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Micropropagation of *Shorea robusta*: an economically important woody plant

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ABSTRACT: *Shorea robusta* is a valuable tree species which provides good quality timber along with other useful materials like seeds which can be used as a source of starch. Woody plants are difficult to regenerate under *in vitro* conditions and only some success has been achieved so far. Here we have presented the data for successful *in vitro* regeneration of *S. robusta* using nodal explants. Shoot proliferation and rooting were also successfully achieved in subsequent subcultures. The best medium for shoot initiation and proliferation was found to be WPM with 1.0 mg·l^{-1} BAP and 0.5 mg·l^{-1} NAA and 1.0 mg·l^{-1} BAP +0.5 mg·l^{-1} NAA, respectively. Likewise for rooting WPM medium with 0.5 mg·l^{-1} IBA was found to be the best medium.

Keywords: WPM medium; BAP medium; IBA medium

Tropical hardwood trees are renewable sources for food and economically important raw material like fodder, fuel wood, timber and other valuable non-timber products. All trees play an important role in the ecosystem (Pijut et al. 2011). Micropropagation of tree species offers a rapid means to produce clonal planting stock for forestation, woody biomass production and conservation of elite germ plasma. In general, the woody plants are difficult to regenerate under *in vitro* conditions but previously some successes were achieved in a few leguminous tree species (Tomar, Gupta 1988; Nanda et al. 2004). *Stereospermum suaveolens* was successfully cultured *in vitro* by Shukla et al. (2012). Amo Marco and Lledo (1996) reported the micropropagation of *Salix tarraconensis*. *Shorea* (sal), belonging to the imbricate-*shorea* group of the *Dipterocarpaceae* family, is widely distributed in India. The sal is a tall handsome tree providing very good quality timber.

Shorea robusta is a large, deciduous tree up to 50 m tall (but these are exceptional sizes), and under normal conditions *S. robusta* trees attain a height of about 18–32 m and girths of 1.5–2 m; the bole is clean, straight and cylindrical, but often bearing epicormic branches; the crown is spreading and spherical. The bark is dark brown and thick, with longitudinal fissures deep in poles, becoming shallow in mature trees; it provides effective protection against fire (Orwa et. al. 2009). The tree develops a long taproot at a very young age. Sal seeds are ground into coarse flour used to make bread, and the plant is used as a famine food. The chemical composition of seeds consists of 10.8% water, 8% protein, 62.7% carbohydrate, 14.8% oil, 1.4% fibre and 2.3% ash. *S. robusta* butter, used in cooking, is derived from the seeds. A defatted kernel powder, popularly known as sal seed cake, contains about 50% starch, in addition to proteins, tannins and minerals. The physicochemical properties of the starch can be exploited for preparing canned food products (Singh, Kushwaha 2005).

In the present communication, we report the development of shoot initiation and proliferation from seedling-derived cotyledonary node explants and their successful subculture and rooting using different types of hormones and different media.

MATERIAL AND METHODS

Explant source. The explant source was plantlets purchased from a local nursery. One month old plantlets were brought to the laboratory and

planted in the greenhouse in polybags containing soil. After acclimatization in the greenhouse for 1 week the nodal segments from the plantlets were used for inoculation as explants.

Initiation of shoots. The nodal segments (3–4 cm) excised from purchased nursery plants were washed gently under running tap water and with diluted (0.5%, v/v) Labolene detergent to remove dust particles. The nodal segments were surface-sterilized with 0.2% mercuric chloride for 5 min and washed 3–4 times with double distilled water. The sterilized nodes were inoculated on basal Murashige and Skoog medium (MS) (Murashige, Skoog 1962), woody plant medium (WPM) (Lloyd, McCown 1981), Gamborg medium (B5) (Gamborg et al. 1968) and Schenk, Hildebrandt medium (SH) (Schenk, Hildebrandt 1972) supplemented with 30 g·l^{-1} and 20 g·l^{-1} sucrose (HiMedia Laboratories Pvt. Ltd., Mumbai, India), respectively. 0.8% (w/v) agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) was used as the gelling agent on the respective media. The pH of all media was adjusted between 5.5 and 5.8 using 0.1N NaOH or 0.1N HCl before solidification. The media were autoclaved at 121°C for 15 min for sterilization. All cultures were incubated in a controlled environment at a temperature of 25 ± 2°C. N-acetylaspartic acid (NAA), indole-3-butyric acid (IBA), and 6-benzylaminopurine (BAP) were different hormone sources used in the present study. The cultures were incubated at 25 ± 2°C under fluorescent light with a 16/8 light/dark photoperiod.

