Micropropagation of *Symplocos racemosa* Roxb., a threatened medicinal tree of India

Symplocos racemosa Roxb. (family: Symplocaceae), commonly known as Lodh, Lodhra or Lodha, is an evergreen forest tree. The plant is known for the immense medicinal values of its stem bark as indicated in the traditional systems of medicine including Ayurveda and Unani. The multifarious use of the stem bark of this medicinal plant has a cure for gynaecological disorders, inflammation, blood diseases, eye diseases and even leprosy. It is also used in the treatment of kapha, dysentery, ulcer, spongy and bleeding gums, etc.[1]. Besides, the bark also has aphrodisiac and anticancer properties[1,2]. Reputed pharmaceutical companies have been reportedly using the bark for various Ayurvedic products. Hence, there is a huge demand for it in local and international market, and the plant is enlisted among the 178 species of India recorded in high volume trade (≥100 Mt/yr). Unfortunately, the materials for trade are being collected mostly from wild habitat in an unsustainable manner that is detrimental to the ecosystem[3]. Besides, *S. racemosa* has a limited geographical distribution, may be due to habitat destruction and anthropological activities. Thus the species is exposed to high risk of extinction in the wild[4]. The Foundation of Revitalisation of Local Health Tradition (FRLHT), Bengaluru has assessed the threat status of this valuable medicinal plant in India through Conservation Assessment and Management Prioritization (CAMP) workshops adopting IUCN Red List category and criteria, and found it to be a threatened species in different Indian states like Odisha, Karnataka and Maharastra[5]. Thus the plant is in urgent need of attention for conservation.

This threatened plant is propagated by vegetative means as well as seeds[4]. Regeneration from vegetative propagation by root segments is low[6] and time-consuming. Seed propagation has its own limitations, as the plants produce non-viable seeds[6]. Besides, the viable seeds lose their viability in three months. In fact, the fruits of this plant are severely infected with larvae of beetles which emerge only one day after collection[4]. Hence the natural and conventional methods of propagation alone cannot meet the demand for commercial utilization. There is a need for reintroduction and conservation of this threatened medicinal tree in its wild habitat. Hence biotechnological intervention through micropropagation is the best alternative to conventional propagation for regeneration of a large number of these plants aiming at their conservation and commercial use by pharmaceutical companies.

To the best of our knowledge, there are no reports available on micropropagation of *S. racemosa*. Here we present a plant regeneration protocol using mature nodal segments for this valuable but threatened medicinal tree.

Young twigs having 4–10 nodal segments were excised from approx. 15-yr-old healthy plant of *S. racemosa* from Katikela Reserve Forest, Odisha, India. The twigs were brought to the laboratory in air-tight polyethylene bags. The nodal segments were excised and kept under constant flow of tap water for 30–45 min and cleaned with a liquid detergent (Teepol; 5% v/v; Rickitt Benckiser Ltd, India) for 15 min. Then they were treated with a fungicide 'ShriramZim 50' (2% (w/v), Shreeji Pesticides Pvt Ltd, India) for 5 min followed by cleaning 3–5 times with double distilled water. After this the nodal segments were surface sterilized with 10 min treatment of 0.1% $HgCl_2$ (Hi-media, India) and then cleaned thoroughly (3–5 times) with sterile double distilled water. Subsequently, both the edges of the nodal segments were trimmed and nodal explants of 1.0–1.5 cm size were used for shoot regeneration experiments.

Murashige and Skoog (MS) medium[7] augmented with various plant growth regulators and adjuvants, including N^6-benzyladenine (BA) (0.5–5.0 mg/l), kinetin (KIN) (0.5–5.0 mg/l), zeatin (Z) (0.5–5.0 mg/l), α-naphthaleneacetic acid (NAA) (0.25–1.0 mg/l), 2,4-dichlorophenoxyacetic acid (2,4-D) (0.25–1.0 mg/l), gibberellic acid (GA_3) (1.0–3.0 mg/l), adenine sulphate (ADS) (25–100 mg/l) and phloroglucinol (PG) (25–100 mg/l), either individually or in different combinations was tested in order to find a suitable medium for axillary shoot proliferation.

