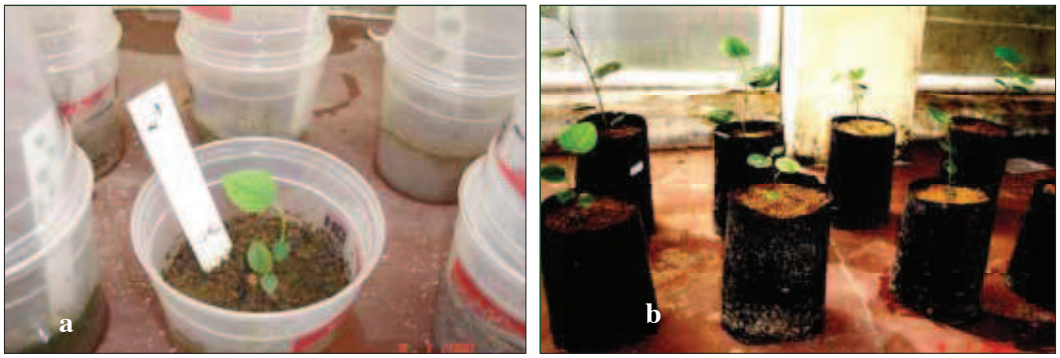


Figure 1: a. Acclimatized plants after transferring to the plant-house; b. Plants transferred from pots into poly bags under plant-house conditions

RESULTS

Surface sterilization of shoots

The highest survival (80 %) was observed in immature cuttings treated with 15 % clorox for 10 minutes. Semi-hard wood cuttings gave the maximum survival rate of 40 % when treated with 10 % clorox for 20 minutes and 15 % clorox for 10 and 15 minutes (data not shown).

Establishment of shoots

There was a combined effect of media type, presence of activated charcoal and explant type on browning, which were evaluated after four weeks ($p \leq 0.05$). Activated charcoal (0.1 %) incorporated WPM medium was the best culture initiation medium for immature and semi-hard wood cuttings where the browning of explants was reduced to zero and 10 %, respectively (Table 1).

Table 1: Effects of medium, activated charcoal and explant type on browning of explants 4 weeks after incubation (n=20)

Medium	Activated Charcoal (+ or -)	Explant type	Browning %
MS	-	Immature	$55.00^{c} \pm 5.77$
MS	-	Semi-hard wood	$70.00^{a} \pm 11.54$
MS	+	Immature	$10.00^{f} \pm 0.00$
MS	+	Semi-hard wood	$35.00^{d} \pm 5.77$
WPM	-	Immature	$20.00^{e} \pm 0.00$
WPM	-	Semi-hard wood	$50.00^{b} \pm 0.00$
WPM	+	Immature	$0.00^{g} \pm 0.00$
WPM	+	Semi-hard wood	$10.00^{f} \pm 0.00$

CV= 6.78, LSD= 0.40

Note: Means within the last column followed by the same letter are not significantly different at $p \leq 0.05$

Table 2: Interaction effects of medium, activated charcoal and explant type on mean number of new leaves and shoot buds formed 4 weeks after incubation

Interaction			
Medium	Activated charcoal (+ or -)	Mean number of new leaves	Mean number of shoot buds per explants
MS	-	$1.22^{d} \pm 0.42$	$1.47^{c} \pm 0.55$
MS	+	$1.97^{b} \pm 0.65$	$1.87^{b} \pm 0.72$
WPM	-	$1.42^{c} \pm 0.50$	$1.42^{c} \pm 0.50$
WPM	+	$2.60^{a} \pm 0.49$	$2.5^{a} \pm 0.50$
Charcoal activated (+ or -)		Explant type	
-	immature	$1.40^{d} \pm 0.49$	$1.85^{c} \pm 0.73$
-	Semi hardwood	$1.25^{c} \pm 0.43$	$1.50^{c} \pm 0.55$
+	immature	$2.70^{a} \pm 0.46$	$2.25^{a} \pm 0.83$
+	Semi hardwood	$1.87^{b} \pm 0.56$	$1.67^{b} \pm 0.47$
		LSD = 0.07	LSD = 0.07
		CV = 12.93	CV = 14.52

