

Rapid Clonal Multiplication Through *In Vitro* Axillary Shoot Proliferation of *Lobelia cardinalis* L., an Ornamental Aquatic Plant

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Abstract

Aquatic plants are grown in aquaria for their beauty and to maintain the quality of water. The explants of *Lobelia cardinalis* L. were obtained from the Aquatic Plant Center Co., Ltd., Thailand. *In vitro* clonal propagation offers alternative strategy for its rapid clonal multiplication. In this study, multiple and rapid propagations of *Lobelia cardinalis* L. with tissue culture techniques were aimed. Highly efficient and repeatable *in vitro* regeneration protocol was established from nodal segments of *Lobelia cardinalis* L. Various concentrations and combinations of various plant growth regulators (PGRs) were employed to induce multiple shoots, shoot elongation, number of leaves and rooting of shoots to obtain complete plantlets of *Lobelia cardinalis* L. Among 2 PGRs such as 6-benzylaminopurine (BAP) and α -Naphthalene acetic acid (NAA) tested for multiple shoot induction. Regenerated shoots had a higher number of shoots on MS medium contained cytokinins with auxins. The maximum shoot proliferation (100 %), number of shoots per explant (6.20 ± 0.51 shoots), shoot length (11.67 ± 0.69 mm) and number of leaves per explant (58.10 ± 0.38 leaves per explant) were observed in MS medium supplemented with 1.0 mg/L BAP and 2.0 mg/L NAA. Rooting was achieved within 3 to 4 weeks on all the rooting media, but MS medium augmented with 1.0 mg/L indole-3-butyric acid (IBA) produced the maximum number of strong and healthy roots (11.70 ± 0.27 roots). The regenerated complete plantlets with healthy roots and shoot system were transferred to 3.5 cm diameter black net plastic pots containing rockwool medium and successfully acclimatized with a survival rate of 100 % in the greenhouse and no phenotypic variations were observed among regenerated plants. The success of this protocol offers highly efficient method of the plant multiplication, which would be beneficial for plant tissue culturist, and nursery people where regular supply of plants is a large number required. This protocol can be utilized for commercial scale propagation.

Keywords: Aquatic plant, Micropropagation, Nodal explant, Regeneration

Introduction

Plant tissue culture is defined as the growth of plant parts isolated from the mother plant in an artificial nutrient environment under aseptic circumstances. The tissue culture medium should contain all the nutrients needed to grow a normal plant. The growth culture contains macro-nutritions, micro-nutritions, vitamins, different organic ingredients, plant growth regulators, carbon source and certain solidification agents. Murashige and Skoog (MS) nutrients [1] are the most used nutrient medium for *in vitro* production of many plant species [2]. In addition, plant growth regulators and nitrogen sources in the nutrient medium are crucial for shoot regeneration [3-5]. The main advantage of tissue culture technology is that high quality plants or herbal products can be obtained, which can be reproduced year-round under sterile conditions, regardless of season and external conditions [6]. Recently, many plant species such as *Allium sativum* L. [7], *Bacopa monnieri* (L.) Wettst [8], *Hemerocallis* sp. L. [9], *Nyctanthes arbor-tristis* L. [10], *Rubus idaeus* [11], *Scoparia dulcis* L. [12], *Vaccinium vitis-idaea* ssp. minus [13], *Cryptocoryne wendtii* [14], *Dendrobium crumenatum* Sw. [15], *Anubias Heterophylla* [16], *Staurogyne repen* [17], *Oxalis triangularis*

A.st.-Hil [18], *Anubias barteri* var. *nana*. [19], *Myriophyllum tuberosum* Jack. [20] and *Anthurium andraeanum* 'HC 028' [21] were propagated quickly and multiple using tissue culture.

Lobelia cardinalis L. grows wild in Northern America. In the nursery this plant is cultivated in marshy conditions, forming dark-green leaves which are purple underneath. In aquariums the leaves turn a beautiful shade of light-green, with stems 10 - 30 cm tall and 5 - 10 cm wide. Widely used in Dutch aquariums in so-called "plant streets". In open aquariums it grows above the water surface, where it forms very beautiful scarlet flowers and the leaves regain their colour. Can be used in garden ponds [22]. *Lobelia cardinalis* L. is a very versatile North American plant that can be grown submerged or emersed in aquariums, palludariums, and terrariums. *Lobelia cardinalis* L. is a very unusual foreground and middle ground plant for the planted freshwater aquarium. It can also be excellent in the background if grown tall enough. Its submerged form is bright green, sometimes with red-purple on the undersides of the leaves, although this red-purple coloration is more typical in its emersed form. This plant is perhaps best known for the bright red flower that grows from its emersed form (**Figure 1**). *Lobelia cardinalis* L. requires moderate to high lighting. Its tops can be trimmed and replanted to increase the size of the mat. It produces side shoot leaves that will be lush with optimal care. It will grow much more quickly in nutrient-rich substrate and will also benefit from CO₂ supplementation, but this is not absolutely required. Fertilizers will often be helpful in assuring that this plant maintains its best coloration and form. This listing is for a single rhizome with multiple stems or a small bunch of 2 - 3 rhizomes with a few less stems of this lovely plant. It is sure to be a standout species in any planted aquarium. It is not too demanding in care, so it is a great option for all aquarists [23].

