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In Vitro Plant Regeneration of *Pterocarpus santalinus* L.f (Red Sanders) – An Endangered Medicinal Plant and Important Timber Tree

K. Padmalatha • M. N. V. Prasad*

Department of Plant Sciences, University of Hyderabad, Hyderabad, Andhra Pradesh, 500 046, India

Corresponding author: * **mnvsl@uohyd.ernet.in**

ABSTRACT

This paper describes multiple shoot regeneration of *Pterocarpus santalinus* L.f, an endangered tree endemic to the Deccan region which is commercially and medicinally most valuable for its heartwood on the international market. Seed germination under *in vivo* (in field conditions) and *in vitro* conditions were 60% and 100%, respectively. The nodal segments from *in vitro* regenerated shoots and from mature trees proliferated into multiple shoots (mean = 6.5) on Murashige and Skoog (MS) medium supplemented with 6-benzyl amino purine (BAP), kinetin (KN) and thidiazuron (3.0 mg L^{-1}) individually. The seed explants (embryonic axis along with the cotyledons), when cultured on MS medium containing a combination of KN and BAP (1.0 + 2.0 mg L^{-1}) formed 19-20 multiple shoots. MS basal medium was found suitable for rooting (70-80%). On transfer to a glasshouse 20% (3 plantlets/explant) survived.

Keywords: conservation, cytokinins, micropropagation, seed germination, seed dormancy

INTRODUCTION

Pterocarpus santalinus L.f (Fabaceae) commonly known as "Red Sanders" is an important commercial tree of international trade. It is an endangered and endemic tree that occurs in Southern regions of the Eastern Ghats (Ahmed and Nayar 1984; Jadhav *et al*. 2001). About 50 t/year (1988-1993) of powdered heartwood was traded internationally and in 2005-2006 an average of 1500 t were exported (Kala 2006). The major exporter is India and the importers are Western Europe, China and Japan, in the latter where it is generally used for the manufacture of a traditional musical instrument, the "Shamisen". Demand by Japan for "wavy grain" quality timber resulted in significant illegal and destructive exploitation of the wild resource in the 1960's and controls were imposed on trading. At an average market rate of Rs 75/Kg and income of Rs. 177.5 lakhs/ha ($375,000/ha) is expected. The market price of wood available during 2003-2006 in India was recorded (**Table 1**) (Kala 2006). Medicinally it is extensively used (**Table 2**).

Table 1 The maximum market price of *P. santalinus* wood (2003-2006). Data from Kala 2006.

Year	Weight (Metric tonnes)	Cost (Crores, Approx.)*
2003-2004	151	6.00
2004-2005	347	13.88
2005-2006	449	17.98

* 1 Crore (= 10 million) Indian rupees = current (2008) exchange is 250,000 US$

Kesavareddy and Srivasuki (1990) reported various limitations involved in propagation of red sanders such as prolonged dormancy, low germinability and poor viability of seeds. In field conditions 60% germination was observed from seeds collected from Balpally. *In vitro* studies showed

Table 2 Medicinal importance of *Pterocarpus santalinus* L.f (based on Parrota 2001).

Part used/alkaloid	Medicinal uses
Wood powder	Astringent, tonic, antipyretic, antihelminthic, antiperiodic, diaphoretic, alexeritic, spider poisoning, freckles, defects of vision, bone fractures, leprosy, scorpion sting, hiccough, ulcers, general debility, mental aberrations, bleeding piles, vomiting, eye diseases, headache, haemophilic disorders, inflammation, ulcers, blood purifier, skin diseases, fever, inflammation, toothache, hemicrania
Wood + Bark brew	Chronic dysentery, worms, blood vomiting, weak vision, hallucination
Wood + Fruit extracts	Astringent, diaphoretics, inflammations, headache, skin diseases, bilious infections, chronic dysentery
Wood powder + Dust	Fish preservative
Wooden chips + Water	Diabetes
Stem bark powder + Soft porridge	Diarrhoea
Bark powder	Astringent, blood purifier, anti helminthic, antipyretic, antidiabetic, curing arecanuts
Condensed bark powder	0.38-0.45% of mild smelling essential oil
Condensed bark powder + alcoholic HCl	1.98-2.25% of mild smelling essential oil
Distil of wood	Medicine for heart diseases, blood purifier
Fruit decoction	Astringent, tonic, chronic dysentery
Pods decoction	Astringent, tonic-chronic dysentery, psoriasis
Santalin	Colouring pharmaceutical preparations, food stuffs, high class alcoholic liquors, paper pulp, etc.
Roots and stumps	Dyeing cotton and leather, staining the woods

