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In vitro plant regeneration of red sanders (*Pterocarpus santalinus* L.f.) from cotyledonary nodes

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A reliable and efficient micropropagation protocol was developed through axillary shoot proliferation from cotyledonary nodes of *Pterocarpus santalinus*. Cotyledonary nodes showed significantly ($P<0.05$) higher shoot multiplication rate and shoot length than leaf nodes on MS medium with 2.5 µ*M* BAP and 2 µ*M* 2-iP after first subculture (i.e. in the second harvest). For rooting, dipping of microshoots in 5 µ*M* IAA solution proved superior to other *in vitro* methods. Of the various hardening media used for the acclimatization of rooted plants, a mixture of coarse sand, clay and farmyard manure (1:1:1) (v/v) supported the maximum percentage of survival (95%). There were no significant differences between the *in vitro* regenerated plants and seedlings of the same age for all the growth parameters measured (i.e. mean plant height, number of leaves per plant, fresh weight and dry weight of leaves, shoot and root per plant) four months after transfer to *ex vitro* conditions.

Keywords: Cotyledonary node, *ex-vitro* rooting, endangered species, leaf nodes, *Pterocarpus santalinus,* Red sanders

Introduction

Red sanders (*Pterocarpus santalinus* L.f.), belonging to the family Fabaceae, is an endangered and endemic taxon in the Eastern Ghats of India. Red sanders is renowned for its characteristic timber of exquisite colour, beauty and superlative technical qualities and ranks amongst the finest luxury wood in the world. Overexploitation without commensurate replenishment of natural stands has posed a severe threat to the very existence of this pretty and precious timber tree[1]. Natural regeneration has major constraints such as prolonged dormancy and low germinability of seeds[2].

It has been reported that conventional vegetative propagation methods like grafting and air-layering have limited scope in large-scale multiplication of this species, while in rooting of cuttings, rooting was observed only in 25 per cent cuttings[3]. Tissue culture techniques have been largely applied when traditional methods of propagation have either failed or proved inadequate. Thus, *in vitro* propagation of endangered plants can offer considerable benefits for the rapid cultivation of at risk species, which have a limited reproductive capacity and exist in threatened habitats[4]. Micropropagation of *P. santalinus* using mature nodal explants as well as seedling explants such as cotyledon, mesocotyl, hypocotyl and roots has been reported earlier[5-8].

The present investigation has been carried out to develop a simple, reliable and an efficient micropropagation protocol through axillary shoot proliferation from cotyledonary nodes and leaf nodes derived from 30-d-old seedlings.

Materials and Methods

After removing the fruit coats manually the seeds were soaked in cold water for 24 h. Then the seeds were sown on seedbed for germination. Cotyledonary nodes (1.5 cm in length) and leaf nodes with single axillary bud (1 cm in length) obtained from 30-d-old seedlings were initially washed in running tap water for 1 h and treated with 0.025% (v/v) tween 20 for 10 min. This was followed by the washing of explants in distilled water thrice and then treating with 0.1% (w/v) aqueous solution of mercuric chloride for 10 min and rinsed with sterile distilled water thrice. After surface sterilization, explants were cultured on Murashige and Skoog medium[9] containing 3% (w/v) sucrose, 0.8% (w/v) bactoagar (Himedia, India) and different concentrations (2.5, 5 and 10 µ*M*) of either

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Abbreviations: BAP–6-benzylaminopurine; 2-iP–N^6- ($\triangle^2$-iso-pentenyl) adenine; IAA- Indole-3- acetic acid.

BAP or Kn or 2-iP to test the effect of explant types and different concentrations of various cytokinins on *in vitro* shoot multiplication.

With regard to shoot multiplication rate (mean number of shoots per responding explant) cotyledonary nodes showed better response than leaf nodes. Therefore, these explants were further used to assess the effect of combination of cytokinins, subculture and the type of basal media on *in vitro* axillary shoot multiplication and shoot development.

Effect of Combination of Cytokinin and Subculture on Shoot Multiplication from Cotyledonary Nodes

In cotyledonary nodes, BAP induced the best response with regard to shoot multiplication rate at a concentration of 2.5 µ*M*. Therefore, BAP (2.5 µ*M*) was combined with different concentrations (0.5, 1 and 2 µ*M*) of various cytokinins viz., 2-iP and Kn to test the effect of combination of different cytokinins on shoot proliferation. In order to test the effect of subculture on shoot multiplication, the explants (after harvesting the shoots) were subcultured on to fresh medium with the same composition for two cycles.

