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Studies on factors influencing node culture establishment during *in vitro* shoot multiplication from mature *Schleichera oleosa* (Lour.) Oken tree

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Schleichera oleosa (Lour.) Oken is a preferred tree-host of *kusmi* form of lac insect, *Kerria lacca*, which produces superior quality lac resin of commercial interest. Very low success rate of clonal multiplication of this tree is one of the major bottlenecks for supply of quality planting material for lac cultivation. *In vitro* multiplication of axillary buds although promise to produce large-scale clones of high-yielding lac hosts, establishment of aseptic node culture is one of the critical steps in standardization of micropropagation from mature *S. oleosa* plant. The seasonal influence on bud emergence, heavy microbial contaminations and phenolic exudations are the important factors observed in the present study that limits the establishment of axillary bud cultures in *S. oleosa*. Predominant fungal and bacterial contaminants were identified through morpho-cytological and DNA sequence analysis. The Murashige and Skoog's (MS) medium with 1.0 mg/L BAP and 1.0 mg/L silver nitrate showed best (83.33±13.61%) shoot initiation. Sub-culturing and elongation of the proliferated microshoots were possible on filter-paper-bridge soaked in liquid MS medium with 0.5-1.0 mg/L BAP, instead of agar-gelled MS media. Rooting of the axillary bud-derived shoots continued to be the major hurdle to achieve success in developing micropropagation protocol in *S. oleosa*.

Keywords: *Schleichera oleosa,* Lac insect host, Node culture, Microbial contamination, Shoot multiplication, Tree.

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Introduction

Schleichera oleosa (Lour.) Oken (Family Sapindaceae), vernacularly known as Lac tree or *kusum* is a large deciduous forest tree species of tropical and subtropical Asian countries like India, Nepal, Sri Lanka, Thailand, Indonesia and Malaysia. The tree is popularly exploited in India, especially in the tribal belts of Jharkhand, Orissa, Madhya Pradesh and Chattisgarh for rearing of *kusmi* infra sub-specific form of lac insects (*Kerria lacca* Kerr.) for production of excellent quality lac resin of various commercial interests[1]. Besides, different plant parts of *S. oleosa*, such as fruits, leaves, bark and seeds are used as tribal food, animal feed, seed-oil and timber. The tree also serves as important source for traditional medicines for curing pruritis, malaria, inflammation and ulcers. Thus, *S. oleosa* is a multipurpose tree with both commercial importance and tribal sentiments which is worth conserving.

The conventional propagation of *S. oleosa* tree is through seeds; resulting into genetic heterogeneity and very long juvenile phase due to slow growth of seedlings, which are discouraged in lac cultivation. The viability and germination rate of seeds of *S. oloesa* is also very poor probably due to high seed lipid content[2]. Clonal multiplication in *S. oloesa* is very difficult and is possible only through air-layering. However, the growth of air-layers is very slow and was found to have very poor field-survival rate, limiting its potential to supply large-scale quality planting material for lac cultivation[3]. Thus, micropropagation of *S. oleosa* is an alternative approach for large-scale multiplication of superior genotypes, their conservation and genetic improvement. Previous report on micropropagation of *S. oleosa* is limited only to the preparation of explants for establishment of node cultures[4]. Very little efforts

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were made to identify the critical factors involved in successful node culture establishment for axillary bud proliferation and multiplication in *S. oloesa*. In the present investigation limitations with reference to axillary bud proliferations, establishment of aseptic bud cultures and optimization of rooting of microshoots from mature *S. oleosa* tree have been worked out.