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100% germination with 3% sucrose solution on a Murashige and Skoog medium, which is highly cost effective (Padmalatha and Prasad 2007). Clonal multiplication through rooted cuttings is also difficult. Poor seed germination was reported by Kalimuthu and Lakshmanan (1995). Although tree species in general and legumes in particular are recalcitrant to regeneration *in vitro*, considerable success has been obtained in achieving organogenesis in tree species (e.g. Mascarenhas and Muralidharan 1989; Jain and Ishii 2003). An earlier investigation on tissue culture of Red Sanders (Sarithapatri *et al*. 1988) reported differentiation of shoots from callus derived from shoot tip cultures and cotyledonary nodes. Lakshmisita *et al*. (1992) obtained a maximum of eight shoots from shoot tip cultures but required numerous steps in preparing the explants. At the same time, four shoots per shoot tip explant were also reported by Kesavareddy and Srivasuki (1992) and Mithila and Srivasuki (1992). Saritha *et al*. (1988) and Sinha *et al*. (2000) reported the formation of shoots with scaly leaves from cotyledonary nodes. Arockiasamy *et al*. (2000) reported the influence of growth regulators and explant type on *in vitro* regeneration.

However in all the above-mentioned cases the number of multiple shoots produced was low. Seed germination and micropropagation has been advocated as one of the most viable biotechnological tools for *ex situ* conservation of threatened germplasm (Kameshwararao 2004). In view of high price of the timber, restricted distribution and slow growth conventionally there is an urgent need to develop methods for rapid regeneration of plantlets of *P. santalinus*. The present study thus focuses on seed germination and standardization of an efficient protocol for multiple shoot regeneration using nodes and seed explant (embryonic axis along with the cotyledon) useful from a conservation point of view.

MATERIALS AND METHODS

Explant source

Pods (seed explant: embryonic axis along with cotyledons) collected from the forests of Cuddapah (home of Red Sanders), Andhra Pradesh, India and nodes (3-4 cm) shoot tips, cotyledonary nodal segments, excised from *in vitro* germinated seedlings and from mature trees on the University of Hyderabad (UH) campus) were used.

Surface sterilization

Mature dried pods were scarified by soaking them in tap water overnight or by using boiling water i.e., at 100°C (5 mins) or sulphuric acid (5% for 10 min) and the seed coat was removed mechanically. The explants (seeds and nodal segments) were initially washed in tap water (30 min) treated with 2% Bavastin® (30 min for seeds), 15 min for nodal segments), 70% (v/v) ethanol (2 min) and with 0.1% (w/v) mercuric chloride (12 min for seeds), 15 min for nodal segments. All the explants were rinsed 4-5 times in sterile double distilled water.

Standardization (media, agar, sucrose, explant orientation, cytokinins)

Initially to find the influence of different types of basal media multiple shoot regeneration attempts were made using MS (Murashige and Skoog 1962), B_5 (Gamborg 1968) and McCown (Lloyd and McCown 1980) media. Initially different parameters like agar and sucrose were tested in all the above mentioned media but since a better response was observed only on MS medium further experiments were continued with MS medium alone. Effect of different percentages of agar (0.5, 0.6, 0.7 and 0.8%), sucrose (1, 2, 3 and 4% w/v) on MS medium apart from the effect of vertical and horizontal orientation of the explants was also checked. For multiple shoot regeneration seed and nodal explants were cultured on MS medium with different cytokinins: 6-benzyl amino purine (BAP) (0.1-7.0 mg L^{-1}), kinetin (KN) (0.1-7.0 mg L^{-1}), thidiazuron (TDZ) (0.1-7.0 mg L^{-1}), TDZ (2.0 mg L^{-1}) in combination with BAP (0.1-7.0 mgL^{-1}) and 2,4-dichloro phenoxyacetic acid (2,4-D) (0.1-7.0 mgL^{-1}) individually and in combination with other cytokinins like BAP (0.1-7.0 mg L^{-1}), KN (0.1-7.0 mg L^{-1}) and TDZ (0.1-7.0 mg L^{-1}). Nodal segments of elongated shoots from seed explants were transferred to MS medium supplemented with 1.0 mgL^{-1} BAP for stage II multiplication. All the cultures were maintained at 25 ± 2°C under a 16/8 h photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 $\mu Em^{-2}s^{-1}$ provided by white fluorescent tubes (Saphirre Scientifics, India).