Effect of Type of Basal Media

The best response in terms of the percentage of explants regenerating shoots and shoot multiplication rate was observed on MS medium supplemented with 2.5 µ*M* BAP and 2 µ*M* 2-iP. Therefore, the different basal media viz., ¼ strength MS, ½ strength MS, full strength MS, B_5 medium[10] and WPM[11] were supplemented with 2.5 µ*M* BAP and 2 µ*M* 2-iP to test their effect on *in vitro* shoot multiplication from cotyledonary nodes.

All the cultures were maintained at 24±2°C under a 16-h light and 8-h dark cycle with the light intensity of 60 µmol m^{-2} S^{-1} provided by cool-white fluorescent tubes (Philips). The percentage of explants regenerating shoots, number of shoots per responsive explant and shoot length were recorded after 4 wks of culture.

***Ex vitro* Rooting**

In *ex vitro* rooting, the basal ends of healthy shoots (1.5-3 cm height) were dipped in different concentrations (1, 5 and 10 µ*M*) of solution of either IAA or IBA or NAA for 25 d. Dipping was carried out by placing two shoots in a test tube containing 15 mL of either IAA or IBA or NAA solution (made up in distilled water). The test tubes were kept in laboratory environment under natural light condition. The percentage of shoots rooted, the percentage of roots with root hairs, the number of roots per responsive explants and root length were measured after 4 wks.

Effect of *ex vitro* Growth Substrates on Plant Survival

At the time of transfer only healthy plantlets (i.e. those with well-developed root and shoot system) were chosen and transferred to plastic pots (10 cm in diameter) containing different growth substrates viz., vermiculite (coarse), a mixture of coarse sand, farmyard manure and clay [2:1:1 (v/v)] and coarse sand to study their effect on the survival rate of rooted shoots after 4 wks at 24±2ºC and 60% relative humidity under the light conditions as described earlier. All the plantlets were supplied with ½ strength MS medium without sucrose twice a wk during acclimatization.

Comparison of Growth Performance of *in vitro* Raised Plants and Seedlings of Same Age

When tissue culture raised plantlets were kept for hardening, seedlings were raised in plastic trays containing coarse sand, farmyard manure and clay (1:2:1) (v/v). Before sowing, seeds were soaked in cold water for 24 h. Seeds germinated within 15 d. Planting stock raised by these two different methods were kept under natural sunlight. The observations on growth and biomass of planting stock were recorded after 4 months.

In all the experiments, 50 explants were used and each experiment was repeated four times. Data were statistically analysed by analysis of variance (ANOVA). The mean data of each treatment were compared by using Tukey's test. The arcsin square root transformation was applied to proportional data prior to ANOVA[12].

Results and Discussion

The percentage of shoot formation was affected significantly ($P<0.05$) by the concentration of various cytokinins but not by the explant type. However, there was a significant interaction between these two factors ($P<0.05$). This indicated that the percentage of shoot formation varies depending upon the concentration of various cytokinins and also by the explant type. Among the various cytokinins tested, BAP induced the maximum response in cotyledonary nodes, whereas in leaf nodes, Kn was found to be superior to other cytokinins (BAP and 2-iP) (Table 1).

Of the two types of explants tested, cotyledonary nodes showed significantly higher shoot multiplication rate and shoot length than leaf nodes

Table 1—Effects of different concentrations of various cytokinins and explant type on *in vitro* shoot multiplication of *P. santalinus*

Concentration of PGR (μ*M*)	% Shoot regeneration Mean±SE		Shoot multiplication rate Mean±SE		Shoot length (cm) Mean±SE	
	CN	LN	CN	LN	CN	LN
BAP						
2.5	95 ± 5.00	65.0 ± 5.00	2.75 ± 0.02	1.37 ± 0.17	2.40 ± 0.10	0.85 ± 0.05
5	75 ± 5.00	69.5 ± 7.07	2.00 ± 0.20	1.18 ± 0.18	1.77 ± 0.22	0.74 ± 0.11
10	95 ± 5.00	75.0 ± 5.00	2.10 ± 0.10	2.07 ± 0.07	1.28 ± 0.22	0.95 ± 0.05
Kn						
2.5	55 ± 5.00	95.0 ± 5.00	1.25 ± 0.35	1.00 ± 0.00	2.32 ± 0.18	1.41 ± 0.09
5	45 ± 5.00	75.0 ± 5.00	1.90 ± 0.14	1.00 ± 0.00	2.05 ± 0.25	1.14 ± 0.05
10	86 ± 5.00	85.0 ± 5.00	1.58 ± 0.17	1.00 ± 0.00	2.18 ± 0.18	0.87 ± 0.12
2-iP						
2.5	76 ± 5.00	75.0 ± 7.07	1.35 ± 0.35	1.00 ± 0.00	1.74 ± 0.55	0.97 ± 0.02
5	77 ± 5.00	57.5 ± 5.00	1.08 ± 0.28	1.00 ± 0.00	2.76 ± 0.14	1.15 ± 0.10
10	68 ± 5.00	75.0 ± 5.00	1.65 ± 0.14	1.0± 0.00	3.52 ± 0.27	1.13 ± 0.06
F value	6.401	4.639	9.134	15.188	11.500	7.962
P value	P = 0.008	P = 0.022	P = 0.003	P = <0.001	P=0.001	P=0.004

SE-Standard error of the mean of four replicated experiments with 50 explants each.