Materials and Methods

Preparation of explants

Forty to sixty years old *S. oleosa* tree known for high lac resin productivity was chosen from the research farm of IINRG, Ranchi (23°19′51″N, 85°22′18″E, elevation~693 m above sea level) as a source for deriving explants. The branches were pruned during January and sprayed with fungicide 0.25 g/L Bavistin (Carbendazim 50%) (BASF Ltd., India), 0. 25 g/L Ridomil MZ 72 WP (mixture of Metalaxyl 8% and Mancozeb 64% WP) (Syngenta) and 1.0 g/L urea to harvest fresh shoots. The terminal buds and nodal segments (3^{rd}-6^{th} node from the branch apex) of 2-3 cm length containing a single axillary bud were collected from the fresh sprouts. The explants were immediately washed with pre-chilled antioxidant-fungicide solution containing 0.3 g/L citric acid, 0.1 g/L bavistin, 0.1 g/L ridomil and few drops of tween-20 for one hour under continuous shaking. The explants were washed with double distilled water for twice followed by surface sterilization of explants by sequential treatment of 70% ethanol for two minutes and 1.0 g/L mercuric chloride and 1.0 g/L SDS for 8 minutes. The surface sterilized explants were washed in sterile distilled water for five times and soaked on sterile paper towel before inoculating on plant culture medium.

Media and culturing conditions

The sterilized nodal segments with single axillary bud were aseptically transferred to test tubes containing about 15 mL of agar-gelled media. Seven different media compositions (MS, ½MS, B_5, S & H, WPM, MS+$AgNO_3$ and MS+¼sucrose) were tested for response to axillary bud emergence. The explant browning and microbial contaminations were prevented by supplementing antioxidants (0.5 g/L ascorbic acid and 0.3 g/L citric acid) and antibiotics (40 mg/L gentamycin or 250 mg/L cefotaxime) to the medium. Different combinations of plant growth regulators (PGRs), like BAP, kinetin, 2ip, TDZ, GA_3, NAA, silver nitrate and adenine sulphate were evaluated with Murashige and Skoog's medium for axillary bud proliferation[5]. The seasonal influence on axillary bud-break was investigated at monthly interval. Elongation and multiplication of shoots were compared on agar-gelled MS medium and on liquid MS medium-soaked bridge made from white filter paper or brown seed germination paper. Both the agar-gelled MS and liquid MS medium used for shoot elongation were supplemented with 0.5-1.0 mg/L of 6-BAP. All the above node cultures were incubated in dark for 72 h followed by incubation at 24±2°C under white fluorescent tube lights with 16 h photoperiod. The shoot cultures were maintained at 24±2°C and 16 h photoperiod for three weeks before data recording and sub-culturing.

Identification of microbial contaminants

Visually different fungal contaminants from *S. oleosa* node cultures were inoculated on potato dextrose agar (PDA) medium and incubated at 26°C for 6 days. The bacterial contaminations were streak-inoculated on nutrient agar medium and incubated at 37°C for 2 days. All the microbial cultures were purified through repeated sub-culturing of the isolates. Identification of fungi isolates were carried out through morphology of the colony shape, size, and color followed by microscopic identification using lactophenol blue dye. The bacterial contaminants were microscopically identified through gram staining and growth on Eosin methylene blue (EMB) agar plate.

For molecular identification of the microbial contaminants, genomic DNA was extracted from five different fungal isolates and one bacterial isolate as per the protocols described by Lee *et al* and Cheng and Jiang, respectively[6,7]. The universal 16S (U3 Forward: GTGCCTGCAGCCGCGGTAAT and U8 Reverse: AAGGAGGGGTGTGTA) and internal transcribed spacer DNA (ITS) primers of 18S rDNA (ITS5 Forward: GGAAGTAAAAGTCGTAACAAG and ITS2 Reverse: GCTGCGTTCTTCATCATCGATGC), respectively for bacteria and fungi were used to amplify the microbial genomic DNA templates[8,9]. The PCR amplified products were sequenced and the BLAST analysis of NCBI (www.ncbi.nlm.nih.gov/blast/) was performed to identify the sequence similarity.

Data analysis

Each treatment of axillary bud emergence consisted of six replications (single explant per tube) and the experiments were repeated thrice. The data on axillary bud emergence were collected after three weeks and the response was determined in terms of percentage of bud emergence. The mean values calculated for bud emergence data were subjected to ANOVA followed by multiple comparison tests of means using Fisher's Least Significant Difference (LSD) at $P<0.05$ level using DSAASTAT ver. 1.022. The experiment on shoot elongation was carried out using 20 explants per treatment and was repeated thrice. The data from the experiment of shoot elongation were collected after four weeks.

