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Propagation Studies of Red Sanders (*Pterocarpus santalinus* L. f.) *In Vitro*—An Endangered Taxon of Andhra Pradesh, India

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ABSTRACT: Red sanders, the pride of Andhra Pradesh, which is confined to a narrow restricted area was selected for the present study based on its major limitations for conventional methods of propagation. Efficient protocols have been established for shoot multiplication *in vitro*. Factors responsible for rapid multiplication were screened at various levels. The highest shoot bud regeneration frequency (10-15%) was achieved by culturing mesocotyl explants on B_5 medium fortified with 3 mg/L 6-benzyl aminopurine (BAP) + 1 mg/L napthalene acetic acid (NAA) within a six week culture period. Shoot tip necrosis expressed in regenerated shoots, was controlled. Shoots treated with indole acetic acid (IAA), NAA and indole butyric acid (IBA) 1 mg/L each prior to transferring them to the rooting medium exhibited better rooting than those with no prior treatment.

KEY WORDS: ***Pterocarpus santalinus*, Endangered taxon, Tissue culture, Micropropagation.**

INTRODUCTION

Red sanders (*Pterocarpus santalinus* L.f.), belonging to the family Fabaceae, is an endangered and endemic taxon to Andhra Pradesh, India, restricted to certain forest tracts of Chittoor and Cuddapah districts. Red sanders is renowned for its characteristic timbers of exquisite colour, beauty and superlative technical qualities and ranks among finest luxury woods in the world. Wavy grained wood is used for making a musical instrument, Shamisen in Japan (Kesava Reddy and Srivasuki, 1990). A natural dye, santalin, extracted from red sanders wood is used for coloring pharmaceutical preparations, food stuffs etc. Extracts of wood and fruit find extensive applications as astringents, diaphoretics, external applications for inflammations, headache, skin diseases, bilious infections and chronic dysentery (Anonymous, 1969). This fabulous tree, which formed an irresistable temptation to the smuggler, deserves greater research attention and extensive organized plantation production.

Over exploitation without commensurate replenishment of natural strands has posed a severe threat to the very existence of this pretty and precious timber tree (Ahmed and Nayar, 1984). Natural regeneration has major constraints, such as prolonged dormancy, low germinability and poor viability of seeds. Clonal multiplication through rooted cuttings is difficult. Dayanand and Lohidas (1988), Kesava Reddy and Srivasuki (1990) reported various limitations involved in propagation of Red sanders by natural and conventional means. Tissue culture offers an immense potentiality for mass multiplication of elite Red sanders. Although tree species in general and legumes in particular are recalcitrant to

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regeneration *in vitro,* considerable success has been obtained in achieving organogenesis in tree species (Mascarenhas and Muralidharan, 1989). In earlier investigations regarding tissue culture of Red sanders, Sarita *et al.* (1988) reported differentiation of shoots from callus derived from shoot tip cultures. Lakshmi Sita *et al.* (1992) cultured shoot tips of aseptically raised seedlings and reported a maximum of eight shoots per explant. Elongation of cultured shoot tips and four shoots per explant were reported in *Pterocarpus santalinus* (Kesava Reddy and Srivasuki, 1992; Mithila and Srivasuki, 1992). However, we found no information regarding factors responsible for inducing rapid multiplication in *vitro.* The objectives of the current research were to (a) evaluate the ability of various explants to regenerate *in vitro*, (b) determine the influence of media, hormones (individual and in combinations), carbon source and coconut milk on regenerative ability and (c) standardize an efficient protocol for rapid multiplication and rooting.

MATERIALS AND METHODS

Mature dried pods were collected from Tirumala hills, Tirupati, Andhra Pradesh, India. After removing wings, pods were soaked in 50% HCl + 50% ethyl alcohol for 2-3 hours. The acid alcohol mixture was discarded and pods were washed thoroughly under running tap water to remove traces of acid, blotted and air dried for two days. Pod coats were separated by hand and seeds were used for aseptic germination on 1/2 strength B_5 medium supplemented with 0.05% activated charcoal and 0.1 mg/L BAP. A germination frequency of 40-70% was recorded within a period of 24 hours. Within 15 days after seeds were started seedlings attaining a height of 6-7 cm and containing 3-4 nodes were used for establishing tissue cultures.