(P<0.05) (Table 1; Figs 1a & b). The cotyledonary nodes of *Pterocarpus marsupium* also showed better response than other types of explants[13]. The presence of expanded cotyledons in the cotyledonary nodal explants might explain the reason for its better response in terms of shoot growth and development as the cell wall polysaccharides stored in them might be degraded and mobilized to the developing shoots and thereby enhancing the growth of developing shoots. It has already been hypothesized that the mobilization of xyloglucan in the cotyledons of *Hymenaea courbaril* a leguminous tree species is strictly controlled by the development of first leaves of the seedling. Further, it has been demonstrated that the endogenous auxin synthesized in the developing shoots is essential for the degradation and mobilization of storage cell wall polysaccharides[14].

In the present study, it was found that at high concentrations (5 and 10 μ*M*) of BAP cotyledonary nodes showed prolific callus growth at the basal cut end, which might have suppressed the shoot bud induction and further shoot growth (Table 1). This kind of inhibitory effect elicited by BAP at higher concentration has also been reported in *P. marsupium*[13]. Cotyledonary nodes cultured in MS medium devoid of plant growth regulator produced single shoot bud in 10% of cultures and showed root morphogenesis in isolated cultures, which indicated the presence of endogenous growth hormones in this explant. In cotyledonary nodes, shoot length was found to be significantly higher in MS medium with 10 μM 2-iP compared to other cytokinins (Table 1).

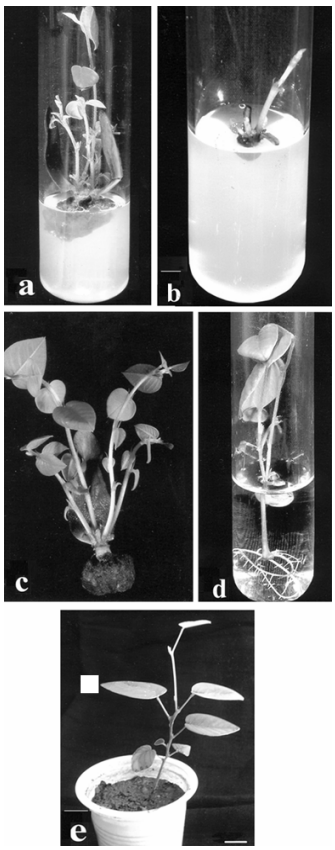

Fig. 1—*In vitro* regeneration of *P. santalianus* from cotyledonary nodes: a, Cotyledonary nodes showing multiple shoot formation on MS medium with 2.5 μ*M* BAP; b, Shoot formation from leaf nodes on MS medium with 10 μ*M* Kn; c, Healthy shoots formed vigorously from cotyledonary nodes on MS medium with 2.5 μ*M* BAP and 2 μ*M* 2-iP after first subculture (i.e. second harvest); d, Rooting of shoots by auxin-dipped method; & e, Tissue culture raised plants 4 months after acclimatization.

Unlike other cytokinins (BAP and Kn) shoot length tends to increase with increasing concentrations of 2-iP (Table 1).

Leaf nodes showed the maximum number of shoots per responsive explant on MS medium with 10 µ*M* BAP and maximum shoot length with 2.5 µ*M* 2-iP (Table 1). However, Kn and 2-iP failed tυ induce multiple shoot formation and produced single shoots of varying lengths depending upon the concentration of the cytokinin added to the medium. It has already been reported that the shoot multiplication rate was very much limited through axillary branching from shoot tip and nodal explants of *P. santalinus*[8].

In order to improve the shoot multiplication rate from leaf nodes and cotyledonary nodes, they were cultured on media containing combination of various cytokinins. However, multiple shoots were not induced from leaf nodes even on media supplemented with combination of different cytokinins (data not shown). On the contrary, cotyledonary nodes showed an improved response in this treatment. The best response in terms of the number of shoots per responsive explant and shoot length was observed on MS medium with 2.5 µ*M* BAP and 2 µ*M* 2-iP after first subculture (i.e., second harvest) (Table 2) (Fig. 1c). However, the shoot length decreased significantly ($P< 0.05$) after second subculture. It has already been reported that 2-iP showed synergistic effect in combination with BAP for the induction of *in vitro* flowering in seedling explants of *Bambusa arundinaceae*[15]. In cotyledon explants of *Glycine max* also, the best response for shoot regeneration was observed on MS medium with BAP and thidiazuron[16].

