Effect of liquid medium on shoots amplification, *in vitro* flowering and *ex vitro* rooting of *Oldenlandia umbellata* L. - A dye yielding medicinal herb

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Abstract. *Oldenlandia umbellata* L. gains importance due to its medicinal properties and the presence of anthraquinones based natural dyes in the roots. Present study describes the effect of Murashige and Skoog's (MS) liquid medium (full strength) on *in vitro* regeneration, flower bud induction and *ex vitro* rooting in *O. umbellata*. Shoot segments with 2-3 nodes (each node with 2 axillary buds) served as explants for establishment of cultures. The liquid medium augmented with 2.0 mg L⁻¹ 6-benzylaminopurine (BAP) with additives (50 mg L⁻¹ of ascorbic acid and 25 mg L⁻¹ each of arginine, adenine sulphate and citric acid) was effective for shoot bud induction (6.4±0.19 shoots per explant within 2-3 weeks). The shoots were further multiplied (89.3±1.07 shoots, 2-3 weeks) when the shoot clusters obtained from the culture initiation directly transferred to the full-strength MS liquid medium incorporated with 1.0 mg L⁻¹ BAP and 0.5 mg L⁻¹ indole-3 acetic acid (IAA) with additives. Flower buds were induced (12.0±0.15 buds per shoot) when the shoots were cultured on 1.0 mg L⁻¹ BAP and kinetin (Kin, 6-furfurylaminopurine) and 0.5 mg L⁻¹ of IAA at 45 µmol m⁻² s⁻¹ SFPD (Spectral Flux Photon Density) light intensity for 14/10h (light/dark) photoperiod. The adventitious roots were induced on 1/4 strength MS medium supplemented with 1.5 mg L⁻¹ indole-3 butyric acid (IBA). *Ex vitro* rooting was achieved (16.0±0.53 roots per shoot) by pulse treatment of the shoots with 300 mg L⁻¹ IBA for 2 min. The *in vitro* produced plantlets were acclimatized in the greenhouse and finally translocated to the *in vivo* conditions with 93 % success rate. This is the foremost (use of liquid MS medium) and cost-effective method for large scale multiplication of *O. umbellata*.

Keywords: Acclimation, *ex vitro* rooting, *in vitro* flowering, liquid medium, *Oldenlandia umbellata*

INTRODUCTION

Oldenlandia umbellata L. is a natural dye yielding plant of the family Rubiaceae. There are ten synonyms of this plant viz. *Gerontogea umbellata* (L.) Cham. & Schltdl., *Hedyotis umbellata* Lam., *H. brevicalyx* Sivar., Biju & P. Mathew, *H. indica* Roem. & Schult., *H. linarifolia* R.Br. ex Wall., *H. puberula* (G.Don) R.Br. ex Arn., *H. umbellata* (L.) Lam., *H. nightii* (Hook. f.) K.K.N.Nair, *H. nightii* (Hook. f.) Sivar., Biju & P. Mathew and *O. puberula* G. Don (The Plant List, 2013). It is a small, prostrate,

profusely branched perennial herb; native to the Indian subcontinent but distributed in the tropical and subtropical regions such as Burma, Sri Lanka, Cambodia, Indonesia, India, Pakistan and the west tropical Africa (Siva, 2007). The plant is commonly known as Indian Madder plant, Chay root and Shaya (Yoganarasimhan and Chelladurai, 2000).

Ethnopharmacological and medicinal potential of this species are explored by the tribal

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people and ethnic communities of India and China (Yoganarasimhan and Chelladurai, 2000). Traditionally, the leaves and roots of *O. umbellata* were used in the treatment of various ailments (Samy *et al.*, 2008). The roots are used to extract chay-root red dye for calico printing and coloring of fur and silk fabrics from centuries (Siva, 2007).