Explant evaluation

Eight types of seedling explants — root, hypocotyl, mesocotyl, cotyledon, shoot tip, nodal, leaf and internodal segments were cultured on B_5 medium fortified with 2 mg/L BAP to identify the most suitable explant for *in vitro* bud multiplication. Explant selected for multiple shoot regeneration were cultured on Murashige and Skoog (MS) (1962), B_5 (Gamborg, *et al.*, 1968) and Woody Plant Media (WPM) (Lloyd and McCown, 1981) supplemented with 2 mg/L BAP. Selected explants were screened by inoculating on selected media, supplemented with BAP or KN or a combination (BAP + KN) or cytokinin along with auxin at various concentrations as shown in tables. The influence of different concentrations of sucrose (1-10%), various carbon sources (2%) and coconut milk (5-20%) were also tested for effects on shoot bud proliferation.

Rhizogenesis

Half and full strength MS medium and White's medium, either liquid or solid, with or without hormones, were employed. Excised shoots, either with prior treatment (NAA, IAA, IBA 1 mg/L each) for 24, 48 and 72 hrs or without treatment were transferred to rooting media.

The basal medium used was that of MS (Murashige and Skoog, 1962), White's medium (White, 1963), B_5 medium (Gamborg *et al.*, 1968) and woody plant medium (Lloyd and McCown, 1981). The pH of the medium was adjusted to 5.7-5.8 before autoclaving at 121℃ for 15 min.

RESULTS AND DISCUSSION

Protocols for organogenesis

Role of explants during organogenesis: The morphogenetic responses of all the explants were studied under identical cultural conditions (Table 1). All explants resumed growth, either by proliferating callus, or by inducing shoot buds after 7-10 days of culture (Fig. 1). Among the various seedling explants, the frequency of organogenesis, as evident from percentage of shoot buds and number of shoot buds differentiated per explant, was maximum from mesocotyl followed by nodal and shoot tip explants.

The general recalcitrance of leguminous tissues for organogenesis has been repeatedly highlighted (Mohan Ram *et al.*, 1981; Lakshmi Sita *et al.*, 1992). It appears that the failures reported by many workers may be partly due to the lack of thorough search for the appropriate explant for each species (Mohan Ram *et al.*, 1981). In this species mesocotyls performed well with high morphogenic potentiality followed by nodal and shoot tip explants.

Table 1. Morphogenesis responses of various seedling explants of *Pterocarpus santalinus* cultured on B_5 medium fortified with 2 mg/L BAP.

Explant	% of explants responding		Nature of response
	Callus	Shoot buds	
Root	50	0	Brown callus by rupturing epidermis
Hypocotyl	90	0	White callus at the cut ends
Mesocotyl	0	100	5-8 shoot buds
Cotyledon	100	0	White friable callus from the cut ends
Shoot tip	0	75	3-4 shoot buds
Nodal	0	80	White callus at the cut ends
Leaf	80	0	2-3 shoot buds
Inter nodal	70	0	White callus at the cut ends

Data represent average values of 20 replicates.

Media evaluation

Because mesocotyl explants showed a high frequency of multiple shoot induction, these were selected to assess the effects of other cultural factors in organogenesis. Choice of media (MS, WPM & B_5) not only influenced the per cent frequency of shoot bud regeneration but also the nature of response of mesocotyl explants (Fig. 2). Results revealed that a higher per cent frequency of shoot bud regeneration (100) and 7-8 multiple shoots per explant could be obtained on B_5 medium than on MS and WP media (Table 2). Although this media dependent variability was noticed in per cent frequency of shoot bud differentiation and number of multiple shoots formed per explant, there was no remarkable difference in length of shoots. High per cent frequency of multiple shoot regeneration was noticed on the B_5 medium but the quality of shoots was inferior as the regenerated shoots showed leaf fall and shoot tip necrosis after the 2nd passage (Fig. 3). Leaf abscission and shoot tip necrosis were not observed on MS medium (Fig. 4); however, delayed leaf fall and necrosis were noticed on WP medium.