In vitro propagation is the best technology for mass production to make the industry sustainable. The present study was aimed to develop shoot organogenesis system and *in vitro* plantlet regeneration in *Lobelia cardinalis* L.

Materials and methods

Plant materials, explant preparation and sterilization

Young plantlets of *Lobelia cardinalis* L. were obtained from the Aquatic Plant Center Co., Ltd., Thailand. Shoots were cut into pieces of 4 - 5 cm long and washed in running tap water to remove impurities. The shoots were surface sterilized using 4 % Clorox® (5.25 % sodium hypochlorite, NaOCl) containing 2 drops of Tween-20 emulsifier per 100 mL solution for 10 min followed by rinsing 3 times with sterile distilled water. They were again surface sterilized for another 2 % Clorox® and 2 - 3 drops of Tween-20 per 100 mL solution for 5 min. The treated plantlets were washed 3 times with sterile distilled water to remove traces of disinfectant. The surface sterilized explants of 1.0 cm in length were excised aseptically and inoculated into bottles containing Murashige and Skoog (MS) medium [1] without plant growth regulators for 7 days. The culture bottles were sealed with Parafilm and incubated under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD).

Culture media and conditions

Murashige and Skoog (MS) medium [1] supplemented with 3.0 % (w/v) sucrose as a carbon source and gelled with 0.76 % (w/v) agar (commercial grade) were used as basal media throughout the experiment. Various concentrations and combinations of plant growth regulators (PGRs) (BAP; 0.0, 1.0 and 2.0 mg/L, NAA; 0.0, 1.0, 2.0 and 3.0 mg/L) were added to different cultures. The pH of the media was adjusted to 5.7 with 1 N KOH or 1 N HCl prior to autoclaving for 15 min at 121 °C. All cultures were aseptically maintained at 25 \pm 2 °C air temperatures under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD). Plant materials were stored in culture bottles each containing 25 mL of medium. Cultures on MS medium devoid of growth regulators were treated as control.

Effects of 6-benzylaminopurine (BAP) and α -Naphthalene acetic acid (NAA) on shoots multiplication of *Lobelia cardinalis* L. after 45 days of culture

The aseptic nodal segments (1.0 cm long) of 7 days old *in vitro* were placed on MS medium supplemented with 6-benzylaminopurine (BAP) at different concentrations (0.0, 1.0 and 2.0 mg/L) either singly or in combination with α -Naphthalene acetic acid (NAA; 0.0, 1.0, 2.0 and 3.0 mg/L). The maximum percentage of shoot proliferation, number of shoots per explant, shoot length (mm), and number of leaves per explant were recorded and compared statistically after 45 days of culture (starting from the initial day of inoculation). A set of cultures without plant growth regulators (PGRs) served as the control.

Effects of α -Naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) on root induction of *Lobelia cardinalis* L. after 45 days of culture

The shoots produced *in vitro* were excised and subcultured as separate shoots on MS medium containing various concentrations of α -Naphthalene acetic acid (NAA; 1.0, 2.0 and 3.0 mg/L) or indole-3-butyric acid (IBA; 1.0, 2.0 and 3.0 mg/L) to induce *in vitro* rooting. The medium without any plant growth regulators (PGRs) was used as a control. After 45 days of culture, the maximum percentage of root formation, number of roots per shoot, and root length (mm) were recorded and compared statistically after 45 days of culture (starting from the initial day of inoculation).

Hardening and establishment of tissue culture plantlets

Plantlets of *Lobelia cardinalis* L. with well-developed shoots and roots (3 - 5 cm in height) were removed from the culture medium, washed gently with a soft brush in tap water to remove the adhering agar-agar with plant tissue. The plantlets were then transplanted into plastic pots containing tap water under greenhouse conditions. The plantlets were grown in the greenhouse with 80 - 90 % relative humidity and about 12 h photoperiod, 300 - 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) (shaded sunlight) and 28 ± 2 to 24 ± 2 °C day/night temperature. After 30 days in the greenhouse, the survival rate of acclimatized plantlets was recorded.

Experimental design and statistical analysis

The data were collected after 45 days from a shoot and root development. All the experiments were conducted in a completely randomized design (CRD) with 5 replicates per treatment and the experiments were repeated 3 times. The results are expressed as mean \pm SE of 1 experiment. The data were analyzed by ANOVA using SPSS version 24 and the mean values were separated using Duncan's multiple range test (DMRT) at a 5 % probability level.

