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Rapid and mass multiplication of *Oldenlandia umbellata* L. from the leaf explants through callus culture

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Abstract
Oldenlandia umbellata L. (Rubiaceae), commonly called as 'Indian Madder', is well known for its dyeing property in addition to medicinal values. This plant is used to extract red dye from its matured roots. Due to diversification and exploitation of this plant for medicinal and dyeing purposes, a simple and reproducible protocol has been established through callus culture in the present study. The leaf explants were cultured aseptically on MS medium nourished with 5-25µM IAA, IBA, NAA or 2,4-D for callus induction. The best response was achieved with 10µM NAA. Higher frequency of sprouting (65 shoots, 98% frequency and 9.5cm length) was observed on MS medium with the combination of 6µM BAP and 2µM KIN. Rooting of isolated shoots was best at 6µM IBA (27 roots, 100% frequency, and 6.5cm length). The well rooted plants were transferred to paper cups containing FYM, red soil and sand in the ratio of 1:2:1 for hardening. Then they were transferred to the field for acclimatization.

Keywords: Natural dyes, Rubiaceae, *Oldenlandia umbellata*, MS medium, auxins, cytokinins

1. Introduction
Every day tons of water wastes are released from the textile manufacturing and dyeing industries that cannot be recycled, thus resulting in ocean and waterway contamination. One possible solution to this major issue is the usage of natural dyes. Colourings from natural sources are very interesting and have wide range of colours. Majority of natural dyes are from plant sources like leaves, flowers, fruits and roots.
Oldenlandia umbellata L. (Rubiaceae) is one of the important dyeing plants widely used in ancient times in addition to the medicinal properties. It is commonly called as "Indian Madder" since it is used to impart red colour to the textile materials. The major dyeing property lies in its roots due to its variety of anthroquinone contents [1, 2].
This plant is also used in traditional medicine and Siddha Medicine in particular for its styptic property [3]. Both leaves and roots are considered as good expectorants, and used for treatment of asthma, bronchitis, and bronchial catarrh [4]. The decoction of leaves is used as a rinse to treat poisonous bites [5] and also used as a febrifuge. Siva *et al*. (2009) reported a novel pH indicator dye from this plant [6]. Extract of the whole plant shows significant antitumor activity [7]. Due to its multipurpose usage, members of this plant are over exploited. So there is a need to develop an alternate method of propagation which is successful for large scale production and reproducibility.
Though the counter search on callus induction in *O. umbellata* revealed some limitations in reported methodology [6, 8, 9] such as long callogenic period, low frequency rate etc. So the present study aimed to develop a speedy and reproducible method for mass propagation of this plant through indirect organogenesis.

2. Materials and Methods
2.1 Creation of aseptic environment
The glasswares, forceps and scalpels used in propagation method were sterilized with chromic acid (potassium dichromate and sulphuric acid, 2:1 w/v), followed by rinsing with tap water and distilled water. Heat sterilization was done in an autoclave at 121°C for 20 minutes at 1.06 kg cm^{-2}. In this study MS basal medium [10] was used for the nourishment along with solidifying material and different concentrations of Plant Growth Regulators for callus induction (IAA, IBA, NAA and 2,4-D), shoot multiplication (BAP and KIN) and rooting (IAA, IBA and NAA). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 1 N HCl prior to autoclaving (121°C at 1.06 kg cm^{-2} for 20 min).

The explants were collected from the field grown healthy and matured plants of *O. umbellata*. The leaves were excised and rinsed with running tap water followed by liquid detergent wash. The explants were then washed with distilled water for 4 times and further sterilization was carried out in the laminar air flow chamber under aseptic condition prior to inoculation. In the chamber the explants were sterilized with 70% alcohol and 0.1% (w/v) $HgCl_2$ for 30-45 sec and 3 min respectively. The explants were then washed 4-5 times with sterile distilled water to remove excess mercuric chloride to avoid the cell damage.

2.2 Callus initiation and plant regeneration

For callus induction, the leaves were wounded with forceps and placed on MS medium supplemented with different concentrations of indole-3-acetic acid (IAA), indole butyric acid (IBA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) ranging from 5-25µM. For shoot regeneration, 3-15 µM of benzylaminopurine (BAP) and kinetin (KIN) were supplemented to the basal medium alone or in combination of boththe hormones. *In vitro* raised shoots were excised from the culture tube and sub cultured on MS medium supplemented with various concentrations of IAA, IBA or NAA ranging from 3-15 µM for root development.