Lakshmi Sita and Raghava Swamy (1993) have presented a detailed discussion concerning shoot tip necrosis and leaf fall in *Dalbergia latifolia* on MS medium. They reported delayed leaf fall associated with necrosis on WP medium when compared to MS medium. This is in contrast to our results. The media-dependent response has been

emphasized by several researchers. Brand and Lineberger (1986) reported better bud proliferation of *Helesia carolina* on WP medium than on MS medium. Bargchi and Alderson (1985) solved this problem in *Pistacia vera* by culturing on MS medium and the effect was attributed to insufficient uptake of calcium due to callusing and low transpiration rate caused by high humidity conditions in the vessel; however this hypothesis was studied and ruled out in *Dalbergia latifolia* by providing orthodox cotton plugs to decrease the humidity (Lakshmi Sita and Raghava Swamy 1993). Shoot tip necrosis in several temperate trees could not be contained by increasing the calcium levels in the medium (Shah *et al.*, 1985), for example, in *D. latifolia*, even by doubling the calcium strength in MS and WP media. WP medium containing NH_4^+ ions, low levels of $N0_3^-$ and enriched sulphur concentration prevented leaf abscission in rosewood cultures (Lakshmi Sita and Raghava Swamy, 1993).

Table 2. Responses of shoot tip, nodal and mesocotyl explants of *Pterocarpus santalinus* on three media fortified with 2 mg/L BAP

Medium	Explant	% frequency	Number of shoots per explant	Nature of response
B_5	Shoot tip	70	3-4	Shoot tip necrosis
	Nodal	80	2-3	Shoot tip necrosis
	* Mesocotyl	100	7-8	Shoot tip necrosis
MS	Shoot tip	50	2	Healthy growth
	Nodal	60	2	Healthy growth
	* Mesocotyl	90	3	Healthy growth
WP	Shoot tip	65	2	Delayed necrosis
	Nodal	65	2	Delayed necrosis
	* Mesocotyl	80	2	Delayed necrosis

Date represent average of 20 replicates.
* Cotyledonary node.

Lakshmi Sita *et al.* (1992) reported healthy shoot formation in *Pterocarpus santalinus* cultured on B_5 medium. Contrary to their observation we could not obtain healthy shoots on B_5 medium.

However in the current investigation, an efficient system for healthy multiple shoot regeneration was standardized by culturing the explants on Bs medium initially. Multiple shoots were later transferred to MS medium for healthy growth with out precocious leaf drop and necrosis. This procedure was further improved by reducing the subculture period to 10 days. This may be regarded as a simple and efficient technique to overcome the bottlenecks and to obtain high rate of multiple shoots without leaf drop and shoot tip necrosis.

Role of hormones and their interaction on organogenesis

For high frequency regeneration of multiple shoots, a range of cytokinins either individually or in combination with other cytokinins or auxins were used and results are shown in Tables 3 and 4. Of all the treatments used individually or in combinations, a medium supplemented with 3 mg/L BAP + 1 mg/L NAA induced the highest numbers of multiple shoots per explant (10-15 % frequency) (Figs. 5-7). The nature of response varied with each treatment and a certain degree of rooting was also noticed on media supplemented with KN + BAP (2 mg/L each), NAA + BAP (1 mg/L each).

Root Hypocotyl Cotyledon Internode Shoot tip Mesocotyl

1 2 3 4 5

Fig. 1. Regeneration response of various seedling explants. Fig. 2. Response of mesocotyl explants cultures on three media. Fig. 3. Shoot tip necrosis of regenerated shoot on B_5 medium. Fig. 4. Healthy shoots on MS medium. Fig. 5. Effect of BAP (mg/L) on shoot regeneration.

Table 3. Effect of different concentrations of BAP and KN on regeneration response of mesocotyl explants of *Pterocarpus santalinus* on B_5 medium.

Hormone	Conc. mg/L	% of response of shoot	Length of shoots (cm) Mean ± S.E.	Nature of response
BAP	1	90	4.22±0.053	Single shoot
	2	100	3.04±0.024	5-8 Shoot buds
	3	100	3.09±0.032	2-3 Shoots, axillary branching
	4	60	2,10±0.047	2 shoots with callus at the cut ends
	5	5	1.25±0.019	Single shoot, friable callus at its cut ends in all the cultured explants
KN	1	85	5.08±0.028	Single shoot
	2	90	3.28±0.042	4 Shoots
	3	80	3.37±0.043	2-3 Shoots
	4	80	3.00±0.033	2 Shoots with branchings
	5	25	2.55±0.050	2 Shoots

Data represent average of 20 replicates.