Note: Means within the columns followed by the same letter are not significantly different at $p \leq 0.05$

Table 3: Effects of medium, BAP and NAA concentrations on mean number of shoots produced 4 weeks after incubation

Treatment combinations			Cotyledonary nodal segments	Mesocotyls	*In vitro* derived shoot tips	*In vitro* derived seedlings	Immature cuttings
Medium	BAP (mg/L)	NAA (mg/L)					
MS	1	0.1	$2.00^{g} \pm 0.00$	$1.35^{d} \pm 0.48$	$1.00^{e} \pm 0.00$	$1.70^{d} \pm 0.70$	$1.00^{d} \pm 0.00$
MS	2	0.1	$2.75^{e} \pm 0.44$	$2.45^{c} \pm 0.51$	$1.50^{c} \pm 0.51$	$1.70^{d} \pm 0.47$	$1.70^{c} \pm 0.47$
MS	2	0.4	$3.25^{c} \pm 0.44$	$3.00^{c} \pm 0.00$	$2.00^{b} \pm 0.00$	$2.00^{c} \pm 0.00$	$2.00^{b} \pm 0.00$
MS	3	0.1	$2.25^{f} \pm 0.44$	$2.25^{c} \pm 0.44$	$1.20^{d} \pm 0.41$	$1.55^{e} \pm 0.51$	$1.45^{c} \pm 0.51$
MS	3	0.4	$3.30^{c} \pm 0.47$	$2.70^{c} \pm 0.47$	$1.50^{c} \pm 0.51$	$1.70^{d} \pm 0.47$	$1.60^{c} \pm 0.50$
B5	1	0.1	$3.00^{d} \pm 0.00$	$3.00^{c} \pm 0.00$	$2.00^{b} \pm 0.00$	$2.00^{c} \pm 0.00$	$2.20^{b} \pm 0.41$
B5	1	0.4	$3.60^{b} \pm 0.50$	$3.35^{b} \pm 0.48$	$2.15^{b} \pm 0.36$	$2.35^{b} \pm 0.48$	$2.25^{b} \pm 0.44$
B5	2	0.1	$3.80^{b} \pm 0.41$	$3.50^{b} \pm 0.51$	$2.10^{b} \pm 0.30$	$2.00^{c} \pm 0.00$	$2.35^{b} \pm 0.48$
B5	2	0.4	$4.95^{a} \pm 0.22$	$4.00^{a} \pm 0.00$	$3.00^{a} \pm 0.00$	$3.25^{a} \pm 0.44$	$2.95^{a} \pm 0.22$
B5	3	0.1	$3.25^{c} \pm 0.44$	$2.65^{c} \pm 0.48$	$2.50^{b} \pm 0.51$	$2.00^{c} \pm 0.00$	$2.40^{b} \pm 0.50$
B5	3	0.4	$3.65^{b} \pm 0.81$	$2.80^{c} \pm 0.83$	$2.35^{b} \pm 0.48$	$2.30^{b} \pm 0.47$	$2.50^{b} \pm 0.51$
CV			9.77	10.40	5.90	8.51	10.21
LSD			0.08	0.24	0.23	0.27	0.22

Note: Means within the columns followed by the same letter are not significantly different at $p \leq 0.05$

Among the three factors tested (media type, presence of activated charcoal and explant type), only two-factor interactions significantly affected ($p \leq 0.05$) the number of leaves and the shoot buds produced. When considering the interaction between the presence of activated charcoal in the medium and type of the medium, significantly higher ($p \leq 0.05$) number of new leaves (2.60 ± 0.49) and shoot buds (2.50 ± 0.50) were recorded in activated

charcoal-incorporated WPM medium, compared to 1.97 ± 0.65 new leaves and 1.87 ± 0.72 shoot buds produced in activated charcoal-incorporated MS medium (Table 2).