The plantlets were cut into smaller segments (0.5 cm each) having one node in each segment and were inoculated on MS, SH, WPM and B5 media. Different concentrations and combinations of hormones including IBA 0.5 mg·l^{-1}, BAP 0.5–2.50 mg·l^{-1}, NAA 0.5–1.0 mg·l^{-1} were tested and hormone-free medium was used as a control. The pH of all media was adjusted between 5.5 and 5.8 before they were autoclaved for 15 min at 121°C. All cultures were placed in a culture room at 25°C and 16 h photoperiod under cool white fluorescent light.

Proliferation of shoots. Shoots were transferred onto a fresh medium for shoot proliferation. For shoot proliferation, MS, WPM, B5 and SH media were used, each supplemented with 1.0 mg·l^{-1} BAP + 0.5 mg·l^{-1} NAA over a period of 4 weeks on.

Each experiment had 5 culture tubes and experiments were repeated thrice. The different treatments were quantified as the mean number of multiple shoots per subculture. The data were statistically analysed by Duncan's multiple range test (DMRT).

Rooting. The apical portion of *in vitro* raised shoots was cut approximately 2–3 cm and cultured on a root initiation medium. The apical portion was used for root initiation as the region of auxin production and with addition of cytokinin (IBA) it provides a balanced auxin and cytokinin ratio for root initiation. For rooting WPM medium supplemented with different concentrations of IBA, indole-3-acetic acid (IAA) and NAA (0.0, 0.2, 0.5 and 1.0 mg·l^{-1}) was applied and hormone-free medium was used as a control. The cultures were observed each week. The culture conditions were similar to the conditions applied for *in vitro* shoot initiation and proliferation.

RESULTS AND DISCUSSION

Initiation of shoots

For the shoot initiation of *Shorea robusta* various media were tested (MS, SH, WPM and B5) supplemented with BAP and NAA. The suitable medium for shoot initiation was WPM medium supple-

Table 1. Effect of different media on shoot bud initiation from nodal segments of *Shorea robusta*

Medium	BAP + NAA (mg·l^{-1})	Shoot initiation (%)	Mean no of shoot ± SE	Mean length of shoot (mm) ± SE
MS	0.0	60	2.15 ± 1.12^{b}	21.0 ± 1.22
	1.0 + 0.5	85	2.34 ± 1.32^{b}	34.0 ± 1.09
	0.5 + 0.5	70	2.25 ± 1.46^{b}	20.7 ± 1.35
	0.5 + 1.0	60	1.20 ± 0.78^{c}	15.6 ± 1.23
WPM	1.0 + 0.5	100	5.09 ± 1.46^{a}	44.2 ± 1.43
	0.5 + 0.5	80	1.96 ± 1.53^{c}	31.1 ± 1.68
	0.5 + 1.0	75	1.60 ± 1.26	14.7 ± 1.72
SH	1.0 + 0.5	80	2.00 ± 0.94^{bc}	14.9 ± 10.60
	0.5 + 0.5	70	2.40 ± 1.64^{c}	16.0 ± 1.89
	0.5 + 1.0	65	1.06 ± 0.51^{cd}	19.0 ± 1.64
B5	1.0 + 0.5	40	1.50 ± 0.28^{c}	18.4 ± 1.20
	0.5 + 0.5	30	1.60 ± 1.56^{c}	17.8 ± 1.13
	0.5 + 1.0	30	2.20 ± 1.50^{bc}	22.4 ± 1.38

BAP – 6-benzylaminopurine, NAA – indole-3-butyric acid, MS – Murashige and Skoog medium, WPM – woody plant medium, B5 – Gamborg medium, SH – Schenk, Hildebrandt medium; SE – standard error, mean ± 1 SE – each experiment consisted of five replicates and repeated three times, means separated using DMR (CoStat; Version 4.02, Pacific Grove, USA), different letters indicate non-significant differences at $P \leq 0.05$