Results and Discussion

Pretreatments and choice of explant

Fungicidal (0.25 g/L bavistin, 0.25 g/L ridomil and 1.0 g/L urea) spraying on the pruned branches of mother plant, before shoot sprouting was found effective for harvesting of fresh and contamination-free explants during March-May. One major hindrance of culturing nodal explants from mature tree is the problem of phenolic substance leaching from the cut ends of explants, which gets oxidized resulting into browning of media and explant death[10-11]. The node cultures pretreated with chilled antioxidant and fungicide mixture (0.3 g/L citric acid, 0.1 g/L bavistin, 0.1 g/L ridomil and few drops of tween-20), inoculation of surface sterilized explants on MS media with 0.3 g/L citric acid and 0.5 g/L ascorbic acid and dark incubation of explants for 72 h was found effective in minimizing browning of axillary bud cultures. The antioxidants, citric acid and ascorbic acid, serve as chelating agent in binding metal ions, thereby rendering oxidative enzymes ineffective and safeguarding the explant tissues to oxidative injury from hydrogen peroxide and other oxygen radicals[12]. The above surface sterilization steps could establish approximately 75-80% contamination-free node cultures during March-May. However, even the stringent surface sterilization procedures remain inefficient to control contaminations from the explants derived during the monsoon (during June-September), producing only 10-15% healthy explants.

The type of bud was found very important for bud emergence in *S. oleosa*. Among the apical and axillary bud, the latter was found appropriate for proliferation. The response of apical buds was very slow and resulted into leaf shedding or browning of tissues. The nodes consisting of dormant axillary bud responded satisfactorily in terms of bud proliferation. The buds from the 3^{rd}-6^{th} axils from the shoot apex were found most responsive to bud proliferation than the rest of the buds from the same shoot (data not shown), which is distinct from the result reported in other hardwood plant, *Macademia* spp., in which the 1^{st}-3^{rd} buds responded best[13].

Seasonal influence on axillary bud emergence

The efficiency of micropropagation through axillary bud culture in mature trees depends greatly on the season of explants culturing. In *S. oleosa*, culturing of nodes at monthly interval revealed dormancy of axillary buds throughout October to February. The axillary bud sprouting on MS medium was achieved only during the late March to June; but was observed best only during the month of April (>80%) and extended to some extent up to mid May (>60%). The axillary bud cultures after June were severely affected due to heavy fungal infestations in the cultures (data not shown). The browning of explants, due to phenolic substance leaching was found severe during the months of September to March, resulting in no bud sprouting. Thus, the best season for initiation of axillary bud sprout from the node cultures of *S. oleosa* under *in vitro* condition is suggested during the April-May (Plate 1a). The physiological state of the axillary buds in most of the forest trees become active during the onset of summer months, which otherwise remain dormant during the winter. Therefore, the response of axillary bud sprouting under *in vitro* condition is almost rare in the winter season despite of extensive media and PGR manipulations[14]. The effect of season on bud-sprouting and contamination-free culture establishment has also been reported in micropropagation of other tree species as well[14-18].

Identification and prevention of microbial contamination

Occurrence of cryptic microbial contamination of endophytic bacteria, micro-arthropod as well as fungi is one of the serious problems associated with the micropropagation of tree species; especially when the explants were derived from field source[19,20]. The severity of contamination is largely dependent on season[15]. The microbial contamination due to endophytic bacteria and fungi was one of the major problems encountered during the culturing of nodal explants in *S. oleosa* (Plate 1b) (Table 1). The