Rooting and acclimatization

For rooting, 5-6 cm-long regenerated shoots from the seed explants and nodal segments were excised and cultured on using different strengths of MS medium alone and in combination with various auxins like indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) (0.1-10.0 mg L^{-1}). After plantlets attained a height of 8-10 cm, in the first week, they were transferred to perforated plastic cups with autoclaved Soilrite® for gradual acclimatization initially in the tissue culture then in the greenhouse and nurtured initially with MS liquid (basal) solution without sucrose, every alternate day. Irrigation with water was done after 15 days. The plants were covered with plastic wrapper to prevent humidity loss and were maintained at 25 ± 2°C and 60-70% relative humidity under light of density (PPFD) of 83.6 $\mu Em^{-2}s^{-1}$ in the tissue culture laboratory. After 15-20 days the plastic wrappers were removed and the plantlets were transferred to a mixture of Soilrite®, manure and sand in a 1:1:1 ratio. The complete hardening process was carried out in the glasshouse. The percentage survival was recorded at this stage.

Statistical analysis

Statistical analysis of the data was carried out by using one-way analysis of variance (ANOVA) by following the procedure of the Student-Newman-Keuls Method to check the significance of the treatments. All the experiments were repeated thrice and 40 explants were used for each treatment.

RESULTS

Initially when the nodal and seed explants were cultured on different basal media like MS, McCown's and Gamborg, they did not show much difference and hence further experiments were carried out on MS medium, which is the most universally accepted standard medium amenable for good and efficient growth of multiple shoots. In *in vivo* conditions a 50-60% seed germination was observed while in *in vitro* conditions 100% was observed on MS (liquid), ½ MS (liquid), 3% sucrose, 1% agar and MS with 0.1% phytagel (Padmalatha and Prasad 2007). Horizontal orientation of the explant, 3% sucrose and 0.6% agar in MS medium fortified with 1.0 mg L^{-1} BAP were found to be effective for multiple shoot regeneration when compared to vertical orientation (**Fig. 1A, 1B**).

Nodal explants, when cultured on MS medium supplemented with 3.0 mg L^{-1} BAP and 3.0 mg L^{-1} KN resulted in more multiple shoots (6.5) whereas MS medium fortified with different concentrations of TDZ developed an average of 4.5 number of shoots at 1.0, 2.0 and 3.0 mg L^{-1} while a combination of TDZ and BAP showed a maximum of 6.5 shoots (**Table 3**) in addition to the development of hard brownish callus.

When seed explants were cultured on MS medium fortified with 2.0 mgL^{-1} KN and 1.0 mgL^{-1} BAP, after 15 days 19-20 multiple shoots with a maximum shoot length of 5.4 cm and with 8-9 nodes per shoot was observed (**Fig. 1C-E; Table 4**). When shoots were cultured in a bunch (3-4 shoots) on MS basal medium rooting was observed (**Fig. 1F**). On an average 4-5 bunches from a single seed explant were obtained. Each node from the regenerated when cultured on MS medium with 1.0 mgL^{-1} BAP, 3-4 multiple shoots were observed (**Fig. 1G**). After acclimatization in the glasshouse, 20% of the shoots survived (3 plantlets per ex-

Fig. 1 (**A**) Seed germination on MS basal medium after 1 week of inoculation; (**B**) Multiple shoot induction on MS basal medium; (**C**) Multiple shoot regeneration on MS supplemented with 1 mg L^{-1} BAP and 2 mgL^{-1} KN; (**D**) Rooting on MS medium solidified using 0.25 % phytagel; (**E**) Multiple shoot regeneration from node after subculturing on MS with 1 mg L^{-1} BAP; (**F**) Acclimatization of plants in glasshouse; (**G**) Acclimatized plant of *Pterocarpus santalinus* in a glasshouse.

Table 3 Influence of different cytokinins on multiple shoot regeneration from nodal segments after five weeks of culture (values are mean ± SD of 20 replicates of 3 experiments).

Medium + PGR mg L^{-1}	BAP Av. length (cm) ± SE	% Response	KN Av. length (cm) ± SE	% Response	TDZ Av. length (cm) ± SE	% Response	BAP (3 mg L^{-1}) +TDZ Av. length ± SE	% Response
MS basal	2.3 ± 0.15	20	-	40	1.5 ± 0.08	40	2.0 ± 0.18	50
0.1	4.0 ± 0.25	35	3.0 ± 0.14	50	2.0 ± 0.12	50	2.0 ± 0.18	50
0.5	4.0 ± 0.25	35	3.5 ± 0.16	35	2.5 ± 0.15	75	2.3 ± 0.15	55
1.0	4.9 ± 0.18	60	4.0 ± 0.25	40	4.5* ± 0.13	40	2.3 ± 0.15	55
2.0	4.9 ± 0.18	60	4.5 ± 0.19	40	4.5* ± 0.13	40	4.0 ± 0.25	60
3.0	6.5* ± 0.21	50	6.5* ± 0.23	40	4.5* ± 0.13	40	6.5* ± 0.32	75
4.0	4.9 ± 0.18	50	3.5 ± 0.16	40	4.0 ± 0.17	40	2.2 ± 0.08	50
5.0	4.9 ± 0.18	50	1.9 ± 0.18	30	4.0 ± 0.17	40	2.0 ± 0.18	40
6.0	2.3 ± 0.15	40	1.9 ± 0.18	-	2.5 ± 0.15	30	2.0 ± 0.18	40
7.0	2.0 ± 0.05	-	-	-	1.5 ± 0.08	30	-	-