Results and discussion

Effects of 6-benzylaminopurine (BAP) and α -Naphthalene acetic acid (NAA) on shoots multiplication of *Lobelia cardinalis* L. after 45 days of culture

The aseptic nodal segments (1.0 cm long) of 7 days old *in vitro* were transferred to MS medium to assess the effect of 6-benzylaminopurine (BAP; 0.0, 1.0 and 2.0 mg/L) and α -Naphthalene acetic acid (NAA; 0.0, 1.0, 2.0 and 3.0 mg/L). The results were summarized in **Table 1**. Various combinations and concentrations of BAP and NAA were tried for the induction of shoots from single nodal segments of *Lobelia cardinalis* L. The results showed that there was a significant difference in the effect of type and concentration of PGRs on vegetative traits of *Lobelia cardinalis* L. in the proliferation stage ($p \leq 0.05$). When single nodal segments were cultured on MS medium supplemented with different concentrations of NAA, they induced adventitious roots from the cut ends of explants after 4 weeks of culture (data not shown).

Addition of BAP with various concentrations of NAA induced adventitious shoots at the cut ends of explants. A significant difference in the number of shoots, shoot length, and number of leaves was detected among the treatments containing BAP and NAA. The lowest shoot proliferation percentage (50.00 ± 0.16) and the average number of shoots multiplied per single nodal segments (2.50 ± 0.85 shoots) developed in the absence of plant growth regulators (**Table 1** and **Figures 2(A)** and **2(B)**). In the case of BAP alone, the highest number of shoots per explant (5.10 ± 0.83 shoots), shoot length (9.40 ± 0.46 mm), and number of leaves (65.80 ± 0.87 leaves) was achieved from aseptic nodal segments on MS medium supplemented with 2.0 mg/L BAP (**Table 1**). The number of shoots was significantly better on medium containing 1.0 mg/L BAP and 2.0 mg/L NAA (6.20 ± 0.51) (**Table 1** and **Figures 2(C)** and **2(D)**) than in the presence of BAP or NAA alone (**Table 1**). Regenerated shoots had a higher number of shoots on MS medium contained cytokinins with auxins. The maximum shoot proliferation (100 %), number of shoots per explant (6.20 ± 0.51 shoots), shoot length (11.67 ± 0.69 mm) and number of leaves per explant (58.10 ± 0.38 leaves per explant) were observed in MS medium supplemented with 1.0 mg/L BAP and 2.0 mg/L NAA (**Table 1** and **Figures 2(C)** and **2(D)**). The leaves of the regenerated shoots were healthy with dark green color and did not show any sign of vitrification.



Figure 1 *Lobelia cardinalis* L. is a very foreground and middle ground plant for the planted freshwater aquarium [24].

Table 1 Effects of BAP and NAA on shoots multiplication of *Lobelia cardinalis* L. after 45 days of culture.

Growth regulators (mg/L)		Shoot proliferation (%)	Number of shoots per explant	Shoot length (mm)	Number of leaves per explant
BAP	NAA	(mean ± SE) ^a	(mean ± SE) ^a	(mean ± SE) ^a	(mean ± SE) ^a
0.0	0.0	50 ± 0.16 ^b	2.50 ± 0.85 ^f	10.36 ± 0.97 ^{bcd}	22.90 ± 0.52 ^d
0.0	1.0	100 ± 0.00 ^a	3.90 ± 0.48 ^{cdef}	13.92 ± 0.13 ^a	30.80 ± 0.39 ^{cd}
0.0	2.0	100 ± 0.00 ^a	3.60 ± 0.49 ^{def}	7.41 ± 0.62 ^{fg}	24.80 ± 0.38 ^d
0.0	3.0	90 ± 0.10 ^a	2.90 ± 0.72 ^{ef}	5.96 ± 0.74 ^g	17.40 ± 0.29 ^d
1.0	0.0	100 ± 0.00 ^a	4.00 ± 0.25 ^{cdef}	9.22 ± 0.67 ^{def}	44.10 ± 0.45 ^{bc}
1.0	1.0	100 ± 0.00 ^a	5.80 ± 0.74 ^{ab}	10.21 ± 0.62 ^{bcd}	54.10 ± 0.55 ^{ab}
1.0	2.0	100 ± 0.00 ^a	6.20 ± 0.51 ^a	11.67 ± 0.69 ^{bc}	58.10 ± 0.38 ^{ab}
1.0	3.0	100 ± 0.00 ^a	5.50 ± 0.60 ^{abc}	9.69 ± 0.52 ^{cde}	53.50 ± 0.56 ^{ab}
2.0	0.0	90 ± 0.10 ^a	5.10 ± 0.83 ^{abcd}	9.40 ± 0.46 ^{cdef}	65.80 ± 0.87 ^a
2.0	1.0	100 ± 0.00 ^a	4.90 ± 0.50 ^{abcd}	9.86 ± 0.65 ^{cde}	53.40 ± 0.96 ^{ab}
2.0	2.0	100 ± 0.00 ^a	4.40 ± 0.37 ^{bcd}	12.36 ± 0.72 ^{ab}	47.40 ± 0.54 ^{bc}
2.0	3.0	100 ± 0.00 ^a	3.90 ± 0.34 ^{cdef}	11.46 ± 0.64 ^{bcd}	43.50 ± 0.43 ^{bc}

Similar letters within the same columns mean no significant difference at $p \leq 0.05$ by DMRT.