The interaction of explant type and the presence of activated charcoal in the medium significantly affected ($p \leq 0.05$) the number of leaves and the shoot buds produced as well. The highest number of new leaves (2.70 ± 0.46) and the shoot buds (2.25 ± 0.83) were formed by immature cuttings established in 0.1 % activated charcoal-incorporated culture media while semi- hard wood cuttings produced 1.87 ± 0.56 new leaves and 1.67 ± 0.47 shoot buds (Table 2). However, the interaction between the type of the medium and the explant type did not significantly affect ($p \leq 0.05$) on number of new leaves and shoot buds produced.

Effects of explant type, culture medium and combination of plant growth regulators on *in vitro* shoot proliferation

Interaction of culture medium type, BAP and NAA concentrations significantly ($p \leq 0.05$) affected the number of shoots produced by all explant types. Significantly higher ($p \leq 0.05$) multiplication rates were observed on B5 medium supplemented with 8.0 µM BAP and 2.0 µM NAA (Table 3). The maximum number of shoots were recorded from cotyledonary nodal segments (4.95 ± 0.22) (Figure 2a), mesocotyl segments (4.00 ± 0.00), *in vitro* derived shoot tips excised from 20 day old seedlings (3.00 ± 0.00), immature cuttings (2.95 ± 0.22) and *in vitro* germinated seedlings (3.25 ± 0.44), respectively at four week intervals.

Interaction of medium type, BAP and NAA concentrations significantly affected the mean shoot height ($p \leq 0.05$) of cotyledonary nodal segments, mesocotyls, *in vitro* derived shoot tips, immature stem cuttings (Table 4) and the control, while semi-hard wood cuttings did not show any height increment during the four week duration of culture.

Significantly higher number of shoot branches were formed by five explant types (i.e. cotyledonary nodal segments, mesocotyls, *in vitro* derived shoot tips, immature and semi-hard wood cuttings) on B5 medium containing 8.0 µM BAP and 2.0 µM NAA at $p \leq 0.05$ level (Table 5). However, *in vitro* germinated seedlings; the control produced significantly ($p \leq 0.05$) higher number of branches (3.09 ± 0.22) on B5 medium with 4.0 µM BAP and 2.0 µM NAA. During the multiplication phase, sub-culturing was practiced at four week intervals up to the sixth sub-cultures. However, after the forth sub-culture, the number of new shoot production was reduced drastically (data not shown).

Effects of pulse treatment, IBA concentration and potting media on root formation of micro cuttings

Pulse treatment and IBA-containing solid medium significantly ($p \leq 0.05$) affected the mean number of roots produced and the mean length of the roots (Figures 3 & 4). The highest (4.10 ± 0.00) mean number of roots per shoot was produced when 2,500.0 ppm IBA solution was used for the pulse treatment with 12 hours exposure time, followed by subsequent culturing in half strength solid MS medium supplemented with 0.5 µM IBA. The

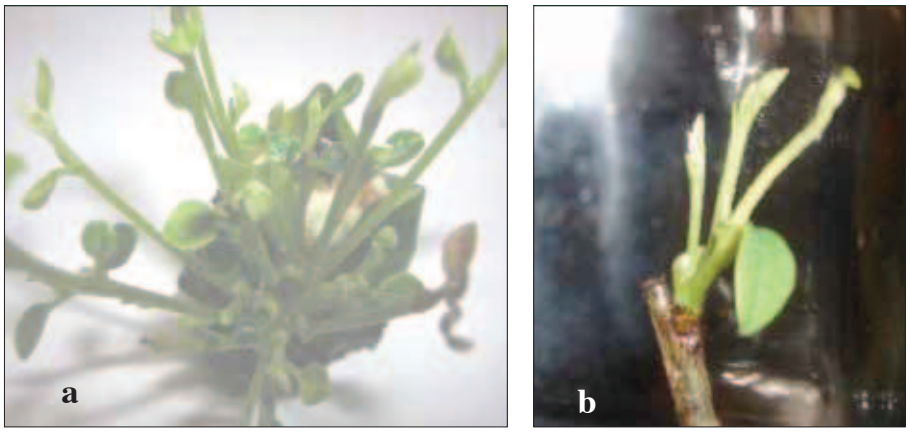


Figure 2: a. Shoots produced by a cotyledonary nodal segment after four weeks; b. New branches formed by a semi- hard wood cutting

