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In vitro flowering in *Oldenlandia umbellata* L.Shuvra Kanta Behera^a, Chandrasekaran Rajasekaran^a, S. Payas^a, Devanand P. Fulzele^b,
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ABSTRACT

Background: *Oldenlandia umbellata* L. (Indian madder) is an antique Ayurvedic Indian herb and a source of various anthraquinone derivatives. The red dye from its roots has been used in diverse applications since ancient times.

Objectives: To establish reliable and effective protocols for *in vitro* flowering of *O. umbellata*.

Materials and methods: For *in vitro* flowering, organogenic calli were subcultured onto Murashige and Skoog (MS) medium supplemented with various concentrations of Naphthalene acetic acid (NAA) (0.15–1.0 mg/l) and Benzyladenine (BA) (0.5–1.5 mg/l) with and without 0.4% of coconut milk (CM).

Results: The highest number of *in vitro* flowers (22.8%) and best response (92.73%) was achieved on MS medium supplemented with 0.7 mg/l NAA + 1.5 mg/l BA with 0.4% CM. It was found that MS medium devoid of BA promoted best root development (47.3 per calli) as well as response (100%). It was also observed that when embryogenic calli grown in depletion of required nutrition transferred to fresh media induced more flowering. *In vivo* and *in vitro* floral comparative analysis revealed that *in vitro* flower induction was required for short time duration (20.67 days) than *in vivo* flower.

Conclusion: To the best of our knowledge, this is the first report on *in vitro* flowering and this study will help to overcome problems associated with flower development and seed production. As a result, this study may be a potent conservation tool to restore innate population size in its natural habitat.

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1. Introduction

Oldenlandia umbellata L., belonging to Rubiaceae is an antique Ayurvedic Indian herb known to yield red dye from its roots [1]. *O. umbellata* flowers are white to lilac in axillary or terminal pedunculate umbels, fruits are loculicidal capsule with reticulate seeds obscurely angled [2]. Heterostyly with distylous flower are seen in *O. umbellata* with short styled and long anthers named “Thrum eyed” and short anthers and long styled “Pin eyed” respectively. Self incompatibility is observed in *O. umbellata* flowers which fail to produce seeds [3,4]. Due to increased utilization by different industries for medicinal and dye extraction purposes, there is an increased exploitation of *O. umbellata* [5–10]. The plant has been used in diverse applications, for instance, decoction of leaves is used to treat poisonous bites, roots are used in asthma,

bronchitis, and bronchial catarrh treatment [5,6] and also considered as good expectorant. In addition, the dye obtained from its roots is reported as a novel pH indicator [7]. These multiple uses have increased utilization and exploitation of *O. umbellata* by different industries for medicinal and dye extraction purposes [8]. Rapid industrialization, urbanization and global warming are the major causes in alteration of phenology, which laid tremendous pressure on natural habitat of *O. umbellata*. As a result, the natural stands of *O. umbellata* (Fig. 1a) are fast disappearing and listed under threatened category [11]. In addition, the above said causes also influencing the poor or non-flowering and seed formation pattern. *O. umbellata* grows wild in forests, barren rocky areas, and there is no propagation system available to replenish these stands [1]. We have reported a reliable protocol for induction of somatic embryogenesis and organogenesis for *O. umbellata* [8]. We have also made successful anthraquinones dye production using the root culture of this plant [11]. But there is no report about the *in vitro* flowering studies on any of the species of *Oldenlandia* till date. Thus, here, an effort has been made to develop an efficient protocol for *in vitro* flowering in *O. umbellata*.