Table 4. Effect of different hormonal combinations on shoot bud regeneration in mesocotyl explants of *Pterocarpus santalinus*.

Hormones (mg/L)			% of Shoot bud regeneration	Average length (cm)	Nature of response
KN	BAP	NAA			
1	1	0	50	4.50±0.065	1-2 Shoots
2	2	0	15	1.00±0,000	Single shoot, Rooting pronounced
2	1	0	85	2.45±0.064	7-10 Shoots
1	2	0	65	4,55±0.050	2 Shoots with branching
0	3	0.1	60	2.05±0.010	Single shoot
0	3	0.5	75	4.40±0.065	2-3 Shoots
0	3	1	100	2.50±0.024	10-15 Shoots
0	1	1	100	6.16±0.060	Single shoot, occasional rooting

Data represent average of 20 replicates.

Although kinetin has been considered as a critical factor in bud initiation and termed highly effective by Miller (1961), the effectiveness of BAP in the present study is in conformity with earlier reports in other tree legumes such as *Acacia* auriculiformis (Mittal *et al.*, 1989), Albizia *lebbeck* (Gharyal and Maheshwari 1983, 1990) and *Parkinsonia* (Mathur and Mukuntha Kumar, 1992). Besides optimizing the concentration of individual cytokinins for high frequency multiple shoot induction the combined effects of BAP + Kinetin and BAP + auxin at varied concentrations were studied and the response was significantly varied when compared to individual cytokinins. Since discovery of the synergistic effect of auxin and cytokinin by Skoog and Miller (1957), it has been used for the induction of shoots and their subsequent development into plantlets, even in many legume trees such as *Leucaena leucocephala* (Datta and Datta, 1985).

In a previous study with *Pterocarpous santalinus* , shoots with scaly leaves were reported when only one cytokinin was used (Sarita *et al.*, 1988). Lakshmi Sita *et al.* (1992), by employing a combination of cytokinins, reported well expanded normal leaves in Red sanders. They extended their work for multiple shoot induction with a combination of cytokinins and obtained eight shoots per explant. In the present study, 10-15 shoots were obtained on a medium containing 2 mg/L KN and 1 mg/L BAP.

Carbon source

The influence of different concentrations of sucrose (1-10%) on morphogenetic response of mesocotyl explants was studied (Table 5). Vigorously growing 8-9 multiple shoots per explant were observed at 2 and 3% sucrose levels (Fig. 8). As the concentration increased steadily stout stems without leaves were seen and at higher concentrations sucrose suppressed shoot regeneration.

In similar studies with *Dalbergia latifolia* Lakshmi Sita and Raghava Swamy (1993) reported 3-4% sucrose as optimal while concentrations above 4% suppressed shoot bud differentiation. Sucrose is one of the more costly ingredients used in media preparation. To reduce the cost of the medium for plant multiplication, cane sugar ranging from 1-4% was added to the medium as a carbon source. Mesocotyls showed comparatively better response at 3% sugar and so this can be preferred for commercial exploitation.

Table 5. Effect of different concentrations of sucrose on regeneration of mesocotyl explants of *Pterocarpus santalinus* cultured on B_5 medium.

Sucrose (%)	% of Cultures responded	Average length (cm) ±S.E.	Nature of response
1	80	4,04±0.043	2 Slender unhealthy shoots
2	100	3.66±0.051	5-8 Healthy shoots
3	100	9.71±0.029	8-9 Healthy Aoots with vigorous growth
4	60	4.00±0.062	3 Unhealthy shoots without leaves
5	35	2,97±0.065	1 Stout shoot without leaves
6	25	3.01±0.042	1 Stout shoot without leaves
7	15	3.05±0.035	1 Stout shoot without leaves
8	10	3.10±0.047	1 Stout shoot without leaves
9	0	0.00±0.000	No response
10	0	0.00±0.000	No response

Data represent average of 20 replicates.