Among the various basal media tested, cotyledonary nodes showed the maximum percentage of explants regenerating shoots and the maximum shoot multiplication rate on full strength MS medium (Table 3). Eventhough the number of shoots was significantly ($P<0.05$) lower in WPM, the shoot length was greater in t' is medium when compared to other two media tested (MS and B_5). It was observed that the shoots induced from cotyledonary nodes on B_5 medium exhibited scale like leaves. In contrast, it has been reported that the best response in terms of the percentage of shoot regeneration and the number of shoots was observed on B_5 medium when compared to MS and WPM[6]. However, it has also been found that the quality of shoots was inferior as the regenerated shoots showed leaf fall and shoot tip necrosis in B_5 medium. Leaf abscission and shoot tip necrosis were not observed on MS medium[6].

Rooting and Acclimatization

Among the different methods used for root induction in shoots of *P. santalinus*, *ex vitro* method proved superior to other *in vitro* methods (data not shown). In *ex vitro* method, the maximum percentage of rooting (82.5%), the maximum number of roots and root length (2.81 and 3.82, respectively) were observed in 5 µ*M* IAA solutions (Table 4; Fig.1d). Dipping of basal end of shoots in IBA solution (2.46 µ*M*) for 7 d induced 100% rooting in *Rotula aquatica*[17]. Successful induction of rooting by dipping of *in vitro* developed shoots in IBA solution has also been reported in *Lagerstroemia parviflora*[18] and *Ceratonia siliqua*[19]. In the present study, it was found that IAA was superior to IBA and NAA, which

Table 2—Effects of combination of cytokinins and subculture on *in vitro* shoot multiplication from cotyledonary nodes of *P. santalinus*

Concentration of PGR (µ*M*)		% Explants regenerating shoots (Mean±SE)			Number of shoots per explant (Mean±SE)			Shoot length (cm) (Mean±SE)		
		Primary culture	Subculture I	Sub culture II	Primary culture (1st harvest)	Subculture I (2nd harvest)	Subculture II (3rd harvest)	Primary culture (1st harvest)	Subculture I (2nd harvest)	Subculture II (3rd harvest)
BAP + 2-iP										
2.5	0.5	75.0±5.0	85.0±5.0	95.0±5.0	1.87±0.12	2.32±0.18	2.28±0.28	3.15±0.33	4.70±0.20	3.53±0.06
2.5	1.0	65.0±5.0	70.0±5.0	75.0±5.0	2.05±0.25	2.16±0.16	2.10±0.20	3.14±0.14	3.07±0.27	3.25±0.25
2.5	2.0	95.0±5.0	85.0±1.2	85.0±2.5	3.90±0.10	4.40±0.50	4.25±0.25	3.64±0.16	3.64±0.16	4.15±0.05
BAP + Kn										
2.5	0.5	77.5±2.5	45.0±2.5	95.0±5.0	2.04±0.24	2.90±0.10	2.91±0.41	2.18±0.18	3.09±0.09	2.78±0.21
2.5	1.0	67.5±2.5	95.0±5.0	97.5±2.5	2.62±0.12	2.17±0.32	2.87±0.12	2.29±0.19	3.29±0.29	3.40±0.10
2.5	2.0	60.0±10.0	75.0±2.5	45.0±2.5	1.92±0.07	3.25±0.25	3.25±0.25	4.83±0.33	3.65±0.15	2.43±0.23
F value		13.556	16.504	11.605	17.855	42.777	7.188	28.782	13.465	10.127
P value		P = 0.006	P = 0.004	P = 0.009	P = 0.003	P = <0.001	P = 0.025	P = 0.001	P = 0.006	P = 0.012

PGR-Plant growth regulator; SE-Standard error of the mean of four replicated experiments with 50 explants each.

Table 3—Effect of different basal media containing 2.5 µM BAP and 2 µM 2-iP on *in vitro* shoot multiplication from cotyledonary nodes of *P. santalinus*.