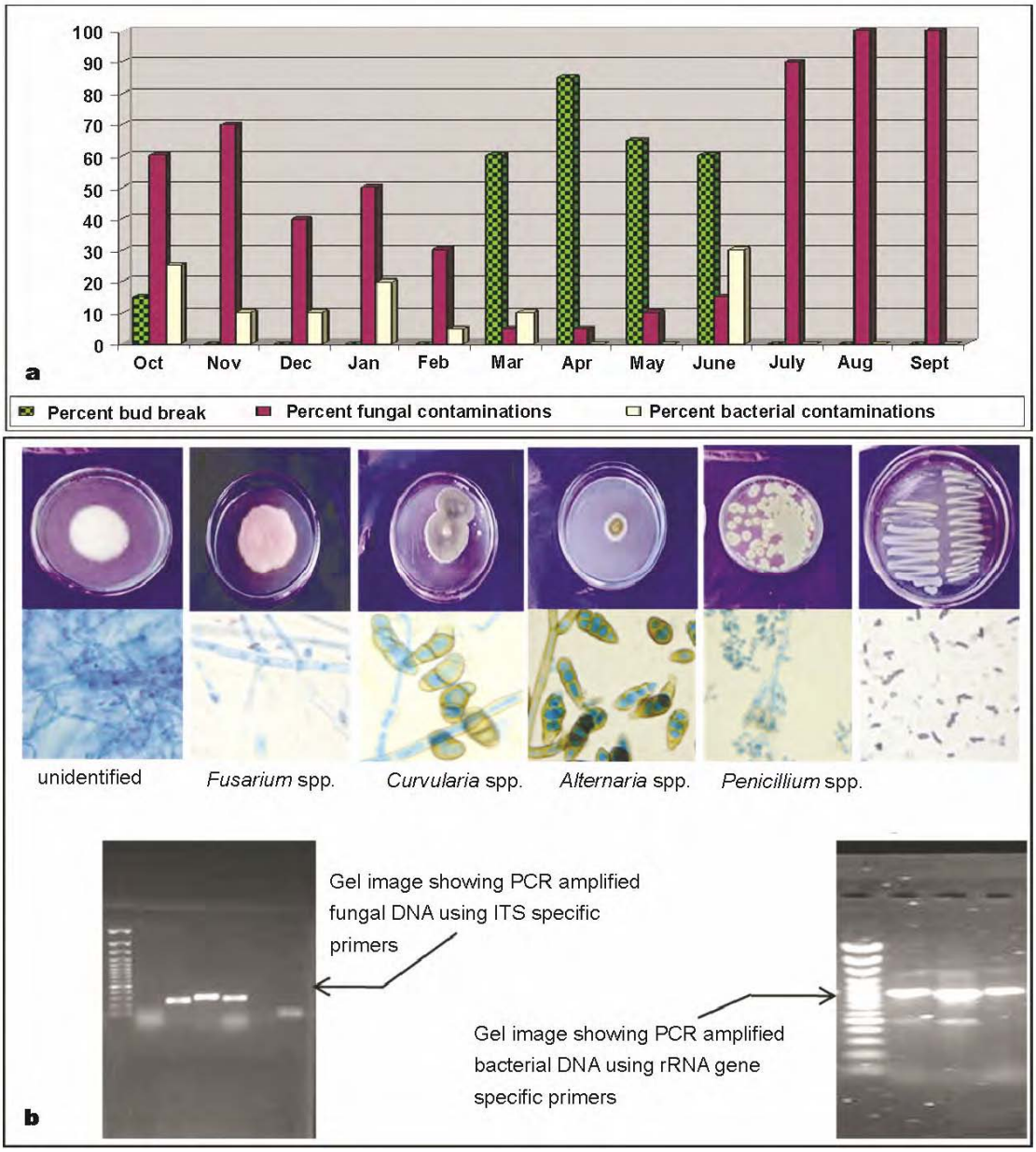

Plate 1—(a) Seasonal influence of axillary bud emergence from node cultures of *S. oleosa* and the occurrence of microbial contaminations. The green, brown and yellow bars indicate percentage of bud-break, phenolic secretion and contaminations, respectively, (b) Identification of predominant microbial contaminants from node cultures of *S. oleosa* through colony morphology (upper panel) cytology (lower panel) and PCR amplification (gel pictures).

bacterial contamination was observed even after several days of culturing, suggesting about the presence of endophytic bacterial contamination in the explants inoculated. Identification of fungal and bacterial contaminants in culture and use of appropriate antimicrobials is the right approach for minimizing contamination in culture. The single gram negative rod-shaped bacteria isolated as contaminant from the node cultures of *S. oleosa* was identified as *Klebsiella pneumonae* through polymerase chain reaction (PCR) studies using universal primers from the conserved rDNA gene. The fungal contaminants of predominant types in the node cultures of *S. oleosa* were identified through morpho-cytological observations and PCR using conserved fungal ITS sequence-specific primers. Four out of the five fungal isolates were identified as *Fusarium* spp. (5% infection rate), *Curvularia* spp. (5%), *Alternaria*