Table 4: Effects of culture medium, BAP and NAA concentrations on mean shoot height (cm) 4 weeks after incubation

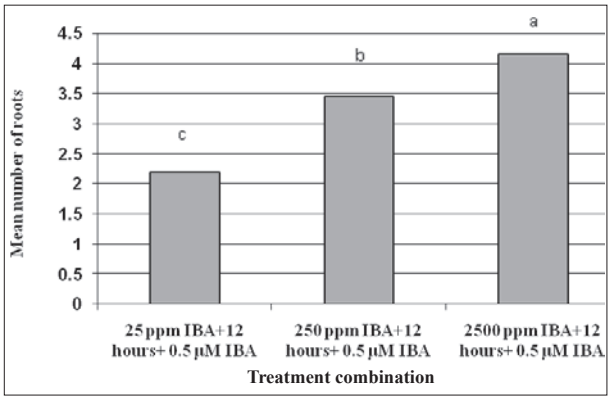
Treatment combinations			Cotyledonary nodal segments	Mesocotyls	*In vitro* derived shoot tips	*In vitro* derived seedlings	Immature cuttings
Medium	BAP (mg/L)	NAA (mg/L)					
MS	1	0.1	$2.80^{c} \pm 0.25$	$3.20^{c} \pm 0.25$	$2.60^{c} \pm 0.20$	$2.50^{d} \pm 0.0$	$2.80^{c} \pm 0.25$
MS	1	0.4	$3.17^{c} \pm 0.24$	$3.25^{b} \pm 0.25$	$2.75^{c} \pm 0.25$	$2.75^{c} \pm 0.25$	$3.12^{c} \pm 0.22$
MS	2	0.1	$3.00^{c} \pm 0.00$	$3.00^{b} \pm 0.00$	$2.85^{c} \pm 0.23$	$2.85^{c} \pm 0.23$	$3.00^{c} \pm 0.00$
MS	2	0.4	$3.20^{c} \pm 0.25$	$3.40^{b} \pm 0.20$	$2.95^{c} \pm 0.45$	$2.95^{c} \pm 0.45$	3.20c ± 0.25
MS	3	0.1	$2.87^{c} \pm 0.22$	$3.12^{b} \pm 0.22$	$2.87^{c} \pm 0.22$	$2.75^{c} \pm 0.25$	$2.87^{c} \pm 0.22$
MS	3	0.4	$3.25^{c} \pm 0.25$	$3.25^{b} \pm 0.25$	$2.75^{c} \pm 0.25$	$2.85^{c} \pm 0.23$	$3.25^{c} \pm 0.25$
B5	1	0.1	$3.10^{c} \pm 0.20$	$3.30^{b} \pm 0.25$	$2.70^{c} \pm 0.25$	$2.70^{c} \pm 0.25$	$3.10^{b} \pm 0.20$
B5	1	0.4	$3.27^{c} \pm 0.25$	$3.27^{b} \pm 0.25$	$2.72^{c} \pm 0.25$	$2.72^{c} \pm 0.25$	$3.27^{c} \pm 0.25$
B5	2	0.1	$3.12^{c} \pm 0.22$	$3.57^{b} \pm 0.43$	$3.20^{b} \pm 0.54$	$3.52^{b} \pm 0.44$	$3.12^{c} \pm 0.22$
B5	2	0.4	$3.62^{a} \pm 0.22$	$4.55^{a} \pm 0.51$	$3.92^{a} \pm 0.18$	$4.00^{a} \pm 0.00$	$3.72^{a} \pm 0.25$
B5	3	0.1	$3.15^{c} \pm 0.23$	$3.07^{b} \pm 0.18$	$2.92^{c} \pm 0.18$	$2.80^{c} \pm 0.25$	$3.07^{c} \pm 0.18$
B5	3	0.4	$3.42^{b} \pm 0.18$	$3.50^{b} \pm 0.00$	$2.50^{c} \pm 0.00$	$2.87^{c} \pm 0.22$	$3.42^{b} \pm 0.18$
CV			7.25	8.36	10.02	9.27	7.0
LSD			0.14	0.17	0.17	0.16	0.13