2.3 Culture maintenance and conditions

All cultures were maintained at 25±2°C in a culture room under cool white fluorescent lamps (Phillips, India) at intensity of 50 µmol m^{-2} s^{-1} with 16 hrs photoperiod.

2.4 Hardening and Acclimatization

Well rooted plantletswere removed and washed gently withrunning tap water. Plantlets were transferred to paper cups (10 cm diameter) containing autoclaved farmyard manure, garden soil and sand (1:2:1). These plants were irrigated with distilled water every 2 days for 2 weeks followed by tap water for one week. Initially the plants were maintained under culture room conditions for 3 weeks and later transferred to normal laboratory conditions for 2 weeks. The relative humidity was reduced gradually in this period. After 30 days the plantlets were allowed to adopt in the field under shade for 3 weeks and then transplanted to the soil for further growth and development.

2.5 Experimental Design, Data Collection and Statistical Analysis

All the experiments were repeated five times and each experiment consisted of one explant per tubes and ten replicates. The different parameters were recorded such as callus induction frequency, nature of callus, shoot induction frequency, number of shoots, shoot length, number of roots per shoot, root length and survival rate (%).

3. Results and Discussion

The leaf explants were cultured on MS medium with different concentration of IAA, IBA, NAA and 2,4-D (5-25µM) for callus initiation (Table 1). All the four hormones induced callus formation. Among them NAA and IAA have the ability to produce green compact calli at the period of 4 weeks. The highest percentage of response (100%) was observed in 10µM NAA (Fig.1 a,b; Table 1).

Table 1: Effect of different concentrations of auxins on callus induction from the leaf explants of *Oldenlandia umbellata* L.

Hormone	Concentrations (µM)	Percentage of Response	Nature of Callus
IAA	5	66	light green friable calli
	10	78	yellowish green friable calli
	15	82	green compact calli
	20	68	yellowish green friable calli
	25	60	pale green,friablecalli
IBA	5	60	white friable calli
	10	72	whitish green friable calli
	15	80	whitish green compact calli
	20	74	light green compact calli
	25	56	light green friable calli
NAA	5	88	yellowish green friable calli
	10	100	green compact calli
	15	94	green compact calli
	20	80	yellowish green friable calli
	25	76	light green firablecalli
2,4-D	5	94	light green compact calli
	10	82	yellowish green compact calli
	15	70	whitish yellow friable calli
	20	62	white friable calli
	25	60	white friable calli

Values are Mean of 5 replicates recorded after 30 days of culture.

Fig 1: Mass propagation of *Oldenlandia umbellata* L. from the leaf explant.a. initiation of callus from the leaf explant; b.callus growth 30 days after inoculation; c. initiation of shoot regeneration from the callus; d. shoot multiplication and shoot elongation after 30 days; e. rooting and *in vitro* flowering from the isolated shoot; f. hardening in paper cup.

The combined and sole influence of NAA on callus induction was observed in many other studies. Thaniarasu *et al*. (2015) reported the maximum callogenic effect was achieved at 1.0 mg l^{-1} NAA, 0.5 mgl^{-1} BA in *Plectranthus bourneae* [11]. The co-performance of NAA with BAP was reported by Hesami and Daneshvar (2018) [12]. In their studies the maximum callus induction (100%) was achieved in MS medium containing 0.5 mg l^{-1} 2,4-D plus 0.05 mg l^{-1} BAP and MS medium containing 1.5 mg l^{-1} NAA plus 0.15 mg l^{-1} BAP in *Ficus religiosa.*

The well developed, green compact calli were transferred to fresh MS medium containing BAP and KIN at different concentration (3-15µM) for shoot regeneration and proliferation (Table.2). The regeneration of shoots was initiated within a week of subculture. Of the two cytokinins, 6µM BAP produced 90% shoot induction frequency. But synergic effect of 6µM BAP with 2µM KIN induced 98% frequency in shoot formation and 65 shoots.The beneficial (4–5 cmlong) shoot length was observed in all the concentration and combinations ofshoot regeneration medium after 4 weeks of culture (Fig.1c, d; Table.2).

Table 2: Effect of different concentrations of cytokinins on shoot organogenesis from callus of *Oldenlandia umbellata* L.