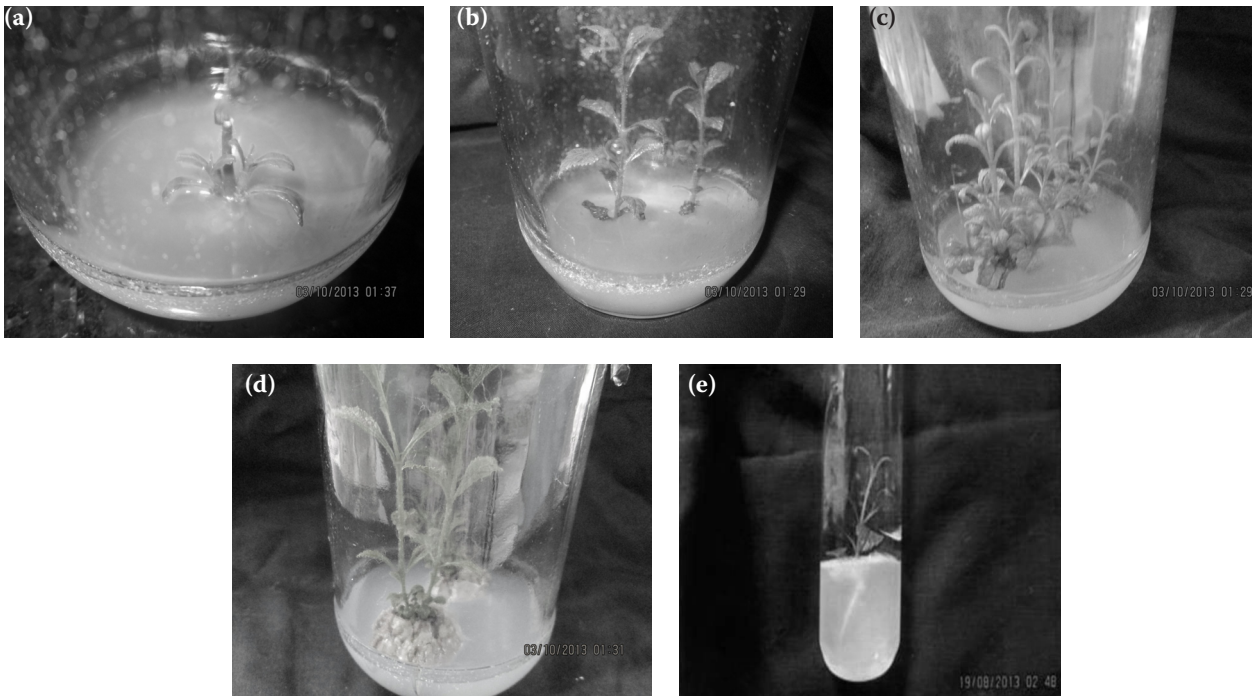

Fig. 1. (a) Formation of single shoots from each axil of the cotyledonary node on WPM basal medium with 1.0 mg·l^{-1} BAP + 0.5 mg·l^{-1} NAA after 4 weeks, (b) elongating shoot buds on WPM with 2.0 mg·l^{-1} BAP, 18 days of culture, (c) multiple shoots on WPM +2.0 mg·l^{-1} BAP after 4 weeks, (d) formation of stunted shoots with profuse basal callus on WPM + 1.0 mg·l^{-1} BAP + 0.5 mg·l^{-1} NAA, (e) rooting on WPM medium with 0.5 mg·l^{-1} IBA after 3 weeks of culture

mented with 1.0 mg·l^{-1} BAP and 0.5 mg·l^{-1} NAA. It gave 100% shoot initiation (Table 1) (Fig. 1a–b). It should be noted here that WPM medium supplemented with BAP and NAA was found to be the best medium for shoot initiation in the case of *S. robusta* in the present study even though the results obtained with MS medium with same concentration of hormones gave good results. The average initiation time for shoots in the present study was recorded as 3.5–4 weeks. It was reported previously that WPM medium supplemented with thidiazuron (TDZ) supported the highest percentage of shoots and bud formation in *Salix nigra* Marsh. (Lyyra et al. 2006). BAP also produced longer shoots as compared to kinetin in *Prosopis cineraria* and *Aegle marmelos* (Kumar, Singh 2009). Similarly with *Salix tarraconensis* Pau ex Font Quer, MS medium with 4.9 μM of 6-γ-γ-dimethylallylaminopurine was found to give the best results (Amo-Marco, Lledo 1996). Shukla et al. (2012) reported that *Stereospermum suaveolens* gave better results of shoot initiation on MS medium supplemented with BAP.