Owing to over exploitation of *O. umbellata* for its roots by the pharmaceutical companies to get herbal drugs (phenolic compounds and their derivatives) and to harvest anthraquinone pigments (red dye), the population of this plant has been depleted (due to uprooting of entire plant system) in the wild (Siva *et al.*, 2012). The natural propagation of this plant is only by seeds and the plants are harvested before seeds setting. Therefore, there is a need of alternate methods of propagation for conservation of this plant species (Krishnan and Siril, 2015). Tissue culture techniques offer powerful tool for large-scale production of important plants which could help in conservation of medicinal plants.

Some reports are available on the *in vitro* studies and biosynthesis of secondary metabolites from *O. umbellata*, but all the researchers used agar-agar as gelling agent (Siva *et al.*, 2009, 2012; Shekhawat *et al.*, 2012; Kumar *et al.*, 2014; Krishnan and Siril, 2015, 2016, 2017). Agar-agar is a conventional gelling agent used in tissue culture experiments and has been reported to possess certain disadvantages in differentiation and growth of the cultures. Agar powder increases the viscosity of the medium and slows down the nutrients intake by the cultures which affect the growth of plantlets (Kuria *et al.*, 2008). Moreover, the gelling agents in plant tissue culture media may constitute about 70% of production cost (Mohamed *et al.*, 2010). Attempts were made with the other alternatives of agar to reduce this cost, but success was always questionable (Babbar and Jain, 2006). The use of liquid medium for *in vitro* propagation is very effective in proliferation of shoots and adventitious roots which reduce time, energy and cost of production of plantlets (Mehta *et al.*, 2014).

In vitro flower induction explains the fundamental mechanism of transition phase from vegetative phase to the reproductive phase and it could further help to study the physiology of *in vitro* flowering process (Cheruvathur *et al.*, 2015). Rooting and acclimatization can be achieved

concurrently through *ex vitro* rooting method. *Ex vitro* rooting produces better adventitious roots which can again reduce the time; cost and labor input during *in vitro* rooting (Ranaweera *et al.*, 2013).

Therefore, experiments were conducted for the first time to explore the effect of liquid medium in bud breaking, shoots multiplication, *in vitro* flowering and *ex vitro* rooting of *O. umbellata*.

MATERIALS AND METHODS

Plant materials and surface sterilization. One-year old *O. umbellata* plants were collected from the natural habitats of the east coast of India (Puducherry Union Territory), the Coromandel coast (11.9416°N, 79.8083°E), maintained in greenhouse and identified by the *Institut Français de Pondichéry* (IFP), Puducherry (Accession number: HIFP 26744). The nodal segments (explants) of 3-4 cm in length (bearing 2-3 nodes, each node with 2 axillary buds) were collected at the flowering stage. Initially, the explants were treated with 0.1% (w/v) broad spectrum antifungal agent Bavistin (BASF India Ltd., India) for 5-7 min and surface sterilization was achieved with 0.1% (w/v) mercuric chloride (Hi-Media, India) solution for 4-5 min and finally rinsed (five times) with sterilized distilled water under aseptic conditions.

Nutrient medium and culture conditions. Full strength Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) incorporated with 3% sucrose, additives (50 mg L⁻¹ of ascorbic acid and 25 mg L⁻¹ each of arginine, adenine sulphate and citric acid) (after Patel *et al.*, 2014) devoid of agar-agar (liquid medium) was used for regeneration of cultures in this study. The pH of the medium was attuned to 5.8±0.02 with 1N HCl or NaOH before autoclaving at 108 kPa pressure and 120°C temperature for 15 min. The culture flasks were kept on a gyratory shaker at 100 rpm (Technico Pvt. Ltd, Chennai, India), incubated at 25±2°C temperature under 12/12 h (light/dark) photoperiod with light intensity of 40-50 µmol m⁻² s⁻¹ Spectral Photon Flux Density (SPFD) provided by cool white fluorescent lamps of 40 Watts each (Philips India Ltd, New Delhi). The 250 mL Borosilicate culture flasks procured from

the Technico Pvt. Ltd., Chennai, India were used in this study. Twenty-five ml liquid MS medium was poured in each culture vessel and the vessels were closed by cotton plugs before autoclaving.