Rooting of *in vitro* shoots (2.5–3.5 cm) was carried out by culturing them either on ½ MS medium alone or ½ MS supplemented with 0.5–8.0 mg/l indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and NAA separately. Due to poor results with MS, we also tried a different basal medium, i.e. woody plant medium (WPM)[8] at half strength (1/2 WPM) alone or supplemented with auxin (IBA; 0.5–8.0 mg/l) for rooting of shoots which were regenerated *in vitro*. All culture media were solidified with 0.7% (w/v) agar. Prior to autoclaving at 121°C for 17 min, the pH of the media was set at 5.8 ± 0.1. All the cultures were incubated at 25 ± 1°C with 16 h photoperiod and 50 µmol m^{-2} s^{-1} photon flux density of light.

In vitro rooted plantlets were removed from the tube and cleaned carefully in water. The plantlets were then planted in small plastic glasses containing sterile sand and soil (1 : 1) moistened with sterile double distilled water. They were maintained in the culture room by covering with polyethylene bags before transferring to larger clay pots having sand, soil and cow dung (1 : 1 : 1).

Twelve nodal segments (six culture flasks with two explants each) and fifteen shoots (one shoot per culture tube) per treatment were taken for shoot proliferation and rooting experiments respectively. All the experiments were carried out thrice. The degree of significance among treatments was determined using analysis of variance (ANOVA) and Duncan's new multiple range test (DMRT) following standard methods[9].

Bud break was observed from the nodal segments after 6–8 days of inoculation, irrespective of the medium tested. Percentage of bud break and shoot proliferation were influenced by the type and concentration of plant growth regulators and growth adjuvants supplementation to MS (Table 1). MS devoid of any plant growth regulators exhibited only a single shoot (Figure 1 *a*). MS medium supplemented with optimum concentration (3.0 mg/l) of BA induced 3.5 shoots/explant. The average shoot length was recorded to be 2.6 cm on this medium. Two other cytokinins, namely KIN and Z responded poorly than BA, for axillary

Table 1. Influence of growth regulators and growth adjuvants on shoot formation from mature nodal segments of *Symplocos racemosa*