Note: Means within the columns followed by the same letter are not significantly different at $p \leq 0.05$

Table 5: Effects of culture medium, BAP and NAA concentrations on mean number of branches formed 4 weeks after incubation

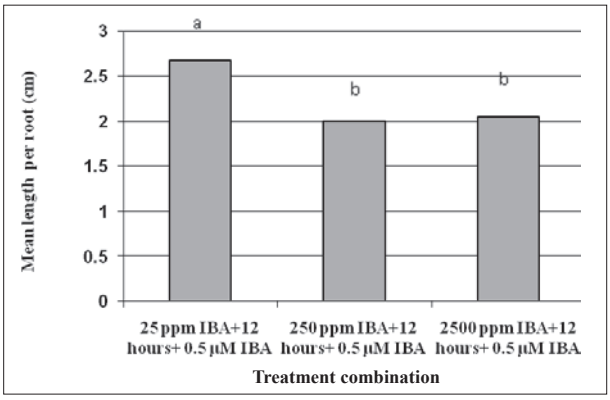
Treatment combinations			Cotyledonary nodal segments	Mesocotyls	*In vitro* derived shoot tips	*In vitro* derived seedlings	Immature cuttings
Medium	BAP (mg/L)	NAA (mg/L)					
MS	1	0.1	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$
MS	1	0.4	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$
MS	2	0.1	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$
MS	2	0.4	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$
MS	3	0.1	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$
MS	3	0.4	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{e} \pm 0.00$	$0.00^{d} \pm 0.00$
B5	1	0.1	$3.00^{c} \pm 0.00$	$2.00^{c} \pm 0.00$	$1.00^{d} \pm 0.00$	$1.30^{d} \pm 0.47$	$1.30^{c} \pm 0.47$
B5	1	0.4	$3.55^{b} \pm 0.51$	$2.55^{b} \pm 0.51$	$1.00^{d} \pm 0.00$	$1.85^{c} \pm 0.36$	$2.00^{b} \pm 0.00$
B5	2	0.1	$3.00^{c} \pm 0.00$	$2.00^{c} \pm 0.00$	$1.25^{c} \pm 0.44$	$2.40^{b} \pm 0.50$	$2.00^{c} \pm 0.00$
B5	2	0.4	$4.20^{a} \pm 0.00$	$3.75^{a} \pm 0.71$	$2.50^{a} \pm 0.51$	$3.15^{a} \pm 0.58$	$3.45^{a} \pm 0.51$
B5	3	0.1	$3.00^{c} \pm 0.00$	$2.00^{c} \pm 0.00$	$1.00^{d} \pm 0.00$	$1.15^{d} \pm 0.15$	$1.00^{c} \pm 0.00$
B5	3	0.4	$3.60^{b} \pm 0.75$	$2.65^{b} \pm 0.81$	$1.30^{b} \pm 0.47$	$1.12^{d} \pm 0.19$	$1.30^{b} \pm 0.47$
CV			10.37	12.03	15.54	17.95	13.67
LSD			0.05	0.05	0.05	0.07	0.05

Note: Means within the columns followed by the same letter are not significantly different at $p \leq 0.05$



CV: 14.15, LSD = 0.03

Figure 3: Mean number of roots as affected by pulse treatment and incorporated IBA concentrations into the culture medium (4 weeks after incubation)



CV: 8.40, LSD = 0.01

Figure 4: Mean root length as affected by pulse treatment and incorporated IBA concentrations into the culture medium (4 weeks after incubation)

Figure 5: Shoots rooted on 0. 5 µM IBA containing ½ strength MS medium after dipping in 25.0 ppm IBA solution for 12 hours

mean root length was the highest (2.67 ± 0.71 cm) in the shoots treated with 25.0 ppm IBA for 12 hours exposure time with subsequent culturing on half strength solid MS medium supplemented with 0.5 µM IBA (Figure 5). However the lowest mean number of roots per shoot (2.20 ± 0.51) was observed in the above treatment.

