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Research article

A modified and improved protocol development for *in vitro* clonal propagation of *Santalum album* L. from internodal explants

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Abstract: Indian sandalwood (*Santalum album*) is a medium-sized evergreen semi root parasitic tree. It is a commercially important species for its heartwood essential oil, extensively used in medicines, cosmetics, perfume and incense industry. Sandalwood harvesting usually involves removal of the entire tree resulting in critical loss of the genetic diversity. Commercial plantation of the species is not widely done due to nonavailability of good quality planting material in sufficient quantity. Production of sandalwood plantlets through vegetative propagation in regular nurseries is also difficult and time-consuming. Hence, there is a need to develop such planting material/plantlets through tissue culture technique, which provide high yielding clones of candidate plus trees of sandalwood. In the present study, an efficient plant regeneration protocol for *in vitro* propagation of *Santalum album* has been developed. Plant propagation using nodal explants was achieved on Murashige and Skoog's (MS) medium. MS solution contains a mixture of macroelement like NH_4, KNO_3, Mg, Ca, KH_2 and microelements like Zn, H_3BO_4, $MnSo_4$, Cu and Co, sucrose as a source of carbohydrate and vitamins. Effect of plant growth regulators (PGR) like 6-Benzylaminopurine (BAP) and Kinetin (Kn) on shoot induction and multiplication and Indole-3 Butyric acid (IBA) and Naphthalene Acetic acid (NAA) on rooting was studied. Highest shoot multiplication with longest shootlets (2.90 cm) was achieved on MS medium containing 0.5 mg L^{-1} BAP and 5.0 ml L^{-1} IBA after 30 days of culture. New shoots were repeatedly harvested up to three subculture passages on the fresh medium of same concentration at 4-week intervals. Microshoots treated with 50.0 ml L^{-1} IBA for 48 h produced roots on growth regulator free, half-strength MS medium followed by one-week incubation in the dark. Hence, this protocol is a simple, rapid and highly reproducible to obtain more number of quality plants of *Santalum album* within a short period.

Keywords: Micropropagation - *In vitro* establishment - Culture medium - Plant growth regulators - Shoot multiplication.

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INTRODUCTION

India has a rich diversity of forest tree species that have been well known for their utility as well as aesthetic value. Our indigenous species like teak, rosewood, and sandalwood are amongst the most valuable timbers in the word. These trees once abundant in the forests throughout the country have been depleted in recent times to meet the increasing demand for wood plantation (Murlidharan 1997).

Sandalwood or Chandan (*Santalum album* L.) belongs to the *Santalaceae* family, a medium-sized evergreen semi root parasitic tree, highly valued for its fragrant heartwood, which contains sandal oil that is used in perfumes, cosmetics, medicine, and also in agarbathi (incense sticks) industries (Srinivasan *et al*. 1992). *Santalum album* has the highest oil content (about 6%) among the species of the genus *Santalum*. The sandalwood and oil demand (80–90%) in the international market has been fulfilled by Indian sandalwood for decades. However, the production of sandalwood declined from 4000 tons in 1950 to 2000 tons in 1990 and to about 1000 tons in 1999 (Ananthapadmanabha 2000). The acute shortage in supply and the demand imbalance resulted in the closure of several sandalwood based industries in India and other Asian countries.

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Santalum album is recalcitrant to *in vivo* and *in vitro* propagation, for which only limited success has been, achieved so far (Sanjaya *et al*. 2003). Natural regeneration and artificial propagation occur mainly by seeds. On the other hand, vegetative propagation is accomplished by grafting, air layering, and with root suckers, but the production of clones is insufficient and time-consuming (Srimati *et al*. 1995).

Previous reports on *in vitro* propagation have focused on adventitious bud regeneration from *in vitro* grown seedling explants such as hypocotyl (Bapat & Rao 1978, Rao & Bapat 1992), endosperm (Sita *et al*. 1979), zygotic embryo (Rai & McComb 2002), and somatic embryogenesis through a callus phase (Sita *et al*. 1980). Although small numbers of plants were regenerated, the methods are still a long way from being optimized for a large number of plantlet productions. Indeed, the bottleneck is *in vitro* rooting, which limits the widespread application of micro propagation techniques in sandalwood. There are very few efforts found for *in vitro* clonal propagation of candidate plus tree (CPT) of *S. album* through axillary shoot proliferation. Sufficient references on this aspect are also not available. There is, therefore, a need to develop clonal techniques to produce disease-resistant and high oil-yielding clones of CPTs of *Santalum album*. The present research study was designed to fill the gap for true to type large scale *in vitro* plantlet production of *Santalum album.*

MATERIAL AND METHODS

Plant material

The plant materials were collected from healthy, disease-free mature trees of *Santalum album*, growing in the nursery of State Forest Research Institute (SFRI), Kanpur (Fig. 1). Nodal explants containing axillary buds were mainly selected for *in vitro* micro propagation of *Santalum album* L.