Induction and proliferation of shoots. The sterilized nodal shoot segments (explants) were inoculated in full strength liquid MS medium (one explant per vessel) without trimming the cut ends. Liquid MS medium fortified with different concentrations of cytokinins, BAP (6-benzylaminopurine) and Kin (6-furfurylaminopurine) ranging from 0.5 to 3.0 mg L⁻¹ (Sigma-Aldrich, St. Louis, USA) alone or in combinations were used for the induction of shoots from nodal explants. The cultures were transferred to the fresh medium after every 2 weeks. The *in vitro* regenerated shoots with optimized length were segmented leaving 3-4 nodes and subcultured on MS medium containing various concentrations of BAP (0.1 to 2.5 mg L⁻¹) combined with various concentrations (0.25 to 0.75 mg L⁻¹) of IAA (indole-3-acetic acid) for proliferation of shoots. The shoots were further multiplied by repeated transfer of mother explants and subculturing of *in vitro* shoots. The cultures on MS medium devoid of growth regulators were treated as control.

In vitro flower buds induction. The *in vitro* produced shoots of 3-4 cm in length were excised and subcultured on full strength liquid MS medium augmented with various concentrations (0.5 to 2.0 mg L⁻¹) of BAP and Kin combined with IAA (0.1 to 0.75 mg L⁻¹) for *in vitro* flower bud induction. Various parameters such as percentage response and average number of flower buds were recorded after 4-5 weeks of incubation. The cultures were maintained at 25±2°C under various photoperiod regimes (10/14 to 16/8h per day light/dark) with light intensity of 40-50 µmol m⁻² s⁻¹ SPFD provided through white fluorescent tubes.

In vitro rooting of the shoots. Healthier shoots were separated from the multiplied shoot clumps and transferred to various strengths of nutrient medium (full, ½ and 1/4 strengths of MS liquid media) augmented with auxins [IAA, IBA (indole-3-butyric acid) and NAA (α-naphthalene acetic acid)] at various concentrations (1.0-4.0 mg L⁻¹)

for induction of roots. The cultures were initially incubated under diffused light for 2-3 days thereafter shifted to the *in vitro* culture environment, and maintained at the light intensity of 40-45 µmol m⁻² s⁻¹ SPFD.

Ex vitro rooting and hardening of plantlets. The end base of the shoots was pulsed with various types and concentrations of auxins (IAA, IBA and NAA at 50-500 mg L⁻¹) for various time durations (1-5 min) for *ex vitro* rooting. The treated shoots were transplanted into 1.5-2.0 cm deep in paper cups (size 150 mL) containing 50 g sterilized soilrite® (Keltech Energy Limited, Bangalore, India) without any antibiotic treatment. Shoots were moistened with 15 mL 1/4 strength MS macro salts solution by the intermission of one week to evade wilting. The set up was capped by perforated transparent polythene cups and maintained in greenhouse under high relative humidity (80-90% RH) and 28±2°C temperature conditions. The caps were gradually removed over a period of two to four weeks. These plantlets were transferred to the nursery poly bags containing organic manure, sand and red soil (1:1:1; w/w) and maintained for 5 weeks. The same plants were next shifted to the earthen pots filled with garden soil, red soil and vermi-compost (2:2:1; w/w), irrigated with tap water and retained in greenhouse for another one and half months for proper hardening.

Experimental design, data collection and statistical analysis. The experiments were laid out in completely randomized block design (CRBD) (Compton and Mize, 1999), conducted with 20 explants for each treatment and repeated thrice (triplicates). The observations on the response, number and length of shoots and roots were recorded at 4 weeks time interval. The data were subjected to the standard error of the mean and single factor analysis of variance (one-way ANOVA). The significance of differences among mean values was carried out using Duncan's Multiple Range Test (DMRT) at P<0.05 using SPSS software, version 16.0 (SPSS Inc., Chicago, USA). The results were expressed as Mean±Standard Error.