BAP: 6-benzyl amino purine; KN: kinetin; TDZ: thidiazuron.
* indicates highest number of multiple shoots in the particular hormone concentration

Table 4 Influence of different hormones on regeneration of multiple shoots from seeds as explants after five weeks of culture (values are mean ± SD of 20 replicates of 3 experiments).

Medium (mg L^{-1})	No. of shoots	Shoot length	No. of nodes	% Response
	Mean ± SE		Mean ± SE	
MS basal	2.33 ± 0.66	4.72 ± 0.65	4.88 ± 0.95	90
MS + 1.0 BAP	3.62 ± 0.46	4.09 ± 1.27	4.75 ± 1.38	80
MS + 1.0 KN + 1.0 BAP	3.66 ±1.13	2.42 ± 0.59	3.14 ± 0.8	60
MS + 2.0 KN + 1.0 BAP	17* ± 2.36	5.04* ±1.13	6.70* ± 1.5	90
MS + 1.0 TDZ	1.6 ± 2.77	1.8 ± 0.34	2.20 ± 0.0	60
MS + 1.0 TDZ+ 1.0 AH	1.0 ± 0.0	3.4 ± 0.23	6.00 ± 0.81	50
MS + 2.0 KN	2.5 ± 0.28	3 .0 ± 0.46	4.60 ± 0.69	60

* indicates highest number of multiple shoots in the particular hormone concentration

Table 5 ANOVA table for the influence of different cytokinins on multiple shoot regeneration from nodal segments in *P. santalinus*.

Source of variation	DF	SS (BAP)	SS (KN)	SS (TDZ)	SS (BAP+ TDZ)
Between treatments	9	190.162	362.76	163.122	272.850
Residual	90	32.025	27.3	27.325	32.40
Total	99	222.188	390.0	190.447	305.250

BAP: 6-benzyl amino purine; KN: kinetin; TDZ: thidiazuron.

plant) (**Fig. 1H-I**).

Other explants viz., leaves with or without petioles (1-4 cm, young and mature), internodal segments (1-2 cm) and shoot tips either collected from plantations in University of Hyderabad (UH) or from *in vitro* raised seedlings when cultured on MS media with different PGRs alone and in combinations, white and creamy friable callus was observed. No multiple shoot regeneration was noticed except with cotyledonary nodal meristem where initiation of 10-12 multiple shoots was observed, which did not elongate further even when treated with different cytokinins. The above data is only a visual observation which was not subjected to statistical analysis as work did not further since no shoot elongation was observed. The data in **Table 3** refers to nodal segments as explants but not to cotyledonary nodal segments.

Table 6 ANOVA table for the influence of different hormones on multiple shoot regeneration (no. of shoots) from seeds in *P. santalinus*.

Source of variation	DF	SS
Between treatments	7	1936.2
Residual	48	741.5
Total	55	2677.7

DF: Degrees of freedom; SS: Sum of Squares.

Statistical analysis using ANOVA in case of multiple shoot regeneration from nodal segments and from seeds as explants showed that the differences in the mean values among the treatment groups are significantly different from each other. In the case of nodal segments the difference in the number of shoots is less compared to that of shoots regenerated from seeds. In the case of seeds as explants the statistically significant difference observed may be due to difference in the composition of media i.e., use of PGRs at different concentrations (**Tables 5, 6**).

DISCUSSION

In *P. santalinus* seeds are highly recalcitrant with a dormancy period of one year. These show poor germination, perhaps due to temperature, water stress, predation, pod (seed) size, light intensity, soil and seed moisture, seasonal variations and site of collection (Kalimuthu and Lakshmanan 1995). This could be overcome to some extent by mechanical or acid scarification (Zodape 1991). Similar findings were also reported in *Olea europaea* and *Podocarpus falcatus* by Teketay and Gränstrøm (1997). As the plant demands strong light, seeds kept in dark did not germinate, which may be due to non-availability of some nutrient reserve to the newly originated seedling, which is a crucial requirement for the germinated seedling (Pullaiah and Anuradha 1999)

Seed size also played a major role in germination capacity as the rate of germination was higher in larger seeds compared to smaller ones as they contain more food reserves to stimulate germination, seedling survival and growth (Milberg and Lamont 1997; Padmalatha and Prasad 2007) from the cotyledons than from the soil and face very heterogeneous situations in field conditions. Low viability and decline in germination percentage may also be due to a loss of moisture, which is associated with a multiple-layered seed coat (3 layers), increased leachate conductivity and decreased fatty acid content due to aging in certain seeds (Thapliyal and Connor 1997). In *in vitro* conditions 100% germination was observed on 3% sucrose (Padmalatha and Prasad 2007), which is cost effective though this level of germination was also observed in other combinations of media. Germination was more effective (visual assessment) when seeds were cultured with the micropylar end touching the medium, which would facilitate direct nutrient uptake. Thus *in vitro* conditions proved to be very useful for enhancing seed germination and building seedling stocks.