Table 1—Morphological and molecular analysis of predominant microbes from *S. oleosa* node cultures

S. No.	Type of microbial contaminant	Mature colony colour	Colony diameter (7 DAI) (cm)	# Percentage distribution of infection during rainy season			Blast result from NCBI database using DNA sequence information
				June	July	August	
1	Unidentified fungus	White	3.4	50	60	55	No amplification of PCR product
2	Fungus (*Fusarium* spp.)	Pink	2.1	5	5	5	99% sequence similarity to *Fusarium oxysporum*
3	Fungus (*Curvularia* spp.)	Black	1.5	5	5	5	91% sequence similarity to *Curvularia pseudorobusta*
4	Fungus (*Alternaria* spp.)	Green	1.2	10	5	10	97% sequence similarity to *Alternaria* spp.
5	Fungus (*Penicillium* spp.)	Olive Grey	4.2	30	25	30	No amplification of PCR product
6	Gram negative Bacteria (*Klebsiella* spp.)	White	Not ascertained	< 5			99% similarity to rRNA gene of *Klebsiella pneumoniae* subsp. *pneumoniae*

Data averaged from 5 observations during the rainy season; DAI – days after innoculation

spp. (10%) and *Penicillium* spp. (30%) through both microscopic studies (Plate 1b) and DNA sequence analysis (Table 1). One of the fungal isolates, which could not be identified either through morphological or molecular studies, was found to affect the *S. oleosa* node cultures most severely (55%). The above unidentified strain of fungus could be unique and specific for *S. oleosa* tissues.

The antibiotics, antimicrobial agents and media manipulations are the different preventive measures used to eliminate contamination from *in vitro* cultures[21]. Bacterial contamination from the node cultures of *S. oleosa* was controlled by addition of either 40 mg/L of gentamycin (effective for Gram +ve & -ve bacteria) or 100 mg/L cefotaxime (effective for Gram-ve bacteria). The occurrence of fungal infection was prevented by incorporation of 0.1 g/L bavistin and 0.1 g/L ridomil in the surface sterilization step. However, the heavy occurrence of fungal infection (55.6 to 94.4%) in the node cultures of *S. oleosa* during the rainy season could not be prevented even using the above supplements in the media.

Effect of different media and PGRs on axillary bud emergence

Response of micropropagation in most of the tree species often depends on specific nutrient media composition. Out of seven different media compositions evaluated, the MS medium produced highest axillary bud emergence from *S. oleosa* node cultures. However, the response of bud emergence and overall growth of the buds was found satisfactory on MS medium with 1.0 mg/L silver nitrate (data not shown). On an average four numbers of fully expanded leaves were observed and the average length of sprouted bud was found 7.0 mm on MS medium consisting of 1.0 mg/L silver nitrate.

The effect of PGRs on initiation of axillary buds from the node cultures of *S. oleosa* was also investigated using 30 different combinations of cytokinins and auxins on MS medium. Out of them, the treatment combination consisting of 1.0 mg/L BAP and 1.0 mg/L silver nitrate produced highest (83.33%) axillary bud (Table 2). The mean length of shoot bud observed was 11.58 ± 2.42 mm for the above treatment and average number of fully expanded leaves recorded was 1.17 ± 0.17 per explants (Plate 2a). The role of silver nitrate in activating axillary buds from nodal explants was reported in tissue culture of other plants as well[22].