RESULTS AND DISCUSSION

Significant improvements were observed in terms of production of shoots with maximum number of nodes and roots with rapid organ developments (within 2 weeks) due to the adoption of liquid MS medium in present study.

Axillary bud emergence from the explants. All the explants yielded shoots on MS liquid medium with various treatments of cytokinins (Figure 1a). The MS medium supplemented with 2.0 mg L^{-1} BAP generated highest number of axillary buds (6.4 ± 0.19 shoots per explant with 4.0 ± 0.21 cm mean length) within 2-3 weeks from the nodal explants (Table 1, Figures 1b and 1c). Liquid MS medium combined with BAP alone significantly enhanced shoots formation from the explants as compared to the other hormonal treatments tested. The MS medium augmented with Kin at 1.5 mg L^{-1} produced 4.2 ± 0.38 shoots. The length of the *O. umbellata* shoots was better in liquid medium as compared to semisolid medium used in previous studies (Shekhawat *et al.*, 2012). The role of liquid medium in axillary bud induction is first time reported in this species. Liquid culture system is advantageous over to the agar gelled medium due to rapid uptake of nutrients by the cells and tissues and minimum negative effect of phenolics leach outs which ultimately enhance the growth of cultured shoots and roots (AlKhateeb and Alturki 2014). Kumar *et al.* (2014) induced 6 shoots (3.5 cm in length) on agar gelled MS medium containing 3.0 mg L^{-1} BAP using shoot tip explants. Krishnan and Siril (2015) observed vitrified shoots with crowning of scaly leaves when nodal explants cultured on agar gelled MS medium with high concentration of 6-benzyladenine (BA).

Proliferation of shoots in liquid MS medium.

The number of shoots was enhanced about 15 folds (from initiation stage) when the regenerated shoots and mother explants were subcultured on MS liquid medium composed of 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} IAA with additives. The maximum shoot numbers (89.3 ± 1.07) with average length of 12.5 ± 0.30 cm were obtained using this media combination (Table 2, Figure 1d). It has been reported that cytokinin and auxin are the major plant growth regulators of core cell cycle

components (Takatsuka and Umeda, 2014). Shekhawat *et al.* (2012) reported shedding of leaves of *O. umbellata* on semisolid media incorporated with optimized growth regulators.

It was observed that liquid media facilitated the production of quality shoots in terms of shoot numbers, shoot length and thickness as compared to agar gelled medium. All the shoots appeared healthy in liquid medium and did not display any symptoms of hyperhydration or vitrification in this experiment. Pati *et al.* (2005) also reported that the liquid medium enhanced rate of shoots multiplication with complete elimination of vitrification in *Rosa damascena* and *R. bourboniana*. Similar results reported in propagation of apple by Mehta *et al.* (2014).

In vitro flowering. Healthy and long shoots (4-5 cm) were cultured on MS medium augmented with combination of cytokinins and auxin resulted in development of terminal and axial congested umbelliform inflorescence. Among the growth regulators tested, the combination of full-strength MS medium containing 1.0 mg L^{-1} BAP and Kin + 0.5 mg L^{-1} of IAA at $45 \mu\text{mol m}^{-2} \text{ s}^{-1}$ SFPD light intensity for 14/10 h (light/dark) photoperiod induced the highest number of flower buds in *O. umbellata* (Table 3). Three inflorescences per shoot were observed and 12.0 ± 0.15 flower buds per inflorescence were emerged when the shoots grown on this medium (Figure 1e). The *in vitro* raised flower buds gradually opened after 2-3 days and expressed similar physical characters as of the mother plants in nature. Flowers were distylous with short pedicel, white corolla, green calyx and glabrous (Figure 1f). *In vitro* flower bud induction is promoted by the plant growth regulators and photoperiods (Taylor *et al.*, 2005). Recently, Behera *et al.* (2017) reported *in vitro* flowers from embryogenic calli of *O. umbellata* on MS semisolid medium. Successful *in vitro* and *ex vitro* flowering is reported to be determined by various intrinsic and extrinsic factors such as florigen coupled with time duration and meristematic activity (Zeevaart, 2006). The results could be used for further analysis of ontogeny of flowers in plants.