In vitro plant regeneration

Even though there are a few reports on organogenesis and micropropagation of legume trees like *Acacia* spp. (Aradhana *et al*. 1989), *Albizia* spp. (Tomar and Gupta 1988), *Dalbergia sissoo* (Suwai *et al*. 1988) and *Sesbania* sp. (Khattar and Mohanram 1983) etc., regeneration has been generally from seedling explants which is not desirable from a tree improvement point of view due to chances for variability and lower multiplication rate. But in the present study among all the explants used i.e., nodal segments, shoot tips, cotyledonary nodal segments and seed, seed explants were found to be the bestt for producing an average of 19-20 shoots per explant within 5 weeks. Thus the seedling material of *P. santalinus* was perfectly suitable for considering its use as an explant for regeneration.

Role of media, sucrose, agar and orientation of the explant

For multiple shoot regeneration, sucrose at 3% was found to be optimum because higher concentrations of sucrose (4 and 5%) may increase the levels of polyphenols, which results in browning of cultures and subsequent growth inhibition. Agar at 0.6% was found to be most effective which might be due to its semi-solid nature, which allows easy uptake of nutrients, in turn causing the profuse proliferation of shoots.

Horizontal orientation was more effective than the vertical because in the former the whole surface of the explant is in contact with the semi-solid medium and sprouting was observed from all the exposed surfaces of the explant profusely (**Fig. 1A**). These were later transferred onto medium with similar hormonal composition in a vertical orientation for proper growth (**Fig. 1B**), as reported in chickpea (Polisetty *et al*. 1997). When 3-4 days-old seed explants were used with the micropylar end of the seed touching the medium, numerous multiple shoots were produced compared to 2-day-old explants. Thus the effect of age and orientation might be attributed to an increased period of starch accumulation during BAP treatment due to a corresponding reduction in solubilization of starch by α-amylase, which might be linked with multiple shoot production (Thorpe and Murashige 1970).

Nodal segments as explants

Successful shoot development was observed when nodal explants were used although fewer multiple shoots formed than seed explants. Around 80% of the nodal explants developed actively growing buds, mostly 6-7 shoots per explant after 30 days of culture on MS medium fortified with different cytokinins like BAP, KN and TDZ, either singly or in combination (**Table 3**). Higher frequencies of bud break and varying degrees of multiple shoot formation that occurred on BAP-supplemented medium compared to MS basal medium might be due to the breaking of dormancy induced by the hormones. Sprouting was better when buds were collected during the rainy season as axillary buds seem to be free from phenolic exudates allowing these explants to proliferate into multiple shoots. Callus developed more at higher and lower concentrations of cytokinins, either singly or in combinations. The dormant axillary bud swelled within a week followed by the differentiation into two to three shoot buds in three weeks was observed accompanied by the development of creamy-brown friable callus at different concentrations of KN, BAP and TDZ.

After five weeks the number of shoots observed were 6.5, 6.5, 4.5 and 6.5 respectively on MS with 3.0 mgL^{-1} BAP, 3.0 mgL^{-1} KN, 1.0, 2.0 and 3.0 mgL^{-1} TDZ and with 3.0 mgL^{-1} BAP + 3.0 mgL^{-1} TDZ. No relation was apparent between the amount of callus and the number of shoot buds per culture but callus and adventitious bud initiation took place simultaneously and varied greatly with the growth regulator composition of the medium. Nodal explants can be used for micropropagation where seed explants are unavailable alhough the number of harvestable shoots is less and rooting is difficult compared to seeds explants. Repeated subculturing helps to achieve continuous production of callus-free, healthy shoots at least through five sub-culture cycles. A similar phenomenon was also observed in *Morus australis* (Pattnaik *et al*. 1996).