MS basal medium + growth regulator/growth adjuvant (mg/l)								Bud break (%)	Shoots/ explant	Average shoot length (cm)
BA	KIN	Z	NAA	2,4-D	GA_3	ADS	PG			
0	0	0	0	0	0	0	0	52.77^{k}	1.00^{w}	1.40^{st}
0.5	–	–	–	–	–	–	–	80.55^{d}	2.10^{o}	1.70^{pqr}
1.0	–	–	–	–	–	–	–	86.11^{b}	2.48^{jklm}	1.90^{nop}
2.0	–	–	–	–	–	–	–	86.11^{b}	3.00^{gh}	2.50^{hij}
3.0	–	–	–	–	–	–	–	91.66^{a}	3.51^{f}	2.60^{ghi}
4.0	–	–	–	–	–	–	–	80.55^{d}	2.00^{op}	2.00^{mno}
5.0	–	–	–	–	–	–	–	80.55^{d}	2.00^{op}	1.90^{nop}
–	0.5	–	–	–	–	–	–	83.33^{c}	1.00^{w}	2.00^{mno}
–	1.0	–	–	–	–	–	–	91.66^{a}	1.20^{uv}	2.00^{mno}
–	2.0	–	–	–	–	–	–	91.66^{a}	1.30^{stu}	2.10^{lmn}
–	3.0	–	–	–	–	–	–	91.66^{a}	1.51^{rs}	1.80^{opq}
–	4.0	–	–	–	–	–	–	86.11^{b}	1.20^{uv}	1.50^{rs}
–	5.0	–	–	–	–	–	–	80.55^{d}	1.00^{w}	1.50^{rs}
–	–	0.5	–	–	–	–	–	69.44^{h}	1.00^{w}	3.00^{cde}
–	–	1.0	–	–	–	–	–	75.00^{f}	1.88^{opq}	2.90^{def}
–	–	2.0	–	–	–	–	–	80.55^{d}	2.51^{jk}	2.60^{ghi}
–	–	3.0	–	–	–	–	–	77.77^{e}	2.00^{op}	2.50^{hij}
–	–	4.0	–	–	–	–	–	72.22^{g}	1.50^{rst}	2.50^{hij}
–	–	5.0	–	–	–	–	–	69.44^{h}	1.00^{w}	2.30^{jkl}
	3.0	–	–	–	–	–	–	91.66^{a}	2.36^{klmn}	2.20^{klm}
	–	–	0.25	–	–	–	–	80.55^{d}	2.10^{o}	1.30^{stu}
	–	–	0.5	–	–	–	–	83.33^{c}	2.80^{hi}	1.50^{rs}
	–	–	1.0	–	–	–	–	77.77^{e}	2.50^{jkl}	1.40^{st}
	–	–	–	0.25	–	–	–	83.33^{c}	3.20^{fg}	2.50^{hij}
3.0	–	–	–	0.5	–	–	–	86.11^{b}	5.20^{bc}	2.9^{def}
	–	–	–	1.0	–	–	–	80.55^{d}	4.48^{d}	3.20^{c}
	–	–	–		1.0	–	–	83.33^{c}	5.33^{b}	3.00^{cde}
	–	–	–	0.5	2.0	–	–	91.66^{a}	5.70^{a}	3.80^{a}
	–	–	–		3.0	–	–	83.33^{c}	4.46^{de}	3.80^{a}
–	–	–	–	–	–	25	–	63.88^{j}	1.00^{w}	2.40^{ijk}
–	–	–	–	–	–	50	–	77.77^{e}	2.00^{op}	2.70^{fgh}
–	–	–	–	–	–	75	–	72.22^{g}	1.50^{rst}	2.90^{def}
–	–	–	–	–	–	100	–	66.66^{i}	1.50^{rst}	2.50^{hij}
–	–	–	–	–	–	–	25	77.77^{e}	1.00^{w}	2.80^{efg}
–	–	–	–	–	–	–	50	83.33^{c}	1.60^{r}	3.10^{cd}
–	–	–	–	–	–	–	75	91.66^{a}	2.70^{hij}	3.70^{ab}
–	–	–	–	–	–	–	100	86.11^{b}	2.00^{op}	3.00^{cde}

Means in a column with different letters are significantly different at $P \leq 0.05$.

shoot regeneration. Augmentation of 0.5 mg/l 2,4-D to 3.0 mg/l BA-supplemented MS medium was beneficial for shoot proliferation, where 5.2 shoots/explant with a mean shoot length of 2.9 cm were recorded. Augmentation of GA_3 to this medium not only promoted multiple shoot regeneration, but also facilitated shoot growth. Thus, MS basal medium augmented with 3.0 mg/l BA, 0.5 mg/l 2,4-D and 2.0 mg/l GA_3 was found to be most favourable for shoot proliferation. About 91.66% shoot regeneration with average shoots of 5.7/explant and mean shoot length of 3.8 cm was observed on this optimal medium (Figure 1 *b* and *c*). Use of growth adjuvants, including ADS and PG, had a positive but not significant effect on axillary shoot proliferation (Figure 1 *d*).

After successful *in vitro* shoot regeneration, the shoots (2.5–3.5 cm) were cultured on different media to test the rooting ability (Table 2). During experiments on rooting of shoots, it was observed that ½ MS containing various concentrations (0.5–8.0 mg/l) of IAA or NAA failed to induce roots (data not shown). IBA at lower concentrations, i.e. 0.5–3.0 mg/l was also unable to regenerate roots from the shoots. Comparatively higher concentrations (4.0–7.0 mg/l) of IBA was found to be essential for root formation of *S. racemosa*, but the rooting was associated with callus formation at the basal portions of the shoots. Beyond 7.0 mg/l IBA, only basal callusing was observed. Forty per cent rooting of shoots, with 8.0 roots/shoot with basal callusing, was observed on 6.0 mg/l IBA augmented ½ MS medium (Figure 1 *e*; Table 2). However best rooting (approx. 49%) without basal callusing was recorded on ½ WPM + 6.0 mg/l IBA medium (Table 2), where rooting initiated after two weeks of culture. On induction of roots (21 days after culture), they were sub-cultured on ½ WPM medium devoid of auxins for further growth. Twelve roots/shoot with mean root length of 4.6 cm were observed on day 42 of culture (Figure 1 *f*). Shoots cultured at lower concentrations (0.5–2.0 mg/l) of IBA augmented with ½ WPM failed to