In vitro rooting of the proliferated shoots.

Liquid MS medium with IBA played an important role in adventitious roots induction from the

shoots (Thakur *et al.*, 2018). The development and proliferation of roots (100%) were more rapid in 1/4th strength of MS liquid medium (Table 4). Quarter strength MS medium with 1.5 mg L⁻¹ IBA resulted in the emergence of 24.2±0.47 roots with the mean length of 6.1±0.22 cm within 3 weeks (Figure 1g). Comparatively, IAA detected as the weakest hormone in the root induction and NAA ranked second next to the IBA. The previous reports on the role of auxins in rooting of shoots of *O. umbellata* are contradictory to the present findings. Siva *et al.* (2012) reported that higher concentration of NAA in semisolid MS medium had better effect in induction of roots and production of anthraquinone pigments as compared to IBA and IAA from the roots of *O. umbellata*. Enhanced induction and proliferation of adventitious roots in the liquid medium with auxins support the concept of the quick absorption of nutrients from the liquid media than the semisolid medium.

***Ex vitro* rooting and hardening of the plantlets.** Comparatively IBA was found most suitable for *ex vitro* root induction than IAA and NAA. The shoots pulsed with 300 mg L⁻¹ IBA for 2 min exhibited 100% rooting response. Maximum 16.0±0.53 roots with 4.2±0.22 cm average length were induced from the cut end of the shoots with IBA at 300 mg L⁻¹ (Table 5, Figure 1h). Krishnan and Siril (2015) observed 14.03 roots/shoot when the shoots were treated with IBA. The roots induced via this method were superior to the *in vitro* roots in terms of thickness and firmness, which helped in hardening processes in order to improve the survival percentage of *O. umbellata* plantlets in the field conditions. The results are in agreed with the reports of Patel *et al.* (2014) in *Caralluma edulis* and Ranaweera *et al.* (2013) in *Camellia sinensis*. According to the cost estimation on *ex vitro* rooting experiments, it is revealed that the plant production cost can be reduced up to 70% in development of a micropropagation protocol using *ex vitro* rooting technique (Ranaweera *et al.*, 2013).

The rooted shoots were placed on the paper cups containing soilrite® and uphold for 5 weeks

in the greenhouse (Figure 2a). Further, the greenhouse hardened plantlets were transferred to nursery poly-bags containing organic manure, sand and red soil (1:1:1; w/w) (Figure 2b). The plantlets were shifted to earthen pots filled with garden soil, red soil and vermi-compost (2:2:1; w/w) after 4 weeks and finally planted in the natural habitat in another 4-5 weeks (Figures 2c and 2d). About 93% plantlets survived in the field conditions after 6 months of transfer. This rate of survival is superior to the earlier reports in this plant. Krishnan and Siril (2015) achieved 81.3% survival rate in quercetin induced shoots of *O. umbellata*.

Figure 1. *In vitro* propagation of *O. umbellata* L. (a) Induction of shoots from nodal explants in liquid MS medium containing 2.0 mg L⁻¹ 6- BAP, (b and c) Elongation of shoots after subculture to fresh liquid MS medium after 2-3 weeks, (d) Proliferation of shoots after 2-3 weeks on MS liquid medium + BAP 1.0 mg L⁻¹ and IAA 0.5 mg L⁻¹, (e) Flower buds induction (axial and terminal) on MS medium augmented with 1.0 mg L⁻¹ BAP and 0.5 mg L⁻¹ each of Kin and IAA at 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, (f) Formation of complete flowers *in vitro* after 2-3 days of formation of inflorescence, (g) *In vitro* root induction within 3 weeks after subculture in 1/4 MS medium containing 1.5 mg L⁻¹ IBA, (h) *Ex vitro* rooted shoots in soilrite® after 3-4 weeks. (Scale bar – 1 cm)

Table 1. Effect of cytokinins (BAP and Kin) on shoots induction from nodal stem segments of *Oldenlandia umbellata* after 3 weeks of cultures in MS liquid medium.