Seeds as explants

According to the previous studies on micropropagation of *P. santalinus* Lakshmisita *et al*. (1992) reported that when shoot tips were cultured on B5 media supplemented with 1 mg L^{-1} BAP and 1 mg L^{-1} KN up to 8 shoots were observed within 4-6 weeks. Rooting was observed on MS media supplemented with 1-2 mg L^{-1} IAA. When cotyledons were used as explants and cultured on MS medium supplemented

with 0.1 mg L^{-1} NAA, 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} KN an average of 10 shoots were observed. Rooting was observed on MS media supplemented with 0.5, 1.0 and 1.5 mg L^{-1} of IAA (Arockiasamy *et al*. 2000). Mithila and Srivasuki (1992) reported 4 multiple shoots when shoot tips were used as explants on MS media with different cytokinins. Rooting was best observed on MS media supplemented with IAA, IBA and NAA in higher concentrations.

Among all the explants and media combinations tried, seed explants responded most favourably in the presence of 2.0 mgL^{-1} BAP and 1.0 mgL^{-1} KN for multiple shoot induction (**Fig. 1C-E**). As in *Albizzia chinensis*, a tree species, seeds, when initially cultured onto MS basal medium these produced 2-3 shoots and when transferred to MS basal media with BAP and KN shoot proliferation was observed while the mother explant remained intact (Sinha *et al*. 1991) An adverse effect of phenols on shoot differentiation was reported by Bhat and Chandel (1991). Increasing or decreasing the concentration of PGRs resulted in a decrease in the rate of shoot regeneration due to a significant increase in phenol content, especially when BAP was used. Superior multiple shoot formation using BAP over KN was demonstrated by several groups (Rech and Pires 1986; Vaneck and Kitto 1990; Kukreja *et al*. 1991; Mishra and Bhatnagar 1995). Significantly longer shoots were obtained in BAP than in KN (**Table 3**). This may also indicate that seed explants contain a sufficient endogenous level of auxin or are capable of its *de novo* synthesis, which can induce shoot formation even in medium containing cytokinin alone (Julliard *et al*. 1992). In the present study, cytokinins like BAP and KN did not aid much in the development of callus.

From a single seed, 80% of the shoots could root on MS without exogenous hormones. The shoots that failed to form roots during this period did not respond later on in culture, suggesting that when seeds are used as explants, a threshold level of endogenous PGRs might have accumulated during the initiation of culture, enabling them to develop an optimum number of multiple shoots initially on MS basal medium, and at reduced levels of BAP and KN without the need to add auxin. Induction of adventitious shoot buds in BAP-treated explants by suppressing the apical dominance was reported by Polisetty *et al*. (1997) in *Cicer arietinum* (green gram). Similar results were observed in case of *P. santalinus* when BAP was used as the phytohormone in MS medium alone and in combination with KN. At the same time maintenance of the auxin:cytokinin ratio was necessary for shoot differentiation in many regeneration protocols but it was avoided in the present study as the addition of auxins in the shooting media may induce variations and true-to-type plants might not be regenerated.

The origin of multiple shoot regeneration may be from embryos with intact embryonic axes and both cotyledons. Individually when an excised embryo alone was cultured on MS media supplemented with different concentrations of cytokinins like BAP, KN and TDZ alone and in combinations, no response was observed. Moreover it was a very difficult task due to the smaller size of the embryo and problems of contamination. Excising of one of the cotyledons, or both while keeping the embryonic axis intact appeared to adversely affect the number of shoots produced per explant (data not shown). Hence it can be concluded that cotyledons served as a support and also as a source of nutrients to the embryo for its proliferation. Similar studies were reported in *Vigna radiata* by Gulati and Jaiwal (1990, 1994) in which cotyledon size affected the regeneration ability of the explant. Their later study clearly demonstrated the requirement of embryonic axis along with cotyledons for inducing multiple shoots. Our results also agree with those of McKently *et al*. (1989) who concluded that cotyledons having intact embryos produced more shoots than other explant types, particularly those in which the embryo axis was excised.

In our experiments, shoot buds developed either at the base or junction of the two cotyledons i.e., possibly from the meristematic cells around the embryo and started elongating simultaneously along with the main shoot. The shoot emergence pattern suggested an axillary origin from the embryo. Adventitious shoots clearly arose sequentially from the basal peripheral region of an already emerged shoot, which was adjacent to a cotyledonary node. The extensive pattern of multiple shoot formation observed also supports that the synergistic effect of both BAP and KN promotes adventitious or axillary bud initiation. However no anatomical studies were done to understand the origin/type and also the extensive multiple shoot regeneration from the embryo as observed (**Fig. 1G**). In the present experiments, in the control treatment i.e., on MS basal media, the main shoot emerged in 3 days and developed into a seedling within 15 days, suppressing the emergence of adventitious buds but when placed in MS in contact with BAP and KN, the main shoot had emerged in 3 days, but its growth was suppressed due to the simultaneous proliferation of adventitious shoots after 3 weeks. Hence suppression of the main shoot growth could have resulted in adventitious bud development. Experimental data presented by Gambley and Dodd (1990) also supported this hypothesis in *Cucumis sativus* L. They demonstrated that if the apical dominance of an existing axillary bud is physically eliminated, a large number of shoots could arise in response to 1.0 µM of BAP and 200 mg L^{-1} of casein hydrolysate in MS medium. Histological evidence of such an adventitious/axillary origin of multiple shoots was reported in other plants in which a cotyledonary node with axillary bud was used as the explant, e.g. *V. radiata* (Gulati and Jaiwal 1994) and *C. cajan* (Prakash *et al*. 1994). Another possible factor may be that the seeds from Cuddapah region could be variants caused by polyembryony or apomoxis and further investigation of the origin of multiple shoots needs to be carried out.