Effect of coconut milk

Coconut milk at three concentrations produced vigorous growth, but did not produce shoot regeneration except for two shoots in a few cultures (Table 6 and Fig. 9). However, branching in the shoot tip region was prevalent and these branches could be excised and used for further multiplication. Of the three concentrations tested, 10% coconut milk produced greatest number of branches at the tip of the shoot.

Table 6. Effect of different concentrations of coconut milk on mesocotyl explants of *Pterocarpus santalinus* cultured on B_5 medium.

Coconut milk (%)	% of cultures responded	Average length (cm) ±S.E.	Nature of response
5	50	4.70±0.032	2 Shoots with 10 axillary branches
10	60	5.04±0.072	Single shoot with terminal branching
15	85	3.50±0.021	Shoots
20	40	0.55±0.050	Single shoot, necrosis

Data represent average of 20 replicates.

After the pioneering attempt of Van Overbeek *et al.* (1941), coconut milk was used for the development of very young embryos of *Datura stramonium* and many tissue culturists exploited this natural extract for regeneration purposes in herbaceous plants. Scant attention was paid to the use of coconut milk in leguminous tree tissue cultures except a few. Mittal *et al.* (1989) reported callus formation with the use of coconut milk, but there was no evidence regarding the shoot or root regeneration. Lakshmi Sita and Raghava Swamy (1993) reported neither positive nor negative influence of coconut milk in *Dalbergia latifolia.*

Rooting of in vitro grown shoots

For successful establishment of in vitro propagation of tree species the most critical and important step is rooting. The regenerated leafy shoots grown on elongation medium were transferred to rooting media to obtain complete plantlets (Fig. 10). Different media were tested for rhizogenesis. Single shoots with 3-4 nodes were transferred to 1/2 strength MS liquid medium fortified with 1 mg/L of NAA, IAA and IBA. After different periods of incubation viz., 12, 24, 48 and 72 hours, the treated shoots were transferred to various rooting media such as RM_1, RM_2, RM_3, and the results are presented the Table 7. There was no rooting response in the shoots treated for 12 hours and hence these were excluded from Table 7. Among all the treatments and the media tested for rhizogenesis, better rooting was noticed from shoots treated with auxin for 24 hours and transferred to RM_3 medium. Shoots treated for more than 24 hours, either initiated callus at the base or showed no rhizogenesis.

Burstrom (1953) entertained the possibility that factors other than auxins be responsible for root initiation, though auxins are the major and most critical factors in root primordial differentiation. Different species require specific auxins at particular concentrations and sometimes root differentiation also depends on endogenous auxin concentration.

Table 7. Rooting response of regenerated shoots in *Ptercarpus santalinus* on various media with different treatments.

Rooting medium	% of rooting			Nature of response
	24 h	48 h	72 h	
RM_1	0	10	0	Adventitious roots
RM_2	0	15	0	Single stout root
RM_3	10	50	25	Single root, with root hairs

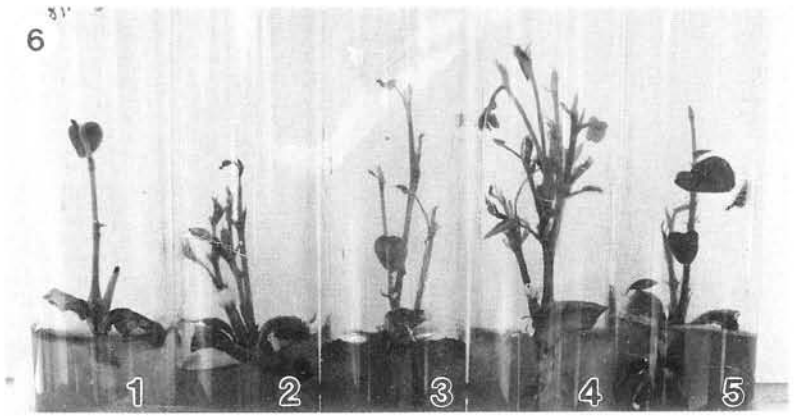
RM_1= $^1/_2$MS (liquid) media without hormones.

RM_2= $^1/_2$White's liquid media without hormones.

RM_3= $^1/_2$ MS (solid) + 1AA, IBA, NAA (0.1 mg/L each).

Data represent average of 20 replicates.