Figure 1. *Santalum album* L. at State Forest Research Institute (SFRI), Kanpur, Uttar Pradesh, India.

Surface sterilization

The long shoots obtained from the mother plant after removing the leaves were gently scrubbed with a cotton swab dipped in 70% alcohol. Cut and trimmed nodal segments containing axillary buds were gently scrubbed all along its length especially around the node bearing the bud (Fig. 2). Explants were sterilized using 0.1% of Bavistin and 0.1% paradigm antibiotic solution for removing bacterial contamination and in Mercuric chloride solution at 0.1% concentration for fungal contamination of each for 5–10 minutes. Surface sterilized explants were thoroughly washed 3–4 times with sterile distilled water to wash off the sterilizing agent. The surface sterilized explants were trimmed at both the ends with the help of scalpel blade and inoculated on Murashige and Skoog's (MS) medium without hormones (Control). 3–4 explants were inoculated in each

culture flask. Finally, the cultures were kept in the culture room under controlled conditions and observations recorded regularly.

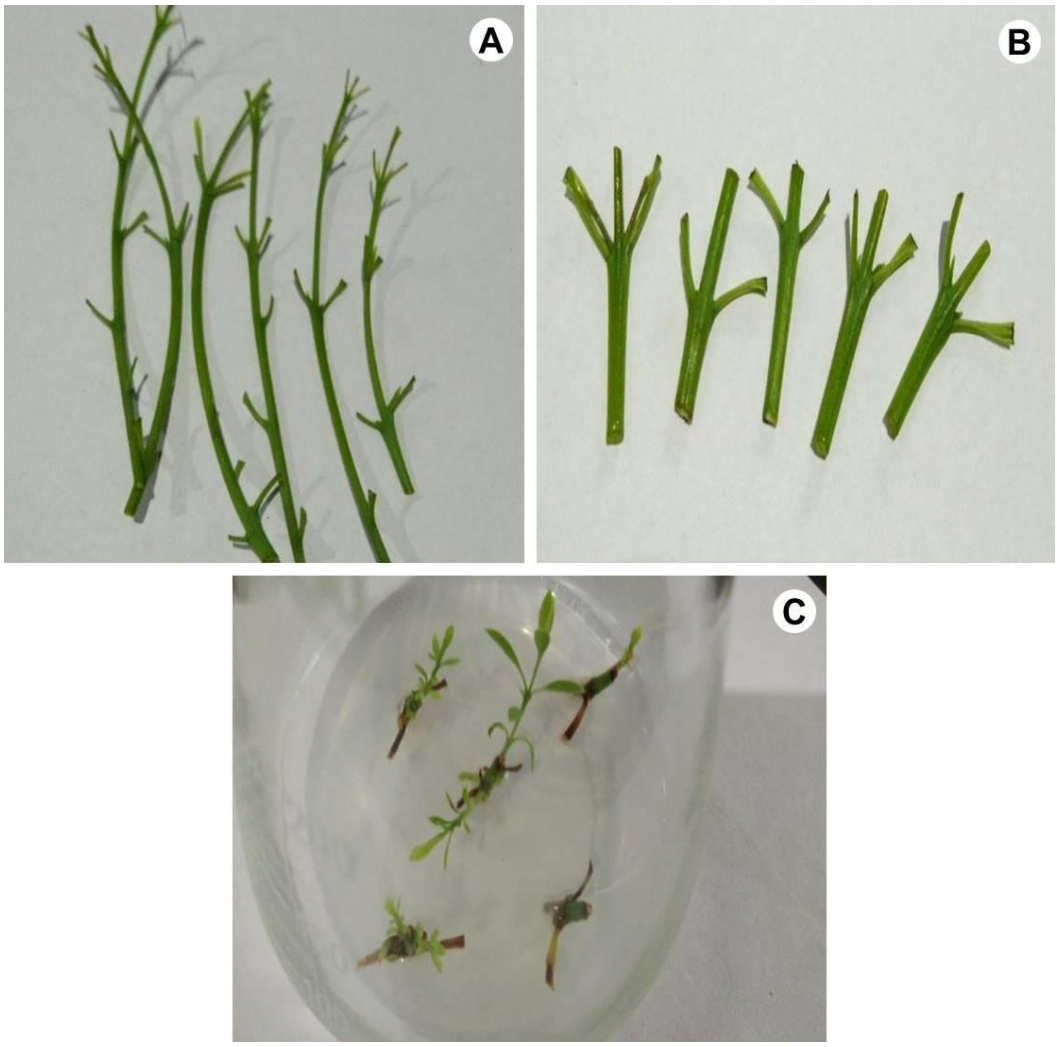

Figure 2. *Santalum album* L.: **A**, Surface sterilized nodal explants; **B**, Surface sterilized trimmed explants; **C**, Proliferated explants.