Rooting

Auxins like IAA, IBA and NAA (0.1-10.0 mg L^{-1}) in MS medium were ineffective in rooting while activated charcoal (absorbent) had no effect. Rooting was observed when the shoots were separated into bunches of 3-4 shoots and cultured on MS basal media. Thus fully-grown plantlets with 30-40 expanded leaves and well-developed roots were transferred into magenta boxes successfully (**Fig. 1H-I**). The survival rate was 20% when the plantlets in Soilrite® were transferred to a glasshouse, forming 3 plantlets per explant.

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REFERENCES

Ahmed M, Nayar MP (1984) Red sanders tree (*Pterocarpus santalinus* L.f.) on the verge of depletion. *Bulletin of the Botanical Survey of India* **26**, 142-143

Aradhana M, Rina A, Gupta SC (1989) *In vitro* development of plantlets from axillary buds of *Acacia auriculiformis* a legume tree. *Plant Cell, Tissue and Organ Culture* **19**, 65-70

Arockiasamy S, Ignaemuthu S, Melchias G (2000) Influence of growth regulators and explant type on *in vitro* shoot propagation and rooting of Red sandal wood *Pterocarpus santalinus* L.f. *Indian Journal of Experimental Biology* **48**, 1270-1273

Bhat SR, Chandel KPS (1991) A novel technique to overcome browning. *Plant Cell Reports* **10**, 358-361

Gambley RL, Dodd WA (1990) An *in vitro* technique for production de novo of multiple shoots in cotyledon explants in cucumber *(Cucumis sativus* L.). *Plant Cell, Tissue and Organ Culture* **20**, 177-183

Gamborg OL Miller RA, Ojima K (1968) Nutrient requirements of suspension culture of soybean root cells. *Experimental Cell Research* **50**, 151-158

Gulati A, Jaiwal PK (1990) Culture conditions affecting regeneration from cotyledon of mungbean (*Vigna radiata* L. W.). *Plant Cell, Tissue and Organ Culture* **23**, 1-7

Gulati A, Jaiwal PK (1994) Plant regeneration from node explants of mungbean (*Vigna radiate* L. W.). *Plant Cell Reports* **13**, 523-527

Jadhav SN, Reddy KN, Reddy CS (2001) Conservation assessment and management planning for medicinal plants of Andhra Pradesh. Medicinal plants conservation center (MPCC) and FRLHT. Hyderabad, India, pp 1-39

Jain SM, Ishii K (Eds) (2003) *Micropropagation of Woody Trees and Fruits*, Kluwer Academic Publishers, Dordrecht, The Netherlands, 840 pp

Julliard J, Sossunlzor L, Habrocot Y, Pelletier G (1992) Hormonal requirement and tissue competency for shoot organogenesis in two cultivars of *Brassica napus*. *Physiologia Plantarum* **84**, 521-530

Kala AP (2006) Directorate of Revenue intelligence Annual Report, Government of India, Ministry of Finance, Department of Revenue, Central Board of Excise and Customs, Annual Report

Kalimuthu K, Lakshmanan KK (1995) Effect of different treatments on pod germination of *Pterocarpus* species. *Indian Journal of Forestry* **18**, 104

Kameshwararao N (2004) Plant genetic resources: Advancing conservation and use through biotechnology. *African Journal of Biotechnology* **3**, 136-145

Kesavareddy K, Srivasuki KP (1990) Vegetative propagation of Red sanders (*Pterocarpus santalinus* L.f). *Indian Forestry* **116**, 536-539

Kesavareddy K, Srivasuki KP (1992) Biotechnological approach for tree improvement in Red sanders. In: Kesavareddy K (Ed) *Vegetative Propagation and Biotechnologies for Tree Improvement*, Reddy Natraj Publishers, Dehradun, pp 141-146

Khattar S, Ram HYM (1983) Organogenesis and plantlet formation *in-vitro Sesbania grandiflora* L. *Indian Journal of Experimental Biology* **21**, 251-253