Bonner (1965) considered that IAA acts as gene activator, i.e., it triggers the early formation of root primordia. Ryogo (1974) proposed that the principal role of IBA (the most effective root inducing auxin in conventional propagation) is to favour conjugation between endogenous IAA and amino acids which leads to synthesis of specific proteins necessary for the formation of root initials. For root elongation, exogenous auxin is usually not required or is even inhibitory. This statement is in agreement with results of the present study since root elongation was vigorous after transferring plantlets to hormone free media, However, IBA, which is considered as the most effective auxin, is ineffective in the case of *Pterocarpus santalinus.* Rooted, regenerated shoots were transferred to sand and soil mixture and high humidity conditions were maintined for 15 days (Fig. 11).



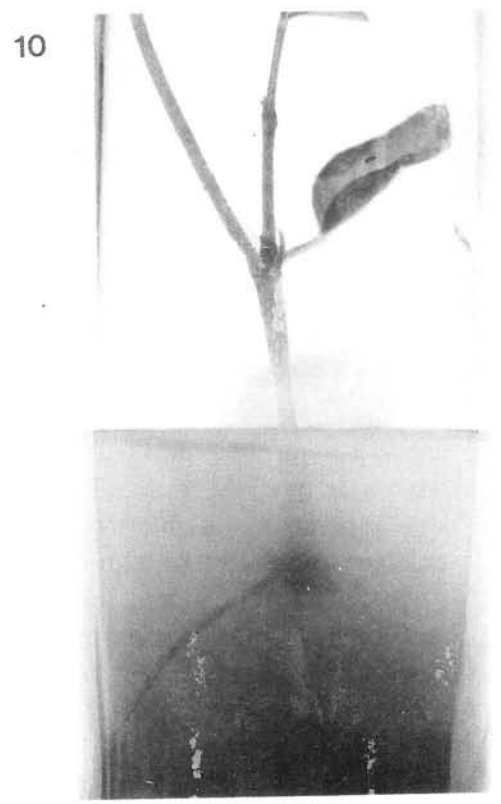


Fig. 6. Effect of KN (mg/L) on shoot regeneration. Fig. 7. Effect of auxin in combination with cytokinin. Fig. 8. Effect of sucrose (%) on shoot regeneration. Fig. 9. Effect of coconut milk (%) on shoot regeneration. Fig. 10. Rooting of in vitro shoots.

Fig. 11. Potted in vitro regenerated plantlet.

Clones of mature trees are generally preferred over seedling explants because it is often not possible to determine whether these embryos or seedlings have the genetic potential to develop the desired qualities in their life cycle (Bonga, 1977). However, most of the studies with trees have used seeds and juvenile tissues that are more amenable to in vitro manipulations than tissue of mature plants (Bonga, 1977). This is because, generally, mature trees or juvenile tissues collected from old trees are not amenable to *in vitro* propagation. The reasons may be accumulation of phenolics, high degree of contamination, physiological variations due to seasonality and loss of morphogenetic potentiality.

Arya and *Shekhawat* (1987) emphasized the recalcitrant nature of woody species with respect to morphogenesis. The principal character of woody species which make them more intractable are short seasonal period of time when any particular tissue or stage of development is available for culture, long period of requirement for regeneration and finally long term commitment required to produce results in woody plants.

In the current study, an efficient system was developed for *in vitro* propagation of seedling explants. In spite of many efforts to adopt the technique to mature tree explants, these efforts failed, except for limited bud proliferation and callus induction. Accumulation of phenolics, browning of callus tissue, 50 % contamination with microbes was observed in cultured tissues.

This established *in vitro* technique for rapid shoot bud proliferation, produced 486 plants from a single aseptically raised seedling of red sanders (Fig. 12). Results produce a means of presentation and propagation of this valuable species.