Effect of plant growth regulator on shoot development and multiplication

To find out the best auxin and cytokinin effect and their optimum concentration for multiple shoot production and subsequent growth, various concentrations of Indole butyric acid (IBA) and 6-benzylaminopurine (BAP) were used in Murashige and Skoog's (MS) medium (Murashige & Skoog 1962). Nodal shoot segments isolated from *in vitro* grown shoots on MS medium without hormones (Control) were subcultured on MS medium supplemented with 0.5 mg l^{-1} BAP and 5 mg l^{-1} IBA. After four weeks, proliferated shoots higher than 2.0 cm of original explants were repeatedly subcultured up to five times on Murashige and Skoog's medium containing the mixture of IBA and BAP of same concentration at 4-week intervals. Data were recorded for each subculture cycle.

In vitro root induction in S. album

In vitro raised shoots of 3.0–4.0 cm in length were used for *in vitro* root induction. Micro shoots were pulse treated (0–72 h) with 50 mg l^{-1} Indole-3-butyric acid (IBA) and subsequently transferred to half-strength Murashige and Skoog's medium without hormones, with 30.0 gm 1^{-1}, sucrose, and 7.0 gm l^{-1} agar-agar. Cultures were incubated in the dark for one week and subsequently shifted to light at 28±1 °C with a 16-h photoperiod provided by cool white fluorescent lamps (Philips, India). The percentage of root induction, number of roots, and root length were recorded at the end of the eighth week.

Pre-hardening and transfer of plantlets to the containers

In vitro rooted shoots were carefully removed from the culture medium and transferred into 400-ml glass bottles containing the autoclaved soilrite mixture. The transplanted plantlets were maintained at 28±1 °C temperature and 16-h photoperiod. The plantlets were sprayed with one-quarter Murashige and Skoog's liquid

medium at 15-day intervals to boost the growth of plantlets at pre-hardening. Once adjusted to these initial shocks these plantlets are ready to move out of the laboratory conditions for further stages of growth at acclimatization in germination house.

Data collection and statistical analysis

Each treatment consisted of 5 replications, and each experiment was repeated twice, to confirm the results. The data were analyzed by one-way analysis of variance (ANOVA).

RESULTS

Shoot initiation

The nodal shoot segments exhibited maximum bud break and the highest number of shoots when cultured on full strength Murashige and Skoog's medium without hormones (Table 1). The nodal shoot segments cultured during November–January yielded maximum bud break (95%) per explants. Poor shoot development was observed when explants were cultured in May–July on optimum shoot induction medium (Fig. 3).

Table 1. Effect of different concentration of growth hormones in MS medium on bud break of *Santalum album* L.

Nutrient medium	Number of explants inoculated	Bud break/explants
MS media without hormones (Control)	**100**	**100**
MS + 1ml BAP	100	85
MS + 1.5 ml BAP	100	85
MS + 1ml Kn	100	70
MS + 1.5 ml Kn	100	60
MS + 1ml BAP + 1ml Kn	100	50
MS + 1.5 ml BAP + 1.5 ml + Kn	100	50

Note: Data recorded after 30 days of culture

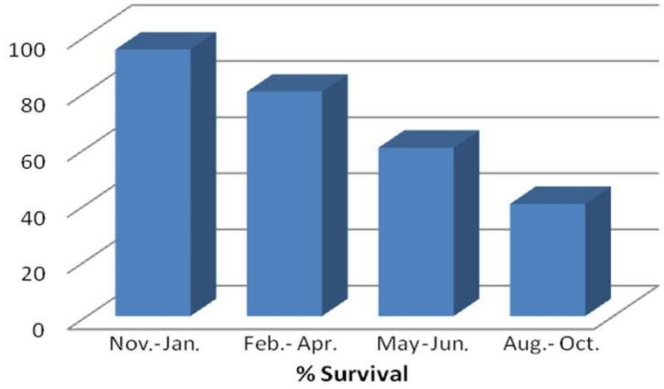

Figure 3. Effect of collection time on the survival of internodeal explants of *Santalum album* L.

