Original Research Article (Experimental)

In vitro flowering in Oldenlandia umbellata L.

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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]. Heterostylism with distylous pedunculate umbels, fruits are loculicidal capsule with reticulate fl

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 has been used in diverse applications since ancient times. The red dye from its roots has been used in diverse applications 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 antheraquinones 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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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 lmol m⁻² s⁻¹, at 22 ± 2 °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/l 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 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).

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
Table 1
Influence 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 callus induction | % response | Duration required for organogenesis | % of Organogenesis induction |
|------------------|----------------------|----------------------------------------|------------|-----------------------------------|-----------------------------|
|                  |                      |                                        |            | Stem                              | Root                        |
| Control          | –                    |                                        | –          | –                                 | –                           |
| NAA              | 3.0                  | 8 weeks                                | 25.3 ± 1.3 | –                                 | –                           |
| BA               | 5.0                  | 6 weeks                                | 89.76 ± 0.7| –                                 | –                           |
| NAA              | 0.15                 | –                                      | –          | –                                 | –                           |
| CM               | 1.0%                 | –                                      | –          | –                                 | –                           |
| NAA + CM         | 0.05 + 1.0%          | –                                      | –          | –                                 | –                           |
| BA               | 1.5                  | –                                      | –          | 6 weeks                           | 93.42 ± 0.3                |
| NAA + BA         | 0.3 ± 1.5            | –                                      | –          | 6 weeks                           | 82.47 ± 0.6                |
| NAA + BA + CM    | 0.3 + 1.5 + 1.0%     | –                                      | –          | 6 weeks                           | 86.87 ± 0.6                |

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

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 *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.

### Table 2
Effect 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                   | 15 ± 0.7        |
| CM               | 4.0                  | 6 weeks           | 96.7 ± 0.7                   | 4.0             |
| NAA + CM         | 0.05 + 0.2%          | 6 weeks           | 92.1 ± 1.9                   | 5.4 ± 0.6       |
| BA               | 1.0                  | 6 weeks           | 98.2 ± 1.8                   | 9.1 ± 0.8       |
| NAA + BA         | 0.3 ± 1.5            | 6 weeks           | 97.1 ± 1.2                   | 8.8 ± 0.3       |
| NAA + BA + CM    | 0.3 + 1.5 + 1.0%     | 6 weeks           | 100.0 ± 0.3                  | 47.2 ± 0.2      |
|                  |                      |                   |                              |                 |

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.

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*.

### Table 3
Effect 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           | –              | –              |
| 0.50             | 2.2 ± 0.4            | 62.34 ± 0.8       |                |                |
| 0.70             | 2.1 ± 0.2            | 63.89 ± 1.6       |                |                |
| 1.0              | 4.5 ± 0.5            | 67.43 ± 1.3       |                |                |
| 0.4%             | 4.2 ± 0.2            | 68.91 ± 0.7       |                |                |
| BA               | 0.5                  | –                 | –              | –              |
| 1.0              | 3.7 ± 0.3            | 70.12 ± 1.7       |                |                |
| 1.5              | 5.5 ± 0.7            | 74.33 ± 1.2       |                |                |
| NAA + CM         | 0.7 + 0.4%           | 6 weeks           | –              | –              |
| 1.0              | 3.4 ± 0.2            | 69.22 ± 1.4       |                |                |
| BA + CM          | 0.5 + 0.4%           | 4 weeks           | –              | –              |
| 1.0              | 4.6 ± 0.3            | 69.87 ± 1.3       |                |                |
| 1.5 + 0.4%       | 12.3 ± 0.8           | 78.65 ± 0.6       |                |                |
| NAA + BA + CM    | 0.7 + 1.0 + 0.4%     | 3 weeks           | –              | –              |
| 0.7 + 1.5 + 0.4% | 22.8 ± 0.7           | 92.73 ± 1.4       |                |                |
| 1.0 + 1.0 + 0.4% | 15.8 ± 0.4           | 85.14 ± 0.8       |                |                |
| 1.5 + 0.4%       | 18.7 ± 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.

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

| Sugrose (%) | In vitro |
|------------|----------|
|            | % 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 | In-vivo | In-vitro |
|------------|---------|---------|
|            | Days required for bud generation |            |
| 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 |
Flowering is a major event in the plant life cycle that has to be precisely timed for reproductive success. Both physiological and genetic 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 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 nobiles [34], Parabitis nil [35], Ammi majus [36], Hypericum brasiliense [37], Bambusa edulis [38,39] and Psymorchis 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 deliver 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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