Cytokinins (mg L ⁻¹)		Response (%)	Shoots number (Mean ± SE)	Shoots length (Mean ± SE)
BAP	Kin			
0.0	0.0	0	0.0 ± 0.00 ^a	0.0 ± 0.00 ^a
0.5	-	62	2.0 ± 0.23 ^c	1.9 ± 0.15 ^b
1.0	-	75	2.7 ± 0.18 ^d	2.5 ± 0.30 ^c
1.5	-	91	4.0 ± 0.39 ^f	3.1 ± 0.16 ^d
2.0	-	100	6.4 ± 0.19 ^h	4.0 ± 0.35 ^f
2.5	-	94	5.1 ± 0.47 ^g	3.8 ± 0.11 ^e
3.0	-	80	3.9 ± 0.24 ^e	3.0 ± 0.20 ^d
-	0.5	57	1.4 ± 0.11 ^b	1.4 ± 0.26 ^b
-	1.0	64	2.0 ± 0.29 ^c	2.0 ± 0.41 ^c
-	1.5	89	4.2 ± 0.38 ^f	3.6 ± 0.11 ^d
-	2.0	80	3.3 ± 0.12 ^e	3.1 ± 0.33 ^d
-	2.5	76	3.0 ± 0.20 ^e	2.7 ± 0.27 ^e
-	3.0	61	2.6 ± 0.36 ^d	2.0 ± 0.25 ^c

Note: The values represented in corresponding column followed by same letters are not significantly different by DMRT at $p < 0.05$.

Table 2. Combined effect of BAP and IAA on proliferation of multiple shoots in liquid MS medium after 2 weeks.

Cytokinins (mg L ⁻¹)		Shoots number (Mean ± SE)	Shoots length (Mean ± SE)
BAP	Kin		
0.0	0.0	00.0 ± 0.00 ^a	0.00 ± 0.00 ^a
0.5	0.25	43.7 ± 0.92 ^b	5.14 ± 0.17 ^b
1.0	0.25	61.0 ± 1.00 ^b	7.15 ± 0.97 ^f
1.5	0.25	58.1 ± 0.80 ^f	6.30 ± 0.80 ^e
2.0	0.25	53.4 ± 0.77 ^d	6.00 ± 0.11 ^d
2.5	0.25	45.9 ± 0.42 ^c	5.72 ± 0.47 ^c
0.5	0.5	60.0 ± 0.29 ^g	9.36 ± 0.39 ^k
1.0	0.5	89.3 ± 1.07 ⁿ	12.5 ± 0.30 ⁿ
1.5	0.5	80.0 ± 1.39 ^m	10.0 ± 0.73 ^l
2.0	0.5	71.6 ± 1.18 ^k	9.14 ± 1.00 ^j
2.5	0.5	63.1 ± 0.94 ⁱ	8.00 ± 0.91 ^h
0.5	0.75	60.3 ± 0.65 ^g	7.63 ± 0.59 ^g
1.0	0.75	82.8 ± 2.13 ^e	10.9 ± 0.82 ^m
1.5	0.75	74.2 ± 1.10 ^l	8.97 ± 0.46 ⁱ
2.0	0.75	69.9 ± 0.49 ^j	8.05 ± 0.50 ^h
2.5	0.75	55.7 ± 1.00 ^e	7.10 ± 0.37 ^f

Note: The values represented in corresponding column followed by same letters are not significantly different by DMRT at $p < 0.05$.

Table 3. Effect of cytokinins (BAP, Kin) and IAA in induction of flowers per inflorescence under 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$ SFPD for 14/10h (light/dark) photoperiod.