Type of basal medium	% explant responded (Mean±SE)	Number of shoots (Mean±SE)	Shoot length (cm) (Mean±SE)
1/4 strength MS	65.0 ± 5.0	2.15 ± 0.15	2.22 ± 0.22
1/2 strength MS	85.0 ± 5.0	2.87 ± 0.12	3.36 ± 0.34
Full strength MS	85.0 ± 5.0	3.9 ± 0.10	3.64 ± 0.16
B_5 medium	47.5 ± 2.5	1.83 ± 0.17	3.56 ± 0.06
WPM	67.5 ± 2.5	2.83 ± 0.17	3.85 ± 0.14
F value	10.450	691.707	10.511
P value	P = 0.022	P = <0.001	P = 0.021

SE-Standard error of the mean of four replicated experiments with 50 explants each.

Table 4—Root induction from shoots dipped in auxin solutions. Values are mean of 20 shoots per treatment, dipping period: 25 d.

Auxins (µ*M*)			% of root induction Mean±SE	% of roots with root hairs Mean±SE	Mean no. of roots Mean±SE	Mean root length (cm) Mean±SE
IAA	IBA	NAA				
1			15.0 ± 5.0	-	1.25 ± 0.25	3.10 ± 0.10
5	1		82.5 ± 2.5	80.5 ± 5.0	2.81 ± 0.19	3.82 ± 0.18
10	5		65.0 ± 5.0	27.0 ± 3.0	3.38 ± 0.18	1.48 ± 0.28
	10		22.5 ± 2.5	47.5 ± 2.5	1.25 ± 0.25	1.75 ± 0.25
			62.5 ± 2.5	58.0 ± 8.0	3.10 ± 0.10	1.81 ± 0.18
			35.0 ± 5.0	37.5 ± 2.5	2.40 ± 0.20	1.90 ± 0.10
		1	22.5 ± 2.5	52.5 ± 2.5	1.25 ± 0.25	1.82 ± 0.17
		5	12.5 ± 2.5	-	2.25 ± 0.25	0.80 ± 0.20
		10	22.5 ± 2.5	-	4.25 ± 0.25	0.77 ± 0.25
F value			47.744	116.226	23.439	37.223
P value			P = <0.001	P = <0.001	P = <0.001	P = <0.001

SE-standard error of the mean of four replicated experiments with 50 explants each. **-** indicates no response.

Table 5—Comparative evaluation of growth of 5-month-old planting stock raised from tissue culture and seedlings

Method of planting stock raised	Height (cm)	Number of leaves/plant	Leaf area (cm^2)	Fresh weight (g/ plant)			Dry weight (g/ plant)		
				Leaves	Shoot	Root	Leaves	Shoot	Root
Tissue culture raised plants	10.0 ± 1.0	5.0 ± 1.0	9.75± 0.75	0.17± 0.03	0.12± 0.02	0.04 ± 0.01	0.06 ± 0.01	0.03 ± 0.10	0.01 ± 0.10
Seedlings	7.5 ± 0.5	3.5 ± 0.5	4.0 ± 0.50	0.09 ± 0.03	0.06 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.03

resulted in the induction of maximum percentage of rooting in shoots of *P. santalinus*. In earlier studies also, it was found that the microshoots of *P. santalinus* showed the maximum percentage of root induction in MS medium supplemented with IAA[7, 20].

Ex vitro rooting accounts for 35-75% reduction of the total cost of plants propagated through tissue culture[21]. As there is a reduction in time, cost and labor and a capability of inducing quality roots in microshoots of *P. santalinus*, auxin dipped *ex vitro* rooting is the most effective method for root induction.

Among the various hardening media used for acclimatization of rooted plants, a mixture of coarse sand, clay and farmyard manure (1:1:1) (v/v) supported maximum percentage of survival (95%). It has already been found that rooted plants of *Azadirachta indica* showed 90% survival when transferred to pots containing soil mixture[22].

In vitro regenerated plants had significantly ($P<0.05$) higher leaf area than seedlings. However, there were no significant differences between the *in vitro* regenerated plants and seedlings for all the other morphological parameters measured (mean plant height, number of leaves per plant, fresh weight and dry weight of leaves, shoot and root per plant) (Table 5; Fig. 1e). In *Quercus leucotrichophora* and *Q. glauca*, it was found that *in vitro* plants of both the species were comparable to seedlings in terms of gas and water vapour exchange characteristics[23].

In the present study, it was observed that cotyledonary nodes showed significantly higher shoot multiplication rate, shoot length and the number of nodes per shoot than leaf nodes. It is concluded that *ex vitro* rooting method was a more suitable for the rooting of shoots. A mixture of coarse sand, clay and farmyard manure (1:1:1) was the best growth substrate for acclimatization of rooted plantlets. The results of comparative growth performance study of tissue culture raised plants and seedlings indicated that the former could be adopted for the successful cloning of *P. santalinus*.

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