^aValues represent means ± standard error.

The present investigation was carried out to establish plant regeneration protocol through multiple shoot induction from nodal segments of *Lobelia cardinalis* L. In the present study, nodal segments were assessed for multiple shoot production in *Lobelia cardinalis* L. using different combinations and concentrations of PGRs. For the successful establishment plant regeneration in different genotypes, various combination and concentration of PGRs and explants play a vital role in ornamental aquatic plant [25-27]. The exogenous addition of PGRs changes the growth of axillary meristems and promotes the meristematic cell proliferation in the axillary buds by mounting the number of shoot bud primordial that originate from the pre-existing axillary meristems [28]. Hence, the present study was conducted to optimize plant regeneration procedure with suitable combinations and concentrations of PGRs in *Lobelia cardinalis* L.



Figure 2 *In vitro* micropropagation of *Lobelia cardinalis* L. after 45 days of culture. (A) - (B) Control and (C) - (D) multiple shoot production from nodal segments cultured on agar-gelled MS medium supplemented with 1.0 mg/L BAP + 2.0 mg/L NAA (Scale bar = 1 cm).

Nodal segments of *Lobelia cardinalis* L. were evaluated for multiple shoot induction and regeneration on twelve combinations and concentrations of BAP (0.0, 1.0 and 2.0 mg/L) and NAA (0.0, 1.0, 2.0, and 3.0 mg/L). Among diverse combinations and concentrations of BAP and NAA tested, BAP (1.0 mg/L) and NAA (2.0 mg/L) combination were found to be the best combination concerning the percentage of response (100 %), number of shoots per explant (6.20 ± 0.51 shoots), shoot length (11.67 ± 0.69 mm) and number of leaves per explant (58.10 ± 0.38 leaves per explant) (Table 1 and Figures 2(C) and 2(D)).

In the present investigation, the exogenous application of BAP in combination with auxin (NAA) in *Lobelia cardinalis* L. resulted in the maximum shoot induction frequency in nodal segments. Auxins are known to promote multiple shoot induction in various plants [28]. NAA (2.0 mg/L) in combination with BAP (1.0 mg/L) enhanced the elongation of healthy shoots with a higher percentage of response when compared to individual treatment of either BAP or NAA. The maximum percentage of response was 100 on medium fortified with NAA (2.0 mg/L) and BAP (1.0 mg/L), with an average shoot length of 11.67 ± 0.69 mm. Rittirat *et al.* [16] observed efficient shoot elongation on medium fortified with BAP (1.0 mg/L) and NAA (1.0 mg/L) in *Anubias heterophylla*. These results suggest that the maximum level of shoot elongation for *Lobelia cardinalis* L. can be achieved from multiple shoots induced in nodal segments on medium containing BAP and NAA. Plant tissues are known to contain different levels of endogenous growth hormones. As a result, they may respond differently to culture medium [29]. Cytokinins were reported to regulate various physiological and development processes lead to shoots proliferation [30]. Similar attempts have also been made to multiply the cultures of *Scadoxus puniceus* [30], *Rhodophiala pratensis* [31] etc. Kanchanapoom *et al.* [27] has reported culture initiation and shoot multiplication from the lateral shoot tip explants of *A. barteri* var. *Nana* on MS medium containing 3.0 mg/L BAP. But in the present study, in *Lobelia cardinalis* L., a combination of BAP and NAA was found to be suitable for plant regeneration. The present finding further indicated that the inclusion of an auxin (IAA or NAA) along with the optimal cytokinin (BAP) concentration improved the multiplication rate. Other research groups also found that the combination of BAP and NAA proved best for the micropropagation of some other ornamental aquatic plants, such as *Anubias Heterophylla* [16], *Aponogeton ulvaceus* Baker [32], *Echinodorus* 'Indian Red' [33], and *Lindernia antipoda* (L.) Alston [34], as well as for the medicinal herb