Conc. of BAP + Kin (mg L ⁻¹)	Conc. of IAA (mg L ⁻¹)	Number of flower buds (Mean $\pm$ SE)
0.00	0.00	0.0 $\pm$ 0.00 ^a
0.50	0.1	4.1 $\pm$ 0.13 ^b
0.75	0.1	4.8 $\pm$ 0.28 ^c
1.00	0.1	6.3 $\pm$ 0.44 ⁱ
1.50	0.1	6.0 $\pm$ 0.37 ^h
2.00	0.1	5.0 $\pm$ 0.15 ^d
0.50	0.5	6.9 $\pm$ 0.11 ^k
0.75	0.5	7.0 $\pm$ 0.53 ^l
1.00	0.5	12.0 $\pm$ 0.15 ^o
1.50	0.5	9.8 $\pm$ 0.12 ⁿ
2.00	0.5	5.1 $\pm$ 0.71 ^e
0.50	0.75	5.5 $\pm$ 0.26 ^g
0.75	0.75	6.7 $\pm$ 0.59 ^j
1.00	0.75	7.2 $\pm$ 0.35 ^m
1.50	0.75	6.0 $\pm$ 0.72 ^h
2.00	0.75	5.4 $\pm$ 0.16 ^f

Note: The values represented in corresponding column followed by same letters are not significantly different by DMRT at $p < 0.05$.

Table 4. Effect of auxins (IAA, IBA and NAA) on *in vitro* root induction from the shoots on 1/4 strength MS liquid medium.

Auxins (mg L ⁻¹)			Response (%)	Roots number (Mean $\pm$ SE)	Roots length (Mean $\pm$ SE)
IAA	IBA	NAA			
0.0	0.0	0.0	00	0.0 $\pm$ 0.00 ^a	0.0 $\pm$ 0.00 ^a
1.0	-	-	47	6.7 $\pm$ 0.11 ^c	2.6 $\pm$ 0.16 ^b
1.5	-	-	62	7.0 $\pm$ 0.39 ^d	3.0 $\pm$ 0.45 ^c
2.0	-	-	68	8.5 $\pm$ 0.35 ^e	3.8 $\pm$ 0.22 ^c
2.5	-	-	81	8.0 $\pm$ 0.72 ^e	4.1 $\pm$ 0.39 ^d
3.0	-	-	70	7.2 $\pm$ 0.41 ^d	4.3 $\pm$ 0.15 ^d
3.5	-	-	64	6.9 $\pm$ 0.19 ^c	3.0 $\pm$ 0.24 ^c
4.0	-	-	59	6.4 $\pm$ 0.25 ^c	3.4 $\pm$ 0.59 ^c
-	1.0	-	92	18.6 $\pm$ 0.18 ^g	5.7 $\pm$ 0.25 ^e
-	1.5	-	100	24.2 $\pm$ 0.47 ^h	6.1 $\pm$ 0.22 ^f
-	2.0	-	95	19.4 $\pm$ 0.33 ^g	5.5 $\pm$ 0.37 ^e
-	2.5	-	89	10.9 $\pm$ 0.49 ^f	5.0 $\pm$ 0.61 ^e
-	3.0	-	83	8.3 $\pm$ 0.82 ^e	4.6 $\pm$ 0.26 ^d
-	3.5	-	78	8.0 $\pm$ 0.16 ^e	4.1 $\pm$ 0.30 ^d
-	4.0	-	70	7.5 $\pm$ 0.22 ^d	3.5 $\pm$ 0.23 ^c
-	-	1.0	50	7.8 $\pm$ 0.10 ^d	3.2 $\pm$ 0.27 ^c
-	-	1.5	66	8.5 $\pm$ 0.32 ^e	3.7 $\pm$ 0.30 ^c
-	-	2.0	79	9.2 $\pm$ 0.67 ^f	4.9 $\pm$ 0.32 ^d
-	-	2.5	88	10.8 $\pm$ 0.44 ^f	5.2 $\pm$ 0.39 ^e
-	-	3.0	75	8.6 $\pm$ 0.19 ^e	5.0 $\pm$ 0.41 ^e
-	-	3.5	70	6.0 $\pm$ 0.61 ^c	4.5 $\pm$ 0.17 ^d
-	-	4.0	67	5.1 $\pm$ 0.36 ^b	3.9 $\pm$ 0.64 ^c

Note: The values represented in corresponding column followed by same letters are not significantly different by DMRT at $p < 0.05$.