Shoot multiplication

The combined use of BAP (0.5 mg l^{-1}) and IBA (5 mg l^{-1}) significantly enhanced the shoot number per shoot segment (Table 2; Fig. 4A). Subculturing within 4 weeks was essential to maintain healthy shoot growth; moreover, a repeated subculture of the original explant (nodal shoot segment) every 4 weeks on the fresh medium of a mixture of BAP and IBA of same concentration produced a crop of shoots (Fig. 4B). Enhanced rate of shoot multiplication was observed up to the fifth subculture, and the gradual decline was recorded after the fifth subculture.

Table 2. Effect of different concentration of growth hormones in Murashige and Skoog's medium on shoot multiplication and shoot length of *Santalum album* L.

Nutrient medium	Mean Number of shoots/explants	Mean shoot length (cm)
MS + 0.1 ml BAP+ 1ml IBA	1.67	1.40
MS + 0.2 ml BAP+ 2ml IBA	1.75	1.40
MS + 0.3 ml BAP+ 3ml IBA	1.80	1.56
MS + 0.4 ml BAP + 4ml IBA	2.00	2.00
MS + 0.5 ml BAP + 5ml IBA	**4.00**	**2.90**
MS + 0.1 ml IBA + 1ml BAP	1.20	1.35
MS + 0.2 ml IBA + 2ml BAP	1.20	1.40
MS + 0.3 ml IBA + 3ml BAP	2.00	1.50
MS + 0.4 ml IBA + 4ml BAP	2.04	1.75
MS + 0.5ml IBA + 5ml BAP	2.04	1.85

Note: Data recorded after 30 days of culture.

Figure 4. *Santalum album* L.: **A**, Development of multiple shoots from nodal shoot; **B**, Shoot multiplication and elongation during subculture.