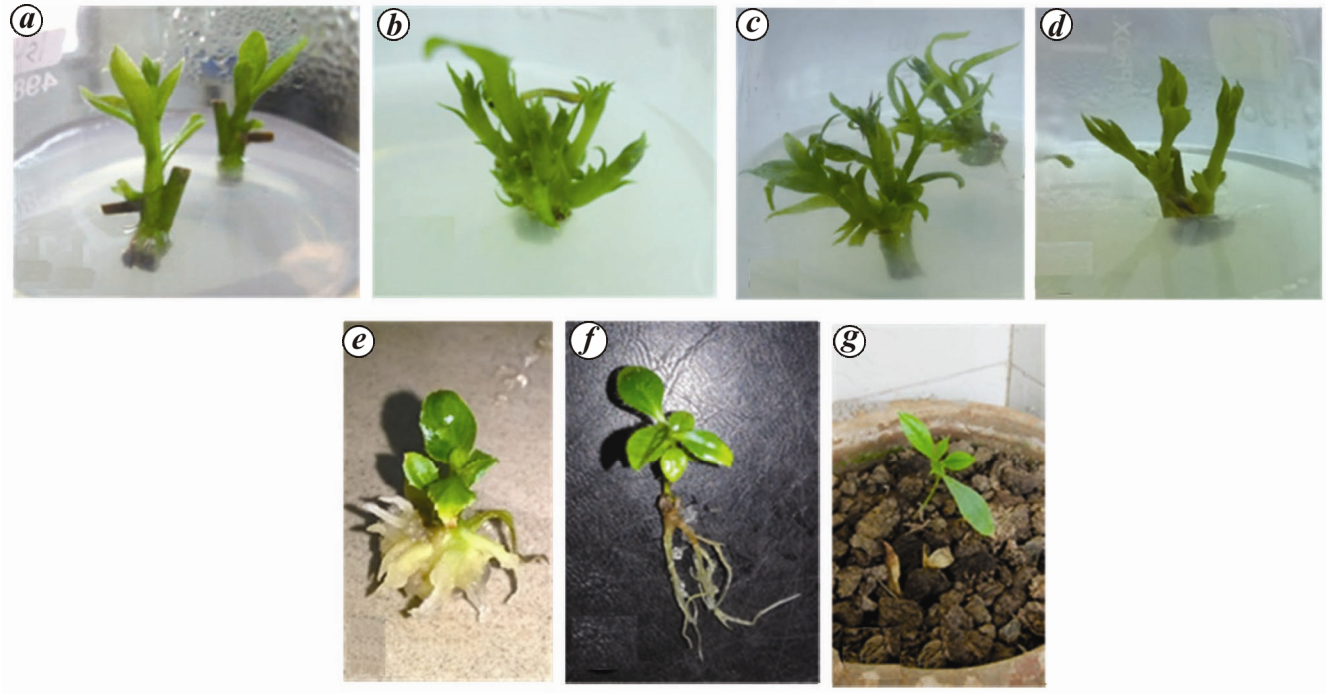

Figure 1. ***a***, Shoot proliferation from nodal segment on MS medium. ***b***, Multiple shoot initiation on MS + 3.0 mg/l BA + 0.5 mg/l 2,4-D + 2.0 mg/l GA_3. ***c***, Multiple shoot proliferation on MS + 3.0 mg/l BA + 0.5 mg/l 2,4-D + 2.0 mg/l GA_3. ***d***, Multiple shoot proliferation on MS + 50 mg/l ADS. ***e***, Rooting of *in vitro* generated shoot on ½ MS + 6.0 mg/l IBA. ***f***, Well-rooted *in vitro* regenerated shoot on ½ WPM medium following transfer from ½ WPM medium + 6.0 mg/l IBA. ***g***, Acclimatized plant in an earthen pot containing sand, soil and cow dung.