IBA concentrations and exposure times used for the pulse treatment had significant effects on the browning of shoots at $p \leq 0.05$ probability level. Exposure times of the pulse treatment and the concentrations of the IBA-incorporated into the rooting medium significantly ($p \leq 0.05$) affected the browning of shoots as well (Table 6). Pulse treatment with 25.0, 250.0 and 2,500.0 ppm IBA for 24 hours followed by culturing on 0.5 or 5.0 µM IBA supplemented media, caused browning and subsequent deaths of shoots (Table 6).

When shoots were pulse treated with 2,500.0 ppm IBA for 12 hours followed by transferring to liquid half strength MS medium fortified with 0.5 µM IBA, 100 % rooting was observed within four weeks of incubation. However, they were fragile in nature and did not show any further growth and turned brown (Figure 6). Shoots pulse treated with 25.0 ppm and 250.0 ppm IBA for 12 hours followed by transferring to liquid half strength MS medium fortified with 0.5 µM or 5.0 µM IBA never produced roots.

Shoots dipped in 1,000.0 ppm IBA solution for 12 hours and subsequently transferred into sand medium produced 40 % rooting while none of the other treatments produced roots (Figure 7).

Acclimatization of plantlets

An 80 % success rate was observed, when the plantlets were acclimatized in sand : coir dust (1:1) potting medium, with increasing plant heights and production of new leaves and shoot branches (data not shown). A two-stage acclimatization procedure (six weeks under high humidity and low light followed by another four weeks in a plant-house under shade) was needed prior to establishment in the field.

Table 6: Interaction effects of exposure time and IBA concentrations on browning of explants 4 weeks after incubation (n=20)

IBA concentration (mg/L)	Exposure time	Browning
25	12 hours	$0.00^{d} \pm 0.00$
25	24 hours	$82.50^{b} \pm 7.07$
250	12 hours	$0.00^{d} \pm 0.00$
250	24 hours	$87.50^{b} \pm 10.35$
2500	12 hours	$0.00^{d} \pm 0.00$
2500	24 hours	$95.00^{a} \pm 9.25$
		LSD = 5.70
Exposure time	**IBA concentration in the medium (mg/L)**	
12 hours	0.1	$0.00^{c} \pm 0.00$
12 hours	1.0	$0.00^{c} \pm 0.00$
24 hours	0.1	$85.00^{b} \pm 9.04$
24 hours	1.0	$91.66^{a} \pm 10.29$
		LSD = 4.66

Note: Means within the last column followed by the same letter are not significantly different at $p \leq 0.05$

DISCUSSION AND CONCLUSION

Surface sterililzation procedure for shoots

During the present study, immature stem cuttings of *P. santalinus* were successfully surface sterilized using 15 % clorox with 10 minutes exposure time followed by 70 % ethanol for 2 minutes exposure time resulting 80 % non-contamination rate, 80 % survival rate and 100 % non-browning rate. The use of mercuric chloride for surface sterilization of explants has been reported in many research articles. Prakash *et al*. (2006) reported that double sterilization using 70 % ethanol for 2 minutes followed by 0.1 % (w/v) aqueous mercuric chloride solution for 7 minutes yielded 80 % of contamination free nodal explants in *P. santalinus*. *S. album* nodal shoot segments surface sterilized with 0.075 % (w/v) mercuric chloride for 5- 6 minutes were reported to have a 90 % survival rate (Sanjaya *et al*., 2006). However, it is a hazardous chemical with problems of disposal. Therefore, it is more eco-friendly and beneficial to find a surface sterilization procedure that does not use mercuric chloride. The present study demonstrates the applicability of 15 % clorox for 10 minutes followed by 70 % ethanol for 2 minutes as surface sterilization agents for immature stem cuttings of *P. santalinus*.