Table 5. Effect of auxins (IAA, IBA and NAA) on *ex vitro* rooting of *in vitro* raised shoots of *Oldenlandia umbellata* in Soilrite®.

Auxins (mg L ⁻¹)			Response (%)	Roots number (Mean ± SE)	Roots length (Mean ± SE)
IAA	IBA	NAA			
0	0	0	00	0.0 ± 0.0 ^a	0.0 ± 0.00 ^a
50	-	-	61	4.6 ± 0.12 ^b	2.3 ± 0.26 ^b
100	-	-	74	5.9 ± 0.26 ^c	2.8 ± 0.39 ^b
150	-	-	79	6.4 ± 0.38 ^d	3.1 ± 0.42 ^c
200	-	-	82	6.9 ± 0.11 ^d	3.6 ± 0.69 ^c
300	-	-	85	8.5 ± 0.35 ^f	3.0 ± 0.23 ^c
400	-	-	71	7.2 ± 0.56 ^e	2.7 ± 0.19 ^b
500	-	-	63	6.7 ± 0.42 ^d	2.0 ± 0.36 ^b
-	50	-	81	7.1 ± 0.39 ^e	2.7 ± 0.27 ^b
-	100	-	87	8.9 ± 0.26 ^f	3.5 ± 0.11 ^c
-	150	-	93	9.4 ± 0.55 ^g	3.8 ± 0.20 ^c
-	200	-	95	12.6 ± 0.60 ^h	4.0 ± 0.39 ^d
-	300	-	100	16.0 ± 0.53 ⁱ	4.2 ± 0.22 ^d
-	400	-	91	14.5 ± 0.29 ⁱ	3.9 ± 0.48 ^c
-	500	-	85	11.3 ± 0.36 ^h	3.3 ± 0.31 ^c
-	-	50	73	5.5 ± 0.72 ^c	2.5 ± 0.20 ^b
-	-	100	79	7.2 ± 0.53 ^e	3.1 ± 0.39 ^c
-	-	150	84	7.7 ± 0.26 ^e	3.3 ± 0.18 ^c
-	-	200	89	8.5 ± 0.45 ^f	3.5 ± 0.10 ^c
-	-	300	92	9.6 ± 0.19 ^g	3.9 ± 0.26 ^d
-	-	400	86	8.0 ± 0.37 ^f	3.4 ± 0.32 ^c
-	-	500	79	7.3 ± 0.22 ^e	2.8 ± 0.35 ^b

Note: Data were recorded after 4 weeks in greenhouse conditions. The values represented in corresponding column followed by same letters are not significantly different by DMRT at $p < 0.05$.

Figure 2. Hardening of plantlets of *O. umbellata*. (a) Hardening of plantlets in paper cups (containing soilrite®) in greenhouse after 5 weeks, (b and c) Hardened plantlets in the nursery polybags (containing organic manure, sand and

red soil (1:1:1; w/w)) and earthen pot (filled with garden soil, red soil and vermi-compost (2:2:1; w/w) after 4-5 weeks, (d) Field transferred plant of *O. umbellata* (6 months old). (Scale bar – 5 cm)

CONCLUSION

The cultures were established from the node explants of *O. umbellata*. The meristematic activity of axillary buds was activated by the liquid MS medium augmented with BAP. The shoots were further multiplied by lowering the concentration of cytokinin and addition of auxin (IAA) to the medium. Well-developed shoots were rooted by *in vitro* and *ex vitro* methods. Comparatively, IBA proved better for induction of roots than other auxins. The present protocol could be implemented for the large-scale production of

plantlets of *O. umbellata* with less input of cost as compared to the semisolid medium.

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