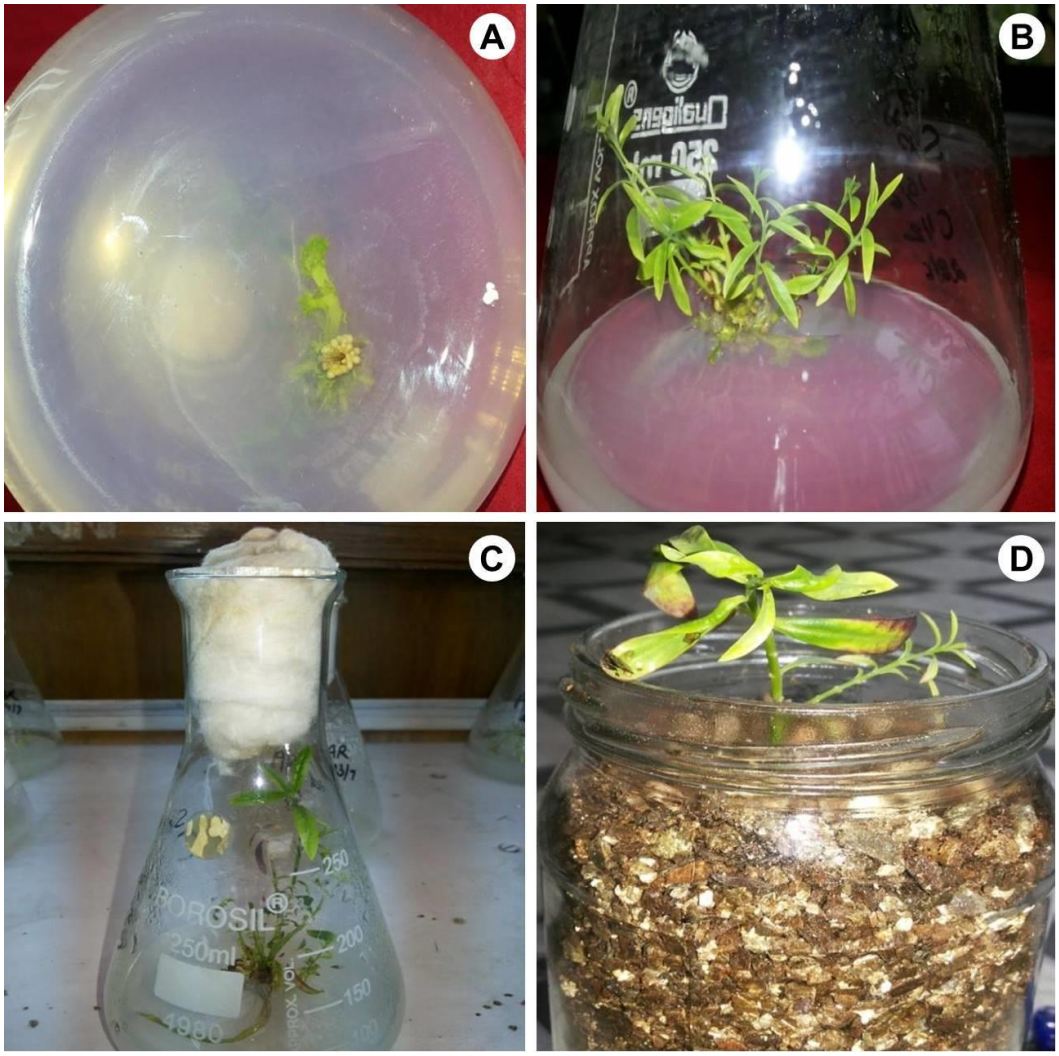

Figure 5. *Santalum album* L.: **A**, *In vitro* rooting of pulse treated shoots after 8 weeks; **B**, *In vitro* rooted microshoots; **C**, Elongation of *in vitro* root and shoot; **D**, *In vitro* microshoots in soilrite.

In vitro rooting

The duration of pulse treatment had a significant effect on rooting capacity; the best results were obtained with micro shoots treated with 50 ml l^{-1} IBA for 48h and subsequently cultured on half-strength Murashige and Skoog's medium (Table 3; Fig. 5). However, shoots exposed to prolonged pulse treatment leads to necrosis, and no root induction was observed in control. Cultures were incubated in the dark for one week and subsequently

shifted to light at 28±1 °C with a 16-h photoperiod provided by cool white fluorescent lamps. Out of the different auxins (IBA, NAA, and IAA) tested, the micro shoots treated with 50 ml l^{-1} IBA showed maximum rooting (50%), and no root induction was noted for IAA and in control without pulse treatment.

Table 3. Effect of duration of pulse treatment of Indole butyric acid (50 ml l^{-1}) on *in vitro* root induction in *Santalum album* L.

Pulse treatment (h)	Root induction %	Root length (cm)
Control (water)	00.00	00.00
1	00.00	00.00
5	04.16	0.15
10	08.33	0.62
24	33.34	0.53
48	**50**	**3.32**
72	08.16	0.45

Note: Data recorded after 30 days of culture.

DISCUSSION

The use of preexisting buds for propagation reduces the possibility of variation among the progeny and therefore can be safely applied for rapid propagation of field-grown CPTs of sandalwood. We optimized shoot multiplication and novel rooting techniques for mass multiplication of the species without the interference of callus. This method is quite common for the propagation of *Fragaria indica* (Indre & Dhar 2000), *Acacia mearnsii* (Marguerite *et al*. 2001), *Tectona grandis* (Mendoza *et al*. 2007) and *Eucalyptus hybrid* (Brondani *et al.* 2011).

The combined use of cytokinin and auxin was emphasized in micropropagation of *Ficus carica* (Kumar *et al*. 1998) and *Syzygium travancorium* (Ajith *et al*. 1999). The season of explant collection also influenced shoot development from individual explants; this fact may be due to long flowering and seed setting habit of trees.

These observations are in concordance with Sharma *et al*. (2003) in *Crataeva adansoni*, where shoot initiation is highly influenced by season. The application of IBA by a pulse treatment was used in adventitious rooting of *Maytenus emarginata* (Rathore *et al*. 1992) and *Tectona grandis* (Siril & Tewari 1999).

The results obtained in this study are very encouraging and the standardized protocol developed in the course of study will also provide a great opportunity to raise high quality *in vitro* clonal plantlets of *Santalum album*. The present investigation will also take another step forward in demonstrating the application of root-trainer technology and compost as a major potting medium ingredient for quality plant production; finally, it will help in the steady establishment of *in vitro* raised plantlets in field conditions. Because *Santalum album* is a slow-growing species, it is too early to evaluate the superiority of *in vitro* raised plants.

CONCLUSION

This study provides an efficient *in vitro* propagation method which could be commercially feasible for *Santalum album*, by providing a protocol for producing genetically uniform plants from selected genotypes. Explants collected in November to January showed maximum survival and explants inoculated on Murashige and Skoog's media without hormones provided the maximum number of bud break or proliferation.

The concentrations of 0.5 mg l^{-1} BAP combined with 5mg/l IBA on full MS medium produced mass multiplication and longest shoots. The effect of pulse treatment of 50 mg l^{-1} IBA on *in vitro* multiplied shoots for 48 hours followed by transferred in half strength MS media provided a maximum percentage of root induction and root length.

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