Effect of liquid and semi-solidified media on shoot establishment and sub-culturing

Complete shedding of foliages was observed as one of the major difficulties associated with establishment of axillary-bud derived shoots of *S. oleosa* on

Table 2—Effect of different plant growth regulators on axillary bud proliferation in *S. oloesa* on MS medium

Sl. No.	Plant growth regulator	Concentration (mg/L)	Percent bud initiated*	Shoot length* (mm)	Leaf number*
1	Control	0	72.22±7.86^{a}	15.00±1.25^{a}	1.50±0.50^{a}
2	BAP	0.5	55.56±15.71^{a}	12.25±2.25^{ab}	0.38±0.38^{ab}
3		1.0	61.11±20.79^{a}	15.75±3.25^{ab}	1.38±0.63^{abc}
4		2.0	27.78±7.86^{ab}	12.50±2.50^{ab}	1.00±0.00^{abc}
5	Kinetin	0.5	50.0±36.0^{ab}	17.42±0.08^{abc}	1.17±0.67^{abcd}
6		1.0	66.67±13.61^{ab}	17.00±2.00^{abcd}	2.00±1.00^{abcd}
7		2.0	44.44±7.86^{ab}	13.75±3.75^{abcd}	1.25±0.75^{abcd}
8	2ip	0.5	27.78±15.71^{ab}	12.83±1.17^{abcd}	2.33±1.33^{abcd}
9		1.0	27.78±7.86^{ab}	18.75±11.25^{abcd}	1.50±0.50^{abcd}
10		2.0	66.67±23.57^{ab}	15.20±5.20^{abcd}	1.50±0.50^{abcd}
11	TDZ	0.025	44.44±34.25^{ab}	12.00±12.00^{abcd}	0.70±0.70^{abcd}
12		0.05	50.0±13.61^{ab}	14.88±6.38^{abcd}	1.25±0.25^{abcd}
13	BAP+NAA	0.5+0.05	77.78±20.79^{ab}	13.33±2.67^{abcd}	0.75±0.08^{abcd}
14		1.0+0.05	77.78±15.71^{ab}	15.21±1.04^{abcd}	1.54±0.71^{abcd}
15	Kinetin+NAA	0.5+0.05	55.56±34.25^{ab}	16.42±1.42^{abcd}	2.00±1.00^{abcd}
16		1.0+0.05	38.89±20.79^{ab}	9.38±4.38^{abcd}	1.63±0.63^{abcd}
17	2ip+NAA	0.5+0.05	44.44±20.79^{ab}	6.38±0.38^{abcd}	0.50±0.50^{abcd}
18		1.0+0.05	38.89±28.33^{ab}	6.50±6.50^{abcd}	1.1±1.13^{abcd}
19	TDZ+NAA	0.025+0.05	27.78±20.79^{ab}	7.50±7.50^{abcd}	1.33±1.33^{abcd}
20		0.05+0.05	66.67±13.61^{ab}	10.45±2.95^{abcd}	0.58±0.18^{abcd}
21	BAP+GA_3	1.0+1.0	55.56±28.33^{ab}	15.30±0.30^{abcd}	2.10±0.90^{abcd}
22	Kinetin+GA_3	1.0+1.0	66.67±13.61^{ab}	14.33±2.67^{abcd}	1.40±0.60^{abcd}
23	TDZ+GA_3	0.05+1.0	44.44±20.79^{ab}	15.25±5.25^{abcd}	0.50±0.50^{bcd}
24	2ip+GA_3	1.0+1.0	38.89±20.79^{ab}	11.63±6.63^{abcd}	0.50±0.50^{bcd}
25	BAP+$AdSO_4$	1.0+1.0	44.44±20.79^{ab}	17.50±2.50^{abcd}	1.50±0.50^{bcd}
26	Kinetin+$AdSO_4$	1.0+1.0	55.56±28.33^{ab}	12.80±2.80^{abcd}	1.50±0.50^{cd}
27	BAP+$AgNO_3$	1.0+1.0	83.33±13.61^{b}	11.58±2.42^{bcd}	1.17±0.17^{cd}
28	Kinetin+$AgNO_3$	1.0+1.0	61.11±20.79^{b}	8.80±1.20^{cd}	0.80±0.20^{cd}
29	BAP+Kinetin+$AdSO_4$	1.0+1.0+1.0	55.56±7.86^{b}	12.83±0.17^{d}	1.83±0.83^{cd}
30	BAP+Kinetin+$AgNO_3$	1.0+1.0+1.0	55.56±28.33^{b}	10.20±5.20^{d}	0.50±0.50^{d}
ANOVA			F value - 1.09 F critical value (α 0.05) - 1.66 P value - 0.39 LSD (p 0.05) = 44.81	F value - 0.49 F critical value (α 0.05) - 1.85 P value - 0.97 LSD (p 0.05) = 9.38	F value - 0.61 F critical value (α 0.05) - 1.85 P value - 0.91 LSD (p 0.05) = 1.54

*Values represents means ±standard errors followed by the same letters within columns are not significantly different by LSD test at $P = 0.05$.