Figure 6: Shoots in ½ strength liquid MS medium treated with 2,500.0 ppm IBA solution for 12 hours, produced roots in fragile nature

Figure 7: Ex vitro rooting of micro shoots after treated with 1,000.0 ppm IBA solution for 12 hours

Establishment of shoots

In the present study, survival rates (number of explants that remained green without contamination for four weeks after culture initiation) were found to be dependent on the type of the medium, presence of activated charcoal in the medium and explant type ($p \leq 0.05$). WPM was found to be the best medium with the highest survival rate when compared to MS medium. Incorporation of activated charcoal into the culture medium reduced the browning by absorbing phenolic compounds and other exudates, enhancing the growth performance of explants and showed higher survival rate when compared to the media without activated charcoal. Immature stem cuttings showed higher survival rate than semi-hard wood cuttings.

It has been demonstrated that leaching of phenolic compounds from cut ends of nodal explants into the liquid nutrient medium could be controlled by the combination of serial transfer technique at intervals of 24, 48, 72 and 96 hours and the incorporation of antioxidants (250.0 ppm ascorbic acid and 50.0 ppm citric acid) into the culture media for *P. santalinus* immature stem cuttings from 10 year old trees with 70 % survival (Prakash *et al*., 2006). In the present study, immature cuttings cultured on 0.1 % activated charcoal incorporated WPM medium achieved 100 % survival rate without browning and with higher numbers of new leaves and shoot buds, thus showing that the WPM medium incorporated with 0.1 % activated charcoal is a better establishment medium for immature stem cuttings of *P. santalinus*.

Effects of explant type, culture medium and combination of plant growth regulators on *in vitro* shoot proliferation

It has been shown that cotyledonary nodal segments cultured on MS medium supplemented with BAP (5.0 µM) achieved maximum shoot multiplication (7.83 ± 0.30) per explant within six weeks and that higher concentrations of BAP and Kinetin decreased the multiplication rates of *Pterocarpus marsupium* while incorporation of low levels (0.25 - 1.00 µM) of indol acetic acid (IAA) or NAA along with BAP induced shoot bud initiation (Anis *et al*., 2005). Prakash *et al* (2006) reported that a combination of BAP and different auxins (IAA or NAA) did not improve the shoot proliferation of *P. santalinus*, whereas BAP and thidiazuron (TDZ) stimulated the production of new shoots with a 3.5 multiplication coefficient in MS medium for nodal explants. However, during the current study, cotyledonary nodal segments produced a higher mean number of shoots, (4. 95 ± 0.22) when compared to mesocotyls (4.00 ± 0.00), *in vitro* germinated seedlings; the control (3.25 ± 0.44), *in vitro* derived shoot tips (3.00 ± 0.00) and immature cuttings (2.95 ± 0.22) on B5 medium containing 8.0 µM BAP and 2.0 µM NAA (Table 3) within four weeks and up to the forth sub-culture while no multiplication was observed in semi-hard wood cuttings.

Anis *et al*, (2005) reported that 5.0 µM BAP and 0.25 µM IAA in MS medium gave the highest mean height (4.01 ± 0.06 cm) per shoot for *P. marsupium* using cotyledonary nodal segments and Prakash *et al*. (2006) showed that a combination of 4.4 µM BAP and 2.2 µM TDZ produced shoots with 6-7 cm mean length per shoot for *P. santalinus* using nodal segments. During the current study, 8.0 µM BAP with 2.0 µM NAA without TDZ gave a mean shoot height of 4.55 ± 0. 51 cm for mesocotyls, 4.00 ± 0.00 cm for immature cuttings, 3.92 ± 0.18 cm for *in vitro* excised shoot tips, 3.72 ± 0.25 for the control and 3.62 ± 0.22 for cotyledonary nodal segments (Table 4). These heights are capable of being transferred to further multiplication or root formation. Semi-hard wood cuttings did not show any height increment during the four week duration of culture.