Table 2. Influence of MS, WPM, B5 and SH media, each supplemented with 1.0 mg·l^{-1} BAP + 0.5 mg·l^{-1} NAA over a period of 4 weeks on multiple shoot initiation of nodal segments of *S. robusta*

2.0 BAP + 0.5 NAA mg·l^{-1}	Mean no of shoot ± SE	Mean of node/ explants	Mean length (mm) of shoot ± SE
MS	4.18 ± 0.75^{b}	12.13^{b}	25.02 ± 1.34^{b}
WPM	7 .0 ± 1.58^{a}	20.45^{a}	44.23 ± 1.38^{a}
B5	2.08 ± 0.90^{c}	10.15^{c}	23.0 ± 1.02^{c}
SH	2.25 ± 0.86^{bc}	7.34^{bc}	20.8 ± 1.21^{bc}

BAP – 6-benzylaminopurine, NAA – indole-3-butyric acid, MS – Murashige and Skoog medium, WPM – woody plant medium, B5 – Gamborg medium, SH – Schenk, Hildebrandt medium; SE – standard error, mean ± 1 SE – each experiment consisted of five replicates and repeated three times, means separated using DMR (CoStat; Version 4.02, Pacific Grove, USA), different letters indicate non-significant differences at $P \leq 0.05$

Proliferation of shoots

For development of multiple shoots MS, WPM, B5 and SH media, each supplemented with 1.0 mg·l^{-1} BAP + 0.5 mg·l^{-1} NAA, were used to inoculate nodal segments of *S. robusta*. The inoculated samples were observed for a period of 4 weeks and data were recorded. The mean number of shoots per explants (7.00 ± 1.58) was recorded after four weeks of culture. The lowest number of shoot proliferations was recorded in B5 medium (Table 2) (Fig. 1c). The cyto-

kinin concentration has been reported several times to be decisive for shoot proliferation and elongation of many medicinal plant species (Saxena et al. 1998; Rout et al. 2000; Rout 2004).

Rooting

Elongated apical portions from nodal segments of *in vitro* shoots were cut and cultured on WPM medium supplemented with different concentrations of IBA, IAA and NAA alone for rooting (Table 3) as it was the most suitable medium for shoot initiation and proliferation found in the present investigation. After 10–15 days 70% root initiation on WPM medium with 0.5 mg·l^{-1} IBA was observed. At this concentration the number and length of roots were also higher in comparison with other concentrations. At the higher concentration (2.0 mg·l^{-1}) of IBA, IAA or NAA, the percentage of rooting was reduced (Table 3) (Fig. 1d–e). Similar results were found out in root induction from microshoots of different *Acacia* species (Vengadessan et al. 2002, 2003; Nanda et al. 2004). The higher concentration of IBA was used in rooting of *Jatropha curcus* (Sujatha et al. 2005). The stimulatory effect of IBA on rooting was earlier reported in *E. agallocha* (Rao et al. 1998), *Melia azedarach* (Thakur et al. 1998), *Ficus carica* (Kumar et al. 1998), *Balanites aegyptica* (Mansor et al. 2003) and *Bambusa vulgaris* (Aliou et al. 2006).

Table 3. Effect of auxins on rooting of excised shoots of *Shorea robusta* within 2 weeks of culture in WPM medium

IBA	IAA	NAA	Root induction (%)	Mean of root ± SE	Mean length of root ± SE
0.0			50	1.5 ± 0.23^{b}	10.21 ± 1.05
0.5			**70**	**3.2 ± 0.83**^{a}	**18.0 ± 0.14**
2.0			40	1.6 ± 0.89^{b}	12.06 ± 0.08
	0.5		30	1.8 ± 1.09^{b}	11.2 ± 0.10
	2.0		44	1.4 ± 0.54^{bc}	15.04 ± 0.05
		0.5	45	1.2 ± 0.44^{bc}	14.02 ± 0.04
		2.0	41	1.4 ± 0.54^{bc}	12.8 ± 0.10

WPM – woody plant medium, , IBA – indole-3-butyric acid, IAA – indole-3-acetic acid, NAA – indole-3-butyric acid; SE – standard error, mean ± 1 SE – each experiment consisted of five replicates and repeated three times, means separated using DMR (CoStat; Version 4.02, Pacific Grove, USA), in bold – significant differences, different letters indicate non-significant differences at $P \leq 0.05$

CONCLUSION

In the present investigation it was shown that WPM medium with 1.0 mg·l^{-1} BAP + 0.5 mg·l^{-1} NAA was the best medium for shoot initiation and number of shoots per explants. It was also found that the longest shoots were formed in WPM medium. In WPM medium IBA alone (0.5 mg·l^{-1}) gave the highest number of roots which were also the longest compared to all the other hormone concentrations. *In vitro* propagation can become an important alternative to conventional propagation for a wide range of plant species. A reproducible protocol for the *in vitro* propagation of *S. robusta* has been developed in this study. For generating true-to-type plants shoot multiplication is preferred to callus mediated regeneration. This paper supports the rapid multiplication of this commercially important plant by an *in vitro* culture technique and provides a simple protocol for the mass propagation of this plant from nodal portions. Based on the data of the present study we can conclude that the reported regeneration system is repeatable and can be easily used to regenerate *S. robusta* plants.