Artemisia abrotanum L. [35]. However, these findings disagree with those of Sheeja *et al.* [36], who reported that BAP alone proved better than the combination of BAP and NAA for the micropropagation of an ornamental aquatic plant *Anubias barteri* Var. Nana petite (Engl.) Crusio. The beneficial effect of BAP on shoot regeneration and proliferation and induction of multiple shoots was reported in other species [37-39]. Some species may require a low concentration of auxins in combination with high levels of cytokinins to increase shoot proliferation [40]. The augmentation of additives in the medium were reported to enhance the rate of shoot proliferation and hinder shoot tip necrosis *in vitro* [41]. A study by Abadi and Kaviani [42] on micropropagation of *Aloe vera* L. using BA, NAA and IBA showed that the best proliferation of shoot per explants was shown on medium supplemented with 0.5 mg/L BA containing 0.5 mg/L NAA. The frequency and response of *in vitro* shoot regeneration are affected by the type of explants and the concentration of PGRs [43-45]. The promoting effect of cytokinin and auxin combinations on organogenic has been well documented [46,47]. *In vitro* shoot multiplication relies largely on medium formulations containing BAP as the major PGRs in combination with a low concentration of NAA [48].

Effects of α -Naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) on root induction of *Lobelia cardinalis* L. after 45 days of culture

Rooting of *in vitro* regenerated *Lobelia cardinalis* L. shoots is regarded as one of the most crucial bottlenecks for successful plant regeneration. In the present investigation, 2 auxins (NAA and IBA) were used with 3 concentrations (1.0, 2.0 and 3.0 mg/L) to optimize the rooting efficiency of the *Lobelia cardinalis* L. The rooting frequency was significantly different in various treatments of auxins. More shoots formed roots on MS medium containing NAA or IBA and the plantlets showed more roots per shoot (up to 11.70 ± 0.27 roots) than those rooted in auxin-free MS medium (7.40 ± 0.13 roots/shoot) but the roots were shorter (4.05 - 12.79 vs. 28.47 mm; **Table 2**). Among diverse concentrations of 2 auxins tested, medium containing 1.0 mg/L IBA was found to best followed by 2.0 mg/L NAA for root induction frequency in *Lobelia cardinalis* L. The percentage response of root formation was also consistently higher in the medium supplemented with IBA at 1.0 mg/L. The average number of roots per shoot was 11.70 ± 0.27 roots and an average length was 12.79 ± 0.72 mm on medium fortified with IBA at 1.0 mg/L (**Table 2** and **Figures 3(C)** and **3(D)**). An increase in concentration of IBA from 1.0 to 3.0 mg/L decreased the rooting percentage to 90 %.

Table 2 Effects of α -Naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) on root induction of *Lobelia cardinalis* L. after 45 days of culture.

Growth regulators (mg/L)		Root Formation (%)	Number of roots per explant	Root length (mm)
NAA	IBA	(mean \pm SE) ^a	(mean \pm SE) ^a	(mean \pm SE) ^a
0.0	0.0	100 \pm 0.00 ^a	7.40 \pm 0.13 ^a	28.47 \pm 0.24 ^a
0.0	1.0	100 \pm 0.00 ^a	11.70 \pm 0.27 ^a	12.79 \pm 0.72 ^b
0.0	2.0	100 \pm 0.00 ^a	8.70 \pm 0.12 ^a	9.04 \pm 0.56 ^c
0.0	3.0	90 \pm 0.10 ^a	8.60 \pm 0.14 ^a	6.60 \pm 0.44 ^{cd}
1.0	0.0	100 \pm 0.00 ^a	10.50 \pm 0.12 ^a	5.32 \pm 0.37 ^d
2.0	0.0	100 \pm 0.00 ^a	11.50 \pm 0.16 ^a	5.79 \pm 0.39 ^d
3.0	0.0	90 \pm 0.00 ^a	9.60 \pm 0.58 ^a	4.05 \pm 0.23 ^d

Similar letters within the same columns mean no significant difference at $p \leq 0.05$ by DMRT.

^aValues represent means \pm standard error.

Among the 2 auxins tested, IBA was excellent in inducing rooting percentage, number of roots per shoot, and root length followed by NAA. IBA has been commonly used for *in vitro* rooting of chickpea [49]. In the present investigation, the response, as well as the nature of roots induced from shoots, different from concentrations of IBA and NAA used. Similar observations were also made in earlier reports on the rooting efficiency of IBA followed by NAA in chickpea [28]. The positive effect of IBA on root induction has also been reported for other species in Asteraceae family such as *Rhaponticum carthamoides* [50] and *Cichorium pumilum* [51]. Our findings demonstrated that the addition of IBA or NAA in culture media was effective for increasing the number of roots. The current study showed the positive effect of IBA on root induction and root length. Barpete *et al.* [52] suggested that PGRs must be used for root initiation in aquatic

plants and legumes. They used IBA for successful rooting in *Lathyrus sativus*. However, they emphasized that plants need lower concentration of IBA at the early stage. Indole-3 butyric acid was reported to induce maximum root number (11.6 per triscalles) as compared with NAA in aquatic plant species *Crinum malabaricum* Lekhak & Yadav [41]. Resetar *et al.* [53] reported roots along with shoot morphogenesis on 2.0 mg/L NAA and IBA in *Galanthus woronowii*. Auxins are routinely used for induction of rooting for *in vitro* shoots [54-57]. The application of auxins promotes the enzyme activities that regulate various pathways of protein, carbohydrates, nitrogen, and polyphenolic metabolism which could influence root formation [55]. Previous studies have shown that in herbaceous plant a very low concentration of strong auxins is effective in induction of rooting [58-60]. Apart from root number, their length is also critical in the survival of plants during hardening [61].