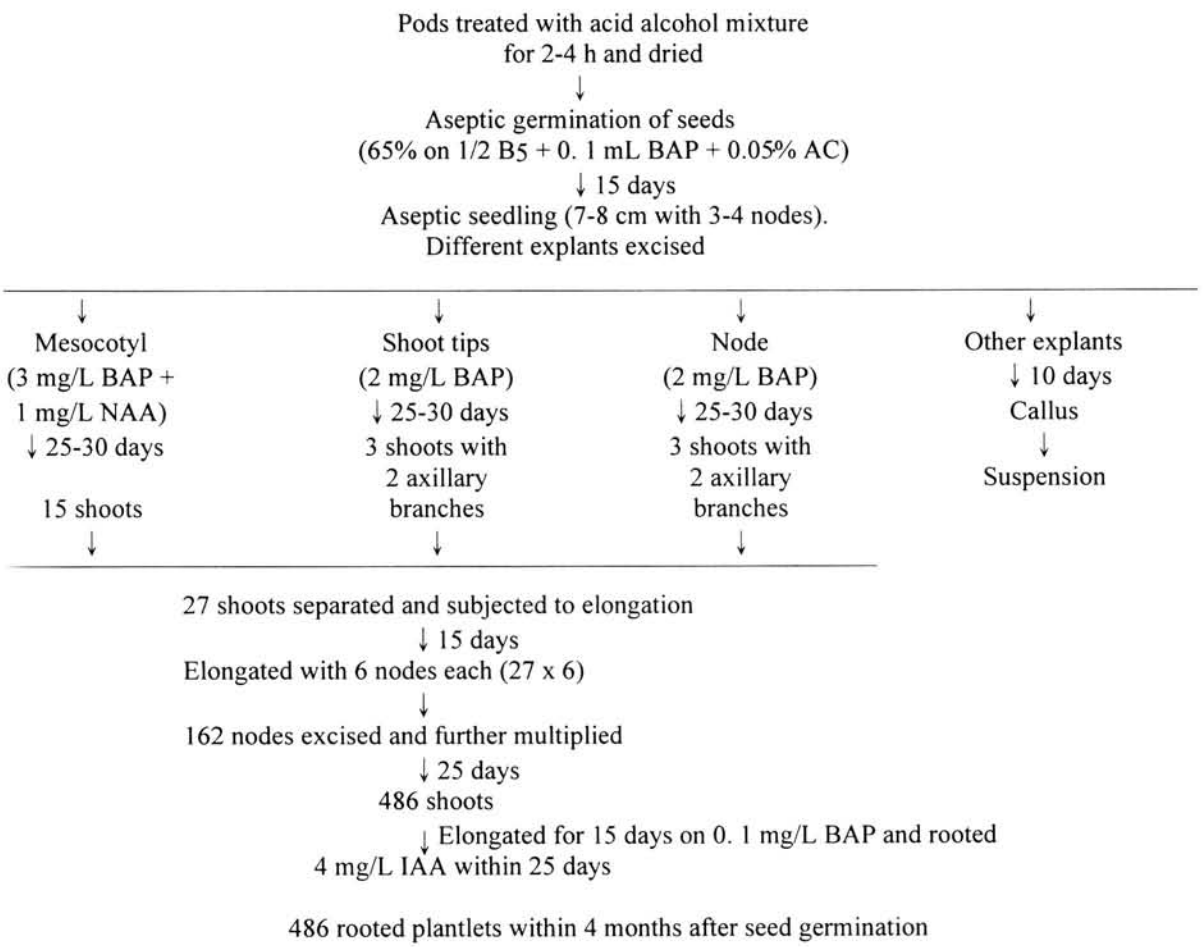


Fig. 12. Flow chart of the established protocol for rapid multiplication of *Pterocarpus santalinus.*

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紅紫檀試管繁殖研究，一種印度 Andhra Pradesh 地區瀕臨絕種的植物

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摘　　要

紅紫檀生長於範圍狹小的特定地區，並且是一種不能使用一般方法繁殖的植物。本研究已建立使用枝梢部份繁殖的有效方法，並且依不同層次篩選出影響其快速繁殖的因子。使莖頂芽能夠快速繁殖（再生頻率 10-15%）的方法就是把中胚軸培養於 B5 培養劑中，額外加入 3 mg/L BAP 及 1 mg/L 的 NAA 培養六星期。再生莖其莖頂壞死現象已被控制。莖部先處理 1 mg/L 的 IAA，NAA，IBA 然後再移入生根培養液中的莖，比事先未處理者能夠長出較好的根。

關鍵詞：紅紫檀、瀕臨絕種、組織培養、微繁殖。

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