Table 2. *In vitro* root formation of shoots of *S. racemosa*

IBA (mg/l)	% Rooting	Roots/shoot	Root length (cm)
½ MS	–	–	–
0.5	–	–	–
1.0	–	–	–
2.0	–	–	–
3.0	–	–	–
4.0	26.66^{f}	4.0^{fg}*	1.5^{hi}
5.0	33.33^{d}	5.3^{de}*	1.9^{fgh}
6.0	40.00^{b}	8.0^{bc}*	2.3^{ef}
7.0	35.55^{c}	5.5^{d}*	1.0^{j}
8.0	–	–*	–
½ WPM	–	–	–
0.5	–	–	–
1.0	–	–	–
2.0	–	–	–
3.0	22.22^{g}	3.4^{fgh}	2.1^{efg}
4.0	31.11^{e}	5.5^{d}	3.7^{c}
5.0	35.55^{c}	9.0^{b}	4.2^{ab}
6.0	48.88^{a}	12.0^{a}	4.6^{a}
7.0	40.00^{b}	4.1^{f}	3.3^{cd}
8.0	40.00^{b}	2.0^{i}*	2.5^{e}

*Callus at the basal end. Means within a column having different letters are significantly different at $P \leq 0.05$.

initiate roots. A comparatively higher (8.0 mg/l) IBA supplemented on ½ WPM showed basal callus with few roots. The rooted plantlets were acclimatized under culture room conditions for three weeks prior to shifting them to larger clay pots (Figure 1 *g*). However, work is still in progress in our laboratory for further refinement of this procedure. In conclusion, we have described an *in vitro* plant propagation protocol for *S. racemosa*, which has the potential to circumvent the

problem of large-scale propagation for conservation of this threatened tree species.

1. Kirtikar, K. R. and Basu, B. D., *Indian Medicinal Plants II*, International Book Distributors, Dehradun, 1975.
2. Raval, B. P., Patel, J. D., Patel, B. A. and Ganure, A., *Rom. J. Biol. – Plant Biol.*, 2009, **54**(2), 135–140.
3. Ved, D. K. and Goraya, G. S., *Demand and Supply of Medicinal Plants in India*, National Medicinal Plants Board (NMPB), New Delhi and Foundation for Revitalisation of Local Health Traditions (FRLHT), Bengaluru, 2007.
4. Acharya, N., Acharya, S., Shah, U., Shah, R. and Hingorani, L., *J. Ethnopharmacol.*, 2016, **181**, 236–251.
5. http://envis.frlht.org/medicinal-plants-conservation-concern-species.php
6. Kalidass, C., *Int. J. Adv. Res.*, 2014, **2**(11), 27–32.
7. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15**, 473–497.
8. Lloyd, G. and McCown, B. H., *Int. Plant Prop. Soc. Comb. Proc.*, 1980, **30**, 421–427.
9. Gomez, K. A. and Gomez, A. A., *Statistical Procedure for Agricultural Research*, Wiley, New York, 1984.

ACKNOWLEDGEMENTS. We thank the Science and Technology Department, Government of Odisha for providing funds. We also thank the Forest and Environment Department, Government of Odisha for permission to use plant materials; the DST-FIST programme of the Department of Botany, Ravenshaw University, Cuttack for providing infrastructural facilities; Dr Pratap Chandra Panda (Regional Plant Resource Centre, Bhubaneswar), Mr Biswa Nath Hota (Retired Divisional Forest Officer) and Mr Himansu Sekhar Muduli (Office of the Divisional Forest Officer, Cuttack) for useful discussions during the course of this work and Dr Subhra Prakash Das (Ravenshaw University, Cuttack) for necessary language corrections. S.K.N. thanks UGC, New Delhi for financial support through UGC-Research Award.