Red sandalwood is a plant with a higher branching habit, producing side shoots frequently near the soil surface, not only due to apical bud damage but also when it bends due to shade conditions. This is common to *in vitro* cultures as well, with cotyledonary nodal segments producing more branches (4.20 ± 0.00) than mesocotyls (3.75 ± 0.71) (Table 5) and controls (3.09 ± 0.22), and semi-hard wood cuttings producing more branches (3.45 ± 0.51) (Figure 2b) than *in vitro* derived shoot tips (2.50 ± 0.51 and immature cuttings (3.15 ± 0.58) (Table 5), which may be governed by the phenomenon of apical dominance. To obtain the above results, explants have to be cultured on B5 medium containing 8.0 µM BAP and 2.0 µM NAA. These branches can also be used as stem cuttings to form roots.

B5 medium fortified with 8.0 µM BAP and 2.0 µM NAA was the best medium and plant growth regulator combination to form the highest number of new shoots and new shoot branches from cotyledonary nodal segments, mesocotyls, *in vitro* derived shoot tips, immature and semi-hard wood cuttings and *in vitro* germinated seedlings, respectively within a four week interval. Mesocotyls gave the highest mean shoot height under the same conditions. The regeneration ability was reduced drastically after the forth sub-culture.

Effects of pulse treatment, IBA concentration and potting media on root formation of micro cuttings

As a woody perennial, red sandalwood is difficult to root. Stem cuttings even treated with higher concentrations of auxins up to 3000.0 ppm failed to produce roots (Subasinghe *et al*., 2004). Sanjaya *et al* (2006) reported that the best results for *S. album* shoots were given when exposed to pulse treatment with 98.4 µM IBA for 48 hours to induce rooting and subsequently cultured on MS medium. Isolated micro shoots of *P. marsupium* have been rooted using a strategy of giving pulse treatment of an auxin (IBA; 200.0 µM) together with a phenolic acid for 5 days and subsequent transfer to lower concentrations of auxin (IBA; 0.5 µM) on half MS medium (Anis *et al*., 2005).

Prakash *et al.* (2006) has reported that *P. santalinus* shoots derived from *in vitro* multiplication were rooted on half strength MS medium supplemented with 4.9 µM IBA, failed to produce more than one root. During the current study, it was seen that without pulse treatment, a range of IBA, IAA and NAA (0.5 µM IBA to 5.0 µM IBA) concentrations were not effective in root formation. Stem cuttings with 2.0 cm length treated with 250.0 ppm IBA solution for 12 hours followed by transferring to half strength solid MS medium containing 0.5 µM IBA formed roots successfully.

Acclimatization of plantlets

The present study revealed that *in vitro* rooted plantlets could be successfully acclimatized in potting media containing sand : coir dust 1:1 ratio. Eighty percent survival was observed when the plantlets were placed in a humid chamber during the first six weeks and maintained under plant-house conditions for another four weeks before repotting or field establishment.

Somewhat similar results were recorded by Prakash *et al*., (2006) where 70 % survival was seen among *in vitro* rooted plantlets of *P. santalinus* acclimatized in a plant-growth chamber at 25 ± 2 ^{0}C and 85 % RH under 16 hour photoperiod provided by cool white fluorescent tubes for two weeks. The plantlets were then transferred to a shady area of a forest nursery for another four weeks after repotting in larger 20 cm pots containing a mixture of soil and farmyard manure (4:1). It is therefore seen that the acclimatization procedure developed in the current study could be effectively applied to acclimatize *in vitro* rooted plantlets of *P. santalinus*. It can be concluded that *Pterocarpus santalinus* could be propagated on mass-scale using the micropropagation protocol reported in this study, which would serve to fulfill the island wide demand for this plant

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