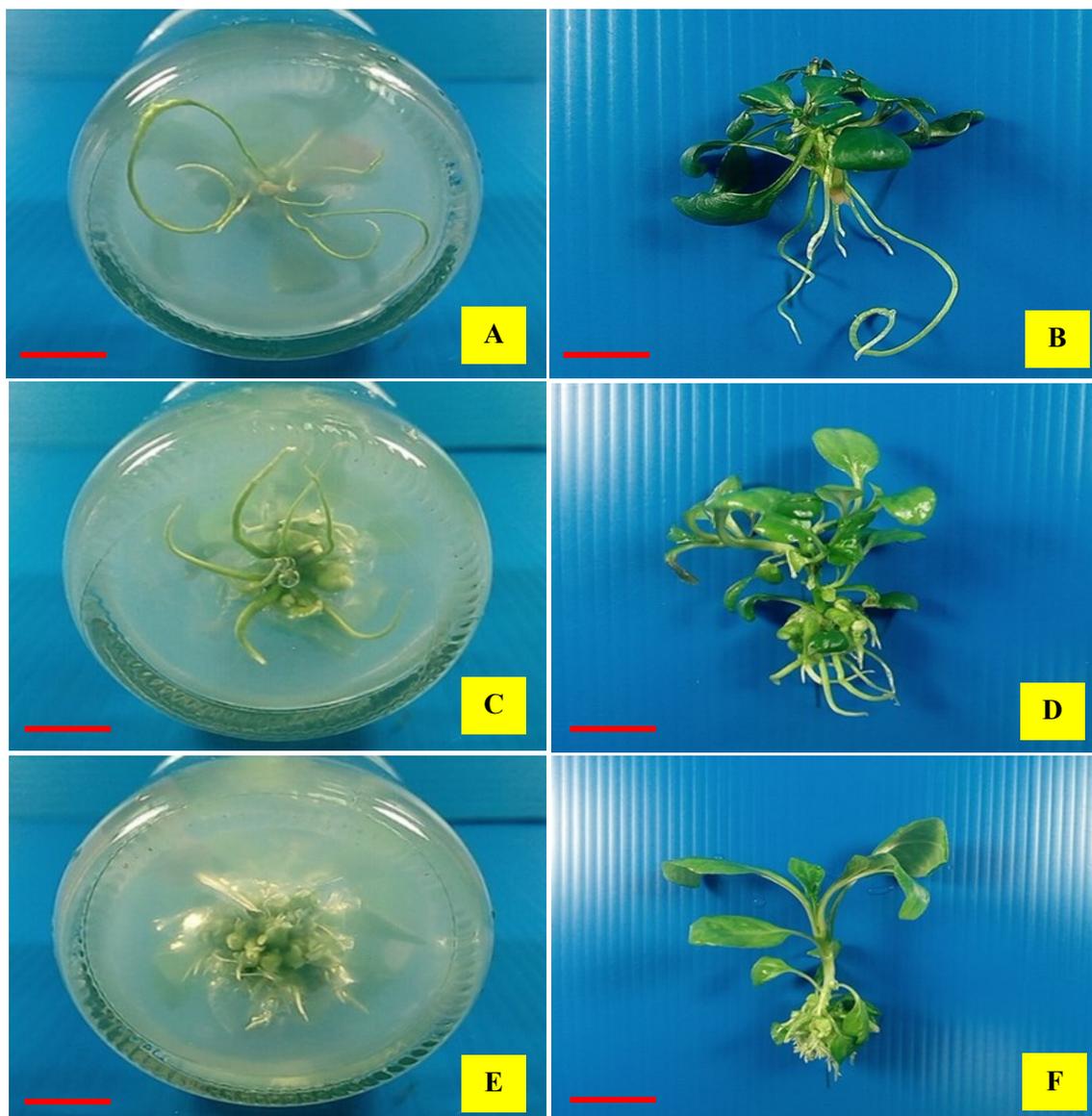


Figure 3 *In vitro* micropropagation of *Lobelia cardinalis* L. after 45 days of culture. (A) - (B) roots developed on MS medium without any plant growth regulators (PGRs) Control, (C) - (D) rooting of regenerated shoots on agar-gelled MS medium augmented with 1.0 mg/L IBA and (E-F) rooted shoot on MS medium supplemented with 2.0 mg/L NAA. (Scale bar = 1 cm).