Received 2 October 2016; revised accepted 31 May 2017

SHASHIKANTA BEHERA
DURGA P. BARIK
SOUMENDRA K. NAIK*

Department of Botany,
Ravenshaw University,
Cuttack 753 003, India
**For correspondence.*
e-mail: sknuu@yahoo.com

Antibacterial activity of some important medicinal plants

The micro-organisms induced diseases are responsible for over 50% deaths and are still a key concern to public health especially in developing countries[1]. Synthetic antimicrobial drugs are expensive and relatively unavailable in developing and under-developed countries[2]. Again, the safety aspect of synthetic drugs is a major concern since they may be responsible for causing cancers and other lethal diseases when used for a long time. This is attributed to the presence of many carcinogenic and teratogenic agents[3]. Emergence of anti-microbial resistance in pathogenic bacteria against antimicrobial drugs is another concern[4,5].

Medicinal plants are considered valuable sources and called nature's 'chemical factories' for providing secondary metabolites that contain anti-microbial properties[6–9]. Plant-based drugs are considered safe for health. Moreover, compounds derived from plants are found more active when compared to well-established antibiotics[10]. For these reasons, scientists are diverting their attention towards the discovery of plant-based anti-microbial drugs[11]. In this communication, we study the anti-bacterial activity of the methanolic extracts of *Swertia chirata*, *Berberis aristata*, *Paris polyphylla*, *Digitalis purpurea*, *Digitalis lanata*, and essential oil of *Cymbopogon flexuosus* against *Citrobacter freundii*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Proteus vulgaris*.

Root/rhizome samples of *S. chirata*, *B. aristata*, *P. polyphylla*, *D. purpurea*, *D. lanta* and fresh leaves of *C. flexuosus* were collected from the herbal garden of Herbal Research and Development Institute, Mandal (1550 m amsl (above mean sea level)). The roots were washed with running tap water and then cut into small pieces. These pieces were then dried at 25°C over glass plate, and were ground through a pulverizer. The root powder passed through 0.35 mm sieve was taken for extraction and anti-bacterial analysis. Exactly 100 mg powdered rhizomes/roots of each species were extracted in 20 ml of methanol on a water bath at 70°C for 6 h and replicated three times. The solvent of the combined extracts was removed to dryness under vacuum. The extract was then dissolved in 10 ml methanol to obtain 10 mg ml^{-1} solution. Syringe filter (0.45 µm) was used to filter the extract used for analysis. The fresh aerial part of *C. flexuosus* was cut into small pieces and put into Clevenger apparatus for hydro-distillation for 5 h. The method described in *British Pharmacopoeia* was followed for isolation of essential oil[12]. Anhydrous sodium sulphate was used to dry the extracted essential oil and was kept in a brown glass jar and stored at 4°C for further analysis.

Six bacterial strains obtained from Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, selected as representative of the class of Gram-positive (*E. faecalis* and *S. aureus*) and Gram-negative (*S. typhimurium*, *E. coli*, *C. freundii* and *P. vulgaris*) were used for assay. Well diffusion method was adopted for screening of anti-bacterial activity of tested extracts/oil following Deshmukh *et al.*[13]. The bacterial strains were maintained on nutrient agar No. 2 (Himedia, India) at 37°C and were pre-cultured in nutrient broth. Sterile saline water (0.85% NaCl) was used to dilute the stock culture suspension. The Mueller Hinton Agar (Himedia, India) was poured in sterile petri plates and kept for solidification. To attend confluent growth of test micro-organism, 0.1 ml of diluted inoculums was spread over in the plates using sterile L spreader. Wells of 6 mm were then bored into the agar using a sterile cork borer which was then filled with exactly 100 µl of different concentrations of crude extracts of different species. The petri plates were incubated in a refrigerator for 2 h to permit diffusion of crude extract in the medium. To obtain colonies of test bacteria, plates were then incubated for