References

Aliou N., Mamadou S.D., Dame N., Yaye Kene G.D. (2006): *In vitro* regeneration of adult trees of *Bambusa vulgaris*. African Journal of Biotechnology, ***5***: 1245–1248.

Amo-Marco J.B., Lledo M.D. (1996): *In vitro* propagation of *Salix tarraconensis* Pau ex Font Quer an endemic and threatened plant. *In Vitro* Cellular and Developmental Biology – Plant, ***32***: 42–46.

Gamborg O.L., Miller A., Ojima K. (1968): Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research, ***50***: 151–158.

Kumar S., Singh S. (2009): Micropropagation of *Prosopis cineraria* (L.) Druce – a multipurpose desert tree. Researcher, ***1***: 28–32.

Lloyd G., McCown B. (1980): Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Proceedings of the International Plant Propagator's Society, ***30***: 421–427.

Lyyra S., Lima A., Merkla S.A. (2006): *In vitro* regeneration of *Salix nigra* from adventitious shoots. Tree Physiology, ***26***: 969–975.

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

Nakamura K. (2006): Micropropagation of *Shorea roxburghii* and *Gmelina arborea* by shoot-apex culture. In:

Suzuki I., Ishii K., Sakurai S., Sasaki S. (eds): Plantation Technology in Tropical Forest Science. Tokyo, Springer-Verlag: 137–150.

Nanda R.M., Das P., Rout G.R. (2004): *In vitro* clonal propagation of *Acacia mangium* Willd. and its evaluation of genetic stability through RAPD marker. Annals of Forest Science, ***61***: 381–386.

Orwa C., Mutua A., Kindt R., Jamnadass R., Simons A. (2009): Agroforestree Database: a Tree Reference and Selection Guide Version 4.0. Avaialable at http://www.worldagroforestry.org/af/treedb

Pijut P.M., Lawson S.S., Michler C.H. (2011): Biotechnological efforts for preserving and enhancing temperate hardwood tree biodiversity, health, and productivity. *In Vitro* Cellular and Developmental Biology – Plant, ***47***: 123–147.

Rajore S., Batra A. (2005): Efficient Plant regeneration via Shoot tip explants in *Jatropha curcas.* Journal of Plant Biochemistry and Biotechnology, ***14:*** 73–75.

Rao C.S., Eganathan P., Anand A., Balakrishna Reddy T.P. (1998): Protocol for *in vitro* propagation of *Excoecaria agallocha* L., a medicinally important mangrove species. Plant Cell Reports, ***17***: 861–865.

Rout G.R. (2004): Effect of cytokinin and auxin on micro propagation of *Cleitoria ternatea* L. Biological Letters, ***41***: 21–26.

Rout G.R., Samantaray S., Das P. (2000): *In vitro* manipulation and propagation of medicinal plants. Biotechnology Advances Journal, ***18:*** 91–120.

Saxena C., Rout G.R., Das P. (1998): Micropropagation of *Psoralea coryfolia*, L. Journal of Medicinal and Aromatic Plant Sciences, ***20***: 15–18.

Schenk R.V., Hildebrandt A.C. (1972): Medium and techniques for induction and growth of monocotyledons and dicotyledons plant cell cultures. Canadian Journal of Botany, ***50***: 199–204.

Shukla S., Shukla S.K., Mishra S.K. (2012) *In vitro* regeneration of multipurpose medicinal tree *Stereospermum suaveolens* – factors controlling the *in vitro* generation. Journal of Biotechnology and Biomaterials, ***13*** (Special Issue): 1–3.

Singh K.P., Kushwaha C.P. (2005): Paradox of leaf phenology: *Shorea robusta* is a semi-evergreen species in tropical dry deciduous forests in India. Current Science, ***88***: 1820–1824.

Thakur R., Rao P.S., Bapat V.A. (1998): *In vitro* plant regeneration in *Melia azedarach* L. Plant Cell Reports, ***18***: 127–131.

Vengadesan G., Ganapathi A., Anand R.P., Anbazhagan V.R. (2002): *In vitro* propagation of *Acacia sinuata* (Lour.) Merr. via cotyledonary nodes. Agroforestry Systems, ***55***: 9–15.

Vengadesan G., Ganapathi A., Anand R.P., Selvaraj N. (2003): *In vitro* propagation of *Acacia sinuate* (Lour.) Merr. from nodal segments of a 10-year-old tree. *In Vitro* Cellular and Developmental Biology – Plant, ***39***: 409–414.

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