Hardening and establishment of tissue culture plantlets

All *Lobelia cardinalis* L. *in vitro* regenerated plants were acclimatized under greenhouse conditions without any mortality. During the transferring process, the regenerated *Lobelia cardinalis* L. plantlets showed normal phenotypic characteristics similar with their donor plant (**Figures 4(A) to 4(G)**). The plantlets regenerated from nodal segments appeared to be morphologically similar to mother plants. Regarding the *in vitro* acclimatization of *Lobelia cardinalis* L. plantlets, our findings are consistent with the 100 % survival rate reported for this genus by several authors [26,62]. These results contrasted with those observed for some plant species; where a high mortality has occurred during the acclimatization process due to stomatal dysfunction, weak root systems, and/or poor cuticle development [63]. Therefore, the protocol established in this study also ensures rapid and inexpensive rooting, as well as an adequate survival rate of this commercially important species.



Figure 4 Acclimatization of the regenerated plantlets. (Scale bar = 1 cm); (A) - (B) 45-day-old regenerated shoots on MS medium supplemented with plant growth regulators, and (C) - (G) plantlets transferred to 3.5×3.5 cm² black net plastic pots containing rockwool medium with tap water under greenhouse conditions and acclimatized for 30 days.

Conclusions

In conclusion, an efficient regeneration protocol for *Lobelia cardinalis* L. has been established, which required 12 weeks from culture initiation to plant regeneration. All plantlets transferred to the *ex-vitro* conditions showed a high homogeneity without obvious morphological avoidance of somaclonal variation. Micropropagation would ensure a continuous supply of plants in limited time and space. This protocol will be helpful for rapid and large-scale propagation, to enrich the ornamental industry.

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References

- [1] T Murashige and F Skoog. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum* 1962; **15**, 473-97.
- [2] MSH Kose, M Dogan and G Sadi. Enhanced *in vitro* shoot proliferation through nodal explants of *Staurogyne repens* (Nees) Kuntze. *Biologia* 2021; **76**, 1053-61.
- [3] N Gupta, V Jain, MR Joseph and S Devi. A review on micropropagation culture method. *Asian J. Pharmaceut. Res. Dev.* 2020; **8**, 86-93.
- [4] S Gupta, AA Mao and S Sarma. Effects of thidiazuron (tdz) on direct shoot organogenesis of *Gymnocladus assamicus*: A threatened and critically endangered species from Northeast India. *Natl. Acad. Sci. Lett.* 2020b; **43**, 85-91.
- [5] A Tazeb. Plant tissue culture technique as a novel tool in plant breeding: A review article. *Am. Eurasian J. Agr. Environ. Sci.* 2017; **17**, 111-8.
- [6] OM Oseni, V Pande and TK Nailwal. A review on plant tissue culture, a technique for propagation and conservation of endangered plant species. *Int. J. Curr. Microbiol. App. Sci.* 2018; **7**, 3778-86.
- [7] YB Wen, XX Liu, HJ Liu, CN Wu, HW Meng and ZH Cheng. High frequency direct shoot organogenesis from garlic (*Allium sativum* L.) inflorescence and clonal fidelity assessment in regenerants. *Plant Cell Tissue Organ. Cult.* 2020; **141**, 275-87.
- [8] M Dogan. Effect of cadmium, chromium, and lead on micropropagation and physio-biochemical parameters of *Bacopa monnieri* (L.) Wettst. cultured *in vitro*. *Rendiconti Lincei Scienze Fisiche e Naturali* 2019; **30**, 351- 66.
- [9] K Matand, M Shoemake and C Li. High frequency *in vitro* regeneration of adventitious shoots in daylilies (*Heemerocallis sp*) stem tissue using thidiazuron. *BMC Plant Biol.* 2020; **20**, 31.
- [10] AK Mishra, KN Tiwari, P Mishra, SK Tiwari, SK Mishra and J Singh. Factors affecting the efficiency of *in vitro* regeneration from seedling-derived nodal explants of *Nyctanthes arbor-tristis* L. and evaluation of genetic fidelity. *Plant Biosyst.* 2020; **154**, 197-205.
- [11] C Kim and W Dai. Plant regeneration of red raspberry (*Rubus idaeus*) cultivars ‘Joan J’ and ‘Polana’. *Vitro Cell. Dev. Biol. Plant* 2020; **56**, 390.
- [12] G Premkumar, T Karuppanapandian, C Sureshpandian, N Arumugam, A Selvam and K Rajarathinam. Optimization of a liquid culture system for shoot regeneration and achieving an enriched level of scopadulcic acid b in the leaf organ cultures of *Scoparia dulcis* L. by response surface methodology. *Vitro Cell. Dev. Biol. Plant* 2020; **56**, 60-71.
- [13] U Arigundam, AM Variyath, YL Siow, D Marshall and SC Debnath. Liquid culture for efficient *in vitro* propagation of adventitious shoots in wild *Vaccinium vitis-idaea ssp. minus* (lingonberry) using temporary immersion and stationary bioreactors. *Sci. Hortic.* 2020; **264**, 109199.
- [14] S Klaoheed, W Jehsu, W Choojun, K Thammasiri, S Prasertsongskun and S Rittirat. Induction of direct shoot organogenesis from shoot tip explants of an ornamental aquatic plant, *Cryptocoryne wendtii*. *Walailak J. Sci. Tech.* 2020; **17**, 293-302.
- [15] S Klaoheed, S Rittirat and K Thammasiri. Plantlet regeneration and multiple shoot induction from protocorm-like bodies (PLBs) of medicinal orchid species, *Dendrobium crumenatum* Sw. *Walailak J. Sci. Tech.* 2021; **18**, 9168.
- [16] S Rittirat, K Thammasiri and S Klaoheed. *In vitro* rapid multiplication of a highly valuable ornamental aquatic plant *Anubias Heterophylla*. *Trends Sci.* 2021; **18**, 3.