Concentration of cytokinins (µM)		Shoot Induction Frequency (%)	Number of Shoots	Shoot Length (cm)
BAP	KIN			
3		72	37.5 ± 1.09	7.7 ± 0.74
6		90	44.2 ± 1.16	8.8 ± 1.23
9		82	40.7 ± 1.28	8.3 ± 0.82
12		68	34.7 ± 1.23	7.0 ± 0.67
15		60	32.1 ± 1.75	6.3 ± 1.13
	3	74	27.2 ± 1.60	4.9 ± 1.06
	6	80	33.4 ± 1.98	5.9 ± 1.12
	9	86	37.8 ± 1.61	6.2 ± 0.91
	12	68	29.2 ± 1.89	5.5 ± 1.34
	15	62	23.3 ± 2.77	4.7 ± 0.87
6.0	1	90	60.6 ± 1.22	7.8 ± 1.12
6.0	2	98	65.4 ± 1.62	9.4 ± 1.09
6.0	3	84	58.4 ± 2.32	8.5 ± 0.92
6.0	4	80	52.7 ± 1.44	6.4 ± 0.85
6.0	5	76	43.2 ± 2.09	6.26 ± 0.61

Values are Mean ± standard error of 5 replicates recorded after 30 days of culture.

The similar synergic effect of BAP with other PGR was reported in many studies. Islam and Alam (2018) stated that shoot regeneration from leaf derived callus was obtained on MS medium supplemented with 2.0 mg l^{-1} BAP+0.5ml l^{-1} NAA in *Mentha piperita* [13]. Hesami and Daneshvar (2018) also reported that the shoot regeneration from callus was highest in MS medium supplemented with 1.5 mg l^{-1} BAP plus 0.15 mg l^{-1} IBA in *Ficus religiosa* [12].Several workers showed that the synergistic combination of two cytokinins was more effective for shoot differentiation [14-16].

Excised shoots were transferred to half strength MS medium containing different concentrations of IBA, IAA and NAA ranging from 3-15 µM, for root induction. The order for root induction was IBA> NAA> IAA. The highest response of rooting was observed at 6µM IBA with 100% root induction frequency, 27 roots per shoot and 6.4cm root length (Fig.1e; Table.3).

Table 3: Effect of various auxins on root induction from the isolated shoots of *Oldenlandia umbellata* L.

Auxins	Concentrations (µM)	Root Induction Frequency (%)	Number of Roots	Root Length (cm)
IAA	3	82	13.7 ± 0.58	5.2 ± 0.78
	6	84	15.5 ± 0.92	5.4 ± 0.66
	9	78	10.7 ± 1.13	4.8 ± 0.63
	12	70	7.5 ± 0.79	3.8 ± 0.81
	15	66	6.3 ± 1.35	2.7 ± 0.51
IBA	3	92	21.1 ± 1.30	6.1 ± 0.58
	6	100	27.3 ± 0.91	6.4 ± 0.65
	9	94	18.7 ± 0.98	5.6 ± 0.45
	12	86	15.2 ± 1.04	4.8 ± 0.52
	15	76	12.6 ± 1.37	4.1 ± 0.52
NAA	3	86	9.8 ± 0.60	4.6 ± 0.73
	6	88	14.2 ± 1.30	5.6 ± 1.40
	9	96	17.6 ± 0.61	6.2 ± 0.81
	12	80	12.6 ± 0.70	3.6 ± 0.20
	15	72	7.2 ± 0.95	2.9 ± 0.43

Values are Mean ± standard error of 5 replicates recorded after 30 days of culture.

The present study revealed that of three auxins tested, IBA was found to be better for root induction. IBA was found better in root induction by many other researchers. Previous studies showed that half strength medium with IBA promotes faster root development [17] than full strength MS medium. These results showed that among all auxins, IBA was better in root induction and growth when compared to IAA and NAA particularly for this plant [18, 19].

In vitro flowering process offers a unique system to understand the molecular basis and hormonal regulation involved in flowering. In this present study the *in vitro* flowering was achieved with longer photoperiod. The 16 hours photoperiod facilitates the flower formation without adding any flowering hormones (Fig. 1e). Similar results were observed in other plants [20-22]. Recently *in vitro* flowering in *O. umbellata* was achieved by supplementing MS medium with 0.7 mg l^{-1} NAA + 1.5 mg l^{-1} BA (Benzyladenine) and 0.4% CM (Coconut milk) [23].

The plantlets with well-developed roots were transferred to paper cups containing FYM, red soil and sand in the ratio of 1:2:1 and maintained inside the culture room at 25±2°C under 16 hr photoperiod and 75-80% relative humidity. Subsequently these plantlets were established in the field through acclimatization (Fig. 1f). Acclimatization is a crucial step to determine the success rate of *in vitro* propagation method. In this study 90% of survival rate was achieved in the field. This indicates the effectiveness of the developed propagation method.

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