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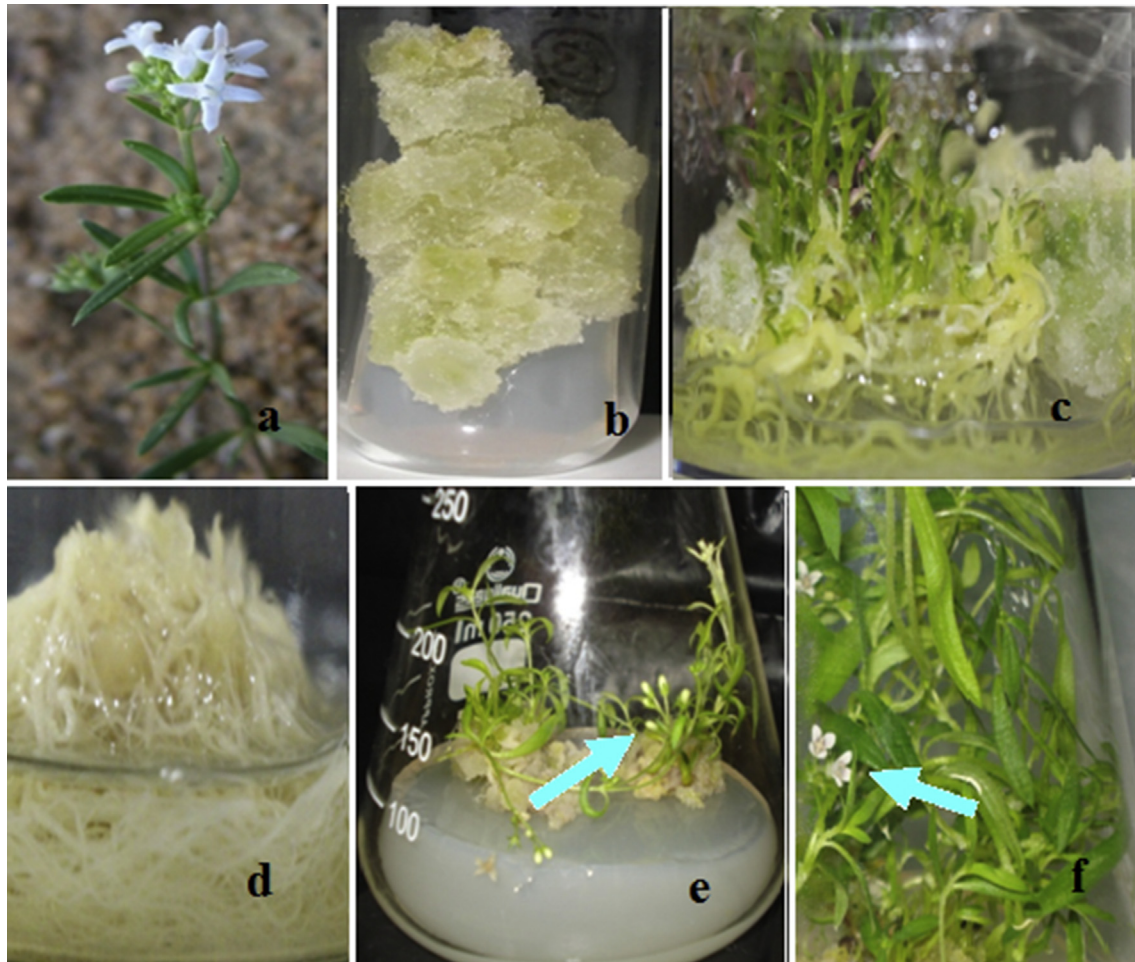


Fig. 1. a – *Oldenlandia umbellata* in natural habitat; b – organogenic calli; c – rapid root induction; d – close –up of roots; e – development of bud formation; f – full-fledged flower.

2. Materials and methods

2.1. Plant material

Plants of *O. umbellata*, located in the vicinity of the VIT University campus were used in this study. The taxonomic identity of the species was confirmed at the Rapinet Herbarium (Tiruchirapalli, India). Young emerging leaves, shoot apices, slender stems, and roots were collected from these plants, and used as source of explants.

2.2. Initiation of cultures

The calli were established as per our previous report [8]. For embryogenic callus induction, MS medium [12] supplemented with NAA (0.3 mg/l), BA (0.5 mg/l), and CM (0.1%) was used.