- [17] S Rittirat, S Klaocheed and K Thammasiri. A reliable protocol for micropropagation of an ornamental aquatic plant, *Staurogyne repen*. *Acta Hortic.* 2022; **1339**, 227-36.
- [18] S Rittirat, S Klaocheed and K Thammasiri. Large scale *in vitro* micropropagation of an ornamental plant, *Oxalis triangularis* A.st.-Hil, for commercial application. *Acta Hortic.* 2022; **1339**, 245-56.
- [19] S Rittirat, S Klaocheed and K Thammasiri. Thidiazuron induces high-frequency plant regeneration from shoot tip explants of an ornamental aquatic plant, *Anubias barteri* var. *nana*. *Acta Hortic.* 2022; **1339**, 273-80.
- [20] S Rittirat, S Klaocheed and K Thammasiri. Micropropagation of an endangered medicinal plant, ant-plant, *Myrmecodia tuberosa* Jack., for conservation in Thailand. *Acta Hortic.* 2022; **1339**, 281-9.
- [21] S Rittirat, S Klaocheed and K Thammasiri. *In vitro* shoot organogenesis in *Anthurium andraeanum* 'HC 028' - a highly valued cut-flower and potted ornamental plant. *Acta Hortic.* 2022; **1339**, 291-9.
- [22] *Lobelia cardinalis*, Available at: <https://tropica.com/en/plants/plantdetails/Lobeliacardinalis%28053CPCS%29/21134>, accessed October 2023.
- [23] Cardinal plant aka cardinal flower (*Lobelia Cardinalis*) tissue culture, Available at: <https://aquaticarts.com/collections/loose-plants/products/cardinal-plant-tissue-culture>, accessed October 2023.
- [24] Cardinals-Lobelie - *Lobelia cardinalis* mini - Dennerle Topf, Available at: <https://www.garnelio.de/en/detail/index/sArticle/28536>, accessed October 2023.
- [25] J Carter and AHLAN Gunawardena. Regeneration of the aquatic monocot *Aponogeton madagascariensis* (lace plant) through callus induction. *Aquat. Bot.* 2011; **94**, 143-9.
- [26] MJ Oh, HR Na, HK Choi, JR Liu and SW Kim. High frequency plant regeneration system for *Nymphoides coreana* via somatic embryogenesis from zygotic embryo-derived embryogenic cell suspension cultures. *Plant Biotechnol. Rep.* 2010; **4**, 125-8.
- [27] K Kanchanapoom, P Chunui and K Kanchanapoom. Micropropagation of *Anubias barteri* var. *Nana* from shoot tip culture and the analysis of ploidy stability. *Not. Bot. Horti. Agrobot. Cluj-Napoca* 2012; **40**, 148-51.
- [28] S Sadhu, P Jogam, RK Thampu, S Abbagani, S Penna and V Peddaboina. High efficiency plant regeneration and genetic fidelity of regenerants by SCoT and ISSR markers in chickpea (*Cicer arietinum* L.). *Plant Cell Tissue Organ Cult.* 2020; **141**, 465-77.
- [29] KJ Palla and PM Pijut. Regeneration of plants from *Fraxinus americana* hypocotyls and cotyledons. *Vitro Cell. Dev. Biol. Plant* 2011; **47**, 250-6.
- [30] D Naidoo, AO Aremu, JV Staden and JF Finnie. *In vitro* plant regeneration and alleviation of physiological disorders in *Scadoxus puniceus*. *S. Afr. J. Bot.* 2017; **109**, 316-22.
- [31] LM Trujillo-Chacon, ER Pastene-Navarrete, L Bustamante, M Baeza, JE Alarcon-