Kukreja AK, Dhawan OP, Mathur AK, Ahuja PS, Mandal S (1991) Screening and evaluation of agronomically useful somaclonal variations in Japanese mint (*Mentha arvensis* L.) *Euphytica* **53**, 183-191

Lakshmisita G, Sreenatha KS, Sujata S (1992) Plantlet production from shoot tip cultures of Red sandal wood *Pterocarpus santalinus* L.f. *Current Science* **62**, 532-534

Lloyd G, McCown B (1980) Commercially feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *International Plant Propagation Society Proceedings* **30**, 421

Masceranhas AF, Muralidharan E (1989) Tissue culture of forest trees in India. *Current Science* **58**, 606-613

Mithila J, Srivasuki KP (1992) *In vitro* growth responses of *Pterocarpus santalinus* L. In: Kesavareddy K (Ed) *Vegetative Propagation and Biotechnologies for Tree Improvement*, Reddy Natraj Publishers, Dehradun, pp 147-152

Milberg P, Lamont BB (1997) Seed/cotyledon size and nutrient content play a major role in early performance of species on nutrient poor soil. *New Phytologist* **137**, 665-672

Mishra AK, Bhatnagar SP (1995) Direct shoot regeneration from the leaf explants of cucumber (*Cucumis sativus* L.). *Phytomorphology* **45,** 47-55

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Padmalatha K, Prasad MNV (2007) Germination studies in *Pterocarpus santalinus* L.f – an endangered and endemic medicinal plant, and relevance to conservation. *Seed Science and Biotechnology* **1**, 32-34

Parrotta JA (2001) *Healing Plants of Peninsular India*, CABI Publishers, New York, USA, pp 97-99, 408

Pattnaik S, Chandan PK (1996) *In vitro* propagation of the medicinal herbs *Ocimum americanum* L. syn. *O. canum* Sims. (Hoary basil) and *Ocimum sanctum* L. (Holy basil). *Plant Cell Reports* **15**, 846-850

Polisetty R, Paul V, Deveshwar JJ, Khetarpal S, Suresh K, Chandra R (1997) Multiple shoot induction by benzyladenine and complete plant regeneration from seed explants of chickpea (*Cicer arietinum* L.). *Plant Cell Reports* **16**, 565-571

Prakash NS, Pental D, Sarin NB (1994) Regeneration of pigeonpea (*Cajanus cajan*) from cotyledonary node via multiple shoot formation. *Plant Cell Reports* **13**, 623-627

Pullaiah T, Anuradha M (1999) Propagation studies of Red Sanders (*Pterocarpus santalinus* L.f.) *in vitro* - an endangered taxon of Andhra Pradesh, India. *Taiwania* **44**, 311-324

Rech EL, Pires MJP (1986) Tissue culture propagation of *Mentha* species by use of axillary buds. *Plant Cell Reports* **5**, 17-18

Sarithapatri, Bhatnagar SP, Bhojwani SS (1988) Preliminary investigations on micropropagtion of a leguminous tree: *Pterocarpus santalinus*. *Phytomorphology* **38**, 41

Sinha RK, Majumdar K, Sinha S (1991) *In vitro* differentiation and plant regeneration of *Albizia chinensis* (Osb.) Merr. *In Vitro Cellular and Developmental Biology – Plant* **36**, 370-373

Sinha P, Govil JN, Singh VK (2000) *Recent Progress in Medicinal Plants, Diseases and Management*, Sci Tech Publishers LLC, Houston, Texas, USA, 205 pp

Suwai B, Karki A, Rajbhandari SB (1988) The *in vitro* proliferation of forest trees. 1. *Dalbergia sissoo* Roxb. Ex DC. *Silvae Genetica* **37**, 26-28

Teketay D, Gränstrøm A (1997) Germination ecology of forest species from the highlands of Ethiopia. *Journal of Tropical Ecology* **14**, 793-803

Tomar UK, Gupta SC (1988) Somatic embryogenesis and organogenesis in callus cultures of a tree legume – *Albizia richardiana* King. *Plant Cell Reports* **7**, 70-73

Thapliyal RC, Connor KF (1997) Effects of accelerated ageing on viability, leachate exudation and fatty acid content of *Dalbergia sissoo* Roxb. seeds. *Seed Science and Technology* **25**, 311-319

Thorpe TA, Murashige T (1970) Some histological changes underlying shoot initiation in tobacco callus cultures. *Canadian Journal of Botany* **48**, 277-285

Vaneck JM, Kitto SL (1990) Callus initiation and regeneration in *Mentha*. *Hortscience* **25**, 804-806

Zodape ST (1991) The improvement of germination of some forest species by acid scarification. *Indian Forester* **3**, 61-66