Embryogenic calli were further subcultured on MS medium fortified with different combination and composition of NAA (0.15–3 mg/l), BA (1.5–5 mg/l) and CM (1%) was used respectively for root and shoot development.

For *in vitro* flowering, organogenic calli were subcultured onto MS medium supplemented with various concentrations of NAA (0.15–1.0 mg/l), BA (0.5–1.5 mg/l) with and without CM (0.4%).

All cultures were incubated under 16 h photoperiod provided by 40 W fluorescent tubes (Philips India Ltd, Mumbai), $45 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$, at $22 \pm 2^\circ \text{C}$ and 65% relative humidity. The cultures were maintained on similar media compositions and subcultured after every six weeks.

Each treatment consisted of five replications, and the whole experiment was repeated thrice. All data were subjected to one-way ANOVA using SIGMA-STAT 3.0 Version. Mean separation comparisons were made using Holm–Sidak test at P value of >0.05 .

3. Results

All the experiments were conducted using slender stem explants. The callus induction and organogenesis was established from our previous report as mentioned in Table 1 [8].

Somatic embryogenic calli were subcultured onto a freshly prepared MS media supplemented with different concentrations of NAA and CM to induce root development. It was observed that within 6 weeks, roots were developed. In our previous report, we have used NAA (0.05–0.3 mg/l) with CM (0.5–1%) for root development and it was observed that 0.3 mg/l NAA with 1.0% CM resulted in maximum conversion of embryogenic calli to produce 18.8 roots per calli with 93% of response. In the present study, treatment with higher concentration of NAA ranging from 0.15 mg to 1.0 mg/l was carried out. Among the various concentrations used, NAA (0.7 mg/l), CM (0.4%) alone and with combination resulted in the highest number of roots (47.2) per embryogenic calli with 100% of response (Fig. 1b–d; Table 2).

For *in vitro* flowering, embryogenic calli were transferred to fresh media containing varying concentrations of BA, NAA and CM alone as well as with the combination. It was found that *in vitro* flowers were developed within three weeks, when MS

Table 1Influence of various growth regulators towards callus induction and organogenesis from slender stem explants of *Oldenlandia umbellata* L. (Siva et al., 2009).

Growth regulator	Concentration (mg/l)	Duration required for calli induction	% response	Duration required for organogenesis		% of Organogenesis induction	
				Stem	Root	Stem	Root
Control	—	10 weeks	21.34 ± 1.3	—	—	—	—
NAA	3.0	8 weeks	90.45 ± 1.3	—	—	—	—
BA	5.0	6 weeks	89.76 ± 0.7	—	—	—	—
NAA	0.15	—	—	—	6 weeks	—	93.42 ± 0.3
CM	1.0%	—	—	—	6 weeks	—	82.47 ± 0.6
NAA + CM	0.05 + 1.0%	—	—	—	6 weeks#	—	86.87 ± 0.6
BA	1.5	—	—	6 weeks	—	74.92 ± 0.6	—
NAA + BA	0.3 + 1.5	—	—	6 weeks	—	67.32 ± 0.5	—
NAA + BA + CM	0.3 + 1.5 + 1.0%	—	—	4 weeks	—	97.22 ± 0.4	—

— No response; # Root initiation was observed at the end of 4th week.

Table 2Effect of NAA and Coconut milk on organogenesis of roots in *O. umbellata* L.