MS agar medium; leading to 100% explant death (Plate 2b). The problem was overcome by sub-culturing of explants on white filter-paper soaked in liquid MS medium with low concentrations of BAP (0.5-1.0 mg/L) (Plate 2c). The survival percentage recorded was 80-90% as compared to agar-gelled MS medium. However, sub-culturing of the axillary shoots on liquid MS medium soaked paper-bridge made from brown seed germination paper resulted into 100% browning of explants followed by death (Plate 2d). The low-cost crude brown paper probably turned toxic for the explants leading to explant death.

Further sub-culturing of explants on filter-paper-bridge soaked with liquid MS medium consisting of 0.5 mg/L BAP resulted into elongation of shoots. The result suggested that the liquid MS soaked white filter-paper-bridge is an ideal support for sub-culturing and elongation of shoots of *S. oleosa* explants. The poor sustenance of microshoots on agar-gelled media compared to filter-paper-bridge could be explained due to the compromised state of physiological status resulted from hypoxic conditions in the agar medium[23]. The shoot elongation and multiplication on liquid MS medium supported by

Plate 2—(a) Axillary bud proliferation from node culture of *S. oleosa* on MS media with 1.0 mg/l BAP and 1.0 mg/l AgNO3. (b) Problem of leaf shedding on agar solidified MS media observed during shoot elongation, (c) Shedding of leaves prevented by culturing of axillary-bud derived shoots on white filter-paper-bridge soaked in liquid MS media with 0.5 mg/l BAP, (d) Effect of white filter paper versus brown seed germination paper as a support for culturing shoots of *S. oleosa* in liquid MS media with 0.5 mg/l BAP. (e) Induction of adventitious roots from the callus cultures derived from axillary bud derived shoots of *S. oloesa* on ½ MS medium with 20 mg/l IBA.

filter-paper-bridge has also been effective in micropropagation of fruit tree, *Litchi chinensis* (Gaertn.) Sonn. which also belongs to the Sapindaceae family[16,24]. The efforts for rooting of microshoots of *S. oleosa* failed to initiate rooting on different treatments like (i) ½ MS medium containing 15.0 g/L glucose, 2.5 g/L activated charcoal and 20 mg/L IBA for three days followed by sub-culturing of shoots on hormone free ½ MS medium; and (ii) pulse treatment of shoots with 20 mg/L IBA for 30 minutes and culturing on sterile cocopeat. However, formation of adventitious roots (58.33%) was observed from the callus derived from shoots of *S. oleosa* cultures on ½ MS medium with 20 mg/L IBA (Plate 2e). The above results suggest that the rooting of *S. oleosa* cultures from mature plant is a difficult task to achieve in comparison to more juvenile tissue source like calli. The problem of root initiation from microshoots is a major obstacle associated with other hardwood tree species too[13,25]. Thus, there is further scope for standardization of methods for *in vitro* root initiation from axillary shoots of *S. oleosa* and acclimatization of explants.

Conclusion

To conclude from this study, few specific limitations associated with the initiation, proliferation and establishment of axillary shoots from node cultures of mature *S. oleosa* tree and their solutions to mitigate those problems have been identified. The dormancy of buds, explants browning and microbial contamination were identified as major limiting factors for axillary bud proliferation and establishment of shoot cultures *in vitro*. The PGR combinations including of cytokinin, auxin and silver nitrate was found to have beneficiary effect on

axillary bud proliferation. The problem of foliage shedding on agar-gelled MS medium was solved by culturing of explants on filter-paper-bridge soaked with liquid MS medium and cytokinin. The rooting of *in vitro* shoots is the major challenge yet to be standardized for micropropagation in *S. oleosa*. Nonetheless, these findings would be extremely helpful in efficient production of shoot cultures for micropropagation studies in *S. oleosa* or other closely related trees for mass multiplication and genetic improvement.

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