Growth regulator	Concentration (mg/l)	Duration required	% of Organogenesis induction	No. Root formed
Control	—	—	—	—
NAA	0.15	6 weeks	93.5 ± 0.5	12.5 ± 0.7 ^c
	0.30		96.7 ± 0.7 ^a	6.4 ± 0.3
	0.50		97.2 ± 0.9 ^a	10.2 ± 0.2 ^d
	0.70		97.9 ± 1.6 ^a	16.7 ± 0.8 ^b
	1.0		96.2 ± 1.8 ^a	17.3 ± 0.4 ^a
CM	0.2%	6 weeks	92.1 ± 1.9	5.4 ± 0.6
	0.4%		98.2 ± 1.8 ^a	9.1 ± 0.8 ^a
	0.6%		97.1 ± 1.2 ^b	8.8 ± 0.3 ^b
NAA + CM (%)	0.7 + 0.2%	6 weeks	85.6 ± 1.5	33.4 ± 0.6
	0.7 + 0.4%		100.0 ± 0.3 ^a	47.2 ± 0.2 ^a
	0.7 + 0.6%		94.3 ± 1.1 ^c	32.6 ± 0.6
	1.0 + 0.2%		90.7 ± 1.1	38.4 ± 0.4 ^b
	1.0 + 0.4%		95.5 ± 1.2 ^c	37.5 ± 0.5 ^c
	1.0 + 0.6%		97.6 ± 1.6 ^b	36.2 ± 0.8 ^c

Values are mean ± SE, five cultures per treatment, repeated thrice. Within a column, means having the same letters are not significantly different at the 5% level according to Holm–Sidak test.

media supplemented with NAA (0.7 mg/l), BA (1.5 mg/l) and 0.4% CM (Table 3). This combination yielded the highest number of flowers (22.8) per calli and best response (92.73%) (Fig. 1e–f). The *in vitro* regenerated plants were subjected to hardening (Fig. 1g).

Table 3Effect of growth regulators for *in vitro* flowering in *O. umbellata* L.

Growth regulator	Concentration (mg/l)	Duration required	No. of flowers	% flower formed
Control	—	—	—	—
NAA	0.15	5 weeks	—	—
	0.30		2.2 ± 0.4 ^c	62.34 ± 0.8 ^c
	0.50		2.1 ± 0.2 ^c	63.89 ± 1.6 ^c
	0.70		4.5 ± 0.5 ^a	67.43 ± 1.3 ^b
	1.0		4.2 ± 0.2 ^b	68.91 ± 0.7 ^a
BA	0.5	—	—	—
	1.0		3.7 ± 0.3	70.12 ± 1.7
	1.5		5.5 ± 0.7 ^a	74.33 ± 1.2 ^a
NAA + CM	0.7 + 0.4%	—	2.3 ± 0.3	71.79 ± 1.1 ^a
	1.0 + 0.4%		3.4 ± 0.2 ^a	69.22 ± 1.4
BA + CM	0.5 + 0.4%	4 weeks	2.2 ± 0.4	63.24 ± 1.8 ^c
	1.0 + 0.4%		4.6 ± 0.3	69.87 ± 1.3 ^b
	1.5 + 0.4%		12.3 ± 0.8 ^b	78.65 ± 0.6 ^a
NAA + BA + CM	0.7 + 1.0 + 0.4%	3 weeks	13.4 ± 0.6 ^a	80.87 ± 0.9 ^a
	0.7 + 1.5 + 0.4%		22.8 ± 0.7 ^a	92.73 ± 1.4 ^a
	1.0 + 1.0 + 0.4%		15.8 ± 0.4	85.14 ± 0.8
	1.0 + 1.5 + 0.4%		16.7 ± 0.3	86.67 ± 1.1

Values are mean ± SE, five cultures per treatment, repeated thrice. Within a column, means having the same letters are not significantly different at the 5% level according to Holm–Sidak test.

4. Discussion

In vitro flowering facilitates understanding the physiology of flower and there are many physio-chemical factors influencing the *in vitro* flowering. According to Heylen and Vendrig [13], various important factors such as carbohydrates, growth regulators, light and pH of the culture medium responsible for *in vitro* flowering. In accordance, Kolar and Senkova [14] noted that *in vitro* flowering in *Arabidopsis thaliana* was accelerated by reduced mineral nutrient availability in medium. Similarly, our studies demonstrated that depletion of essential nutrients in culture medium boosted more flowering in *O. umbellata*.

In the present study, when embryogenic calli grown in depletion of required nutrition transferred onto a fresh media induce more flowering. This finding is in accordance with other studies [15,16].

The nature of carbon source is an important factor for *in vitro* flowering. It has been reported by many researchers that sucrose is the best carbon source for formation of flowering [17,18]. Sucrose is not only a source of carbon and energy for plant growth and development, but also has a signaling function and modulates expression of genes that encodes enzymes, transporter and storage proteins [19]. In addition, sucrose controls growth [20] and flowering [21]. For analyzing the effect of sucrose on *in vitro* flowering, 2% to 5% of sucrose concentrations were used (Table 4). Among that, 2% showed maximum flower production (22.8) and good response (92.73%). The results of this study are in agreement with earlier reports [22,23].

The *in vitro* flowers were compared with *in vivo* flowers based on various parameters viz., days required for bud generation, length, breadth, bud color, flower color, calyx and corolla. As mentioned in Table 5, the *in vitro* flowers are like those *in vivo* except the number of days required for bud generation, which is shorter (20.67 days) under *in vitro* condition. This too was observed before in species as wide apart as pea [19] and *Arabidopsis* [24] and was used to accelerate generation cycles for a faster breeding.

Flowering is a major event in the plant life cycle that has to be precisely timed for reproductive success. Both physiological and genetical studies have revealed the complexity of the mechanisms that tightly control the apical meristem switch from vegetative to reproductive growth [25]. This distinctive phase in plant developmental stage is controlled by various abiotic factors [26,27]. The

Table 4Effect of sucrose on *in vitro* flowering in *O. umbellata* L.

Sucrose (%)	<i>In vitro</i>	
	% of cultures producing flower buds	Number of flowers per culture
Control (2%)	92.73	22.8 ± 0.7
3	90.73	13.2 ± 0.3
4	85.73	13.5 ± 0.2
5	72.73	10.6 ± 0.5

Table 5
Comparison of *in-vivo* and *in-vitro* flowers in *O. umbellata* L.

Characters	<i>In-vivo</i>	<i>In-vitro</i>
Days required to for bud generation	34.18 ± 0.4	20.67 ± 0.3
Length	3.5 ± 0.3	3.7 ± 0.1
Breath	1.8 ± 0.2	1.9 ± 0.3
Bud color	Pinkish white	Pinkish white
Flower color	White	White
Calyx	1.2 ± 0.5	1.3 ± 0.3
Corolla	2.3 ± 0.3	2.4 ± 0.2

transformation of a vegetative meristem into a reproductive/flowering meristem involves various molecular and hormonal changes; this can be studied under an established *in vitro* flowering system [28]. Flower induction and regulation of flowering in *in vitro* condition helps us to study the flowering mechanism [29]. *In vitro* flowering depends on various abiotic and genetic factors [27]. Insights on flower physiology and development can be easily accessed by *in vitro* flowering study [30,31] and premature seed setting and self incompatibility can also be studied. An ideal system to develop *in vitro* flowering if established can lead to rapid breeding of new varieties [32,33] and it has been reported in a number of plant species, for example in *Withania somnifera* [32], Kinnow mandarin [33], *Streptocarpus nobilis* [34], *Pharbitis nil* [35], *Ammi majus* [36], *Hypericum brasiliense* [37], *Bambusa edulis* [38,39] and *Psycmorchis pusilla* [40]; however, there is no report to date on any species of *Oldenlandia*. A flowering system *in vitro* is considered to be a suitable tool to understand the detailed aspects of flowering, floral initiation, floral organ development, and floral senescence [17,41].

5. Conclusion

Flowering is an important stage of plant life and complicated to understand. Though this plant has been used since ancient time in Ayurvedic medicine, no attempt has been made on *in vitro* flowering and this is the first report on *in vitro* flowering of this precious medicinal cum dye yielding plant. This study will help to conserve and incessant delivery of plant material throughout the year by knowing its *in vitro* flowering nature. This study will also helpful to understand fruit formation and seed production. This protocol also can be extended to plant breeding studies for the purpose of quick flowering and fruit formation under *in vitro* conditions.

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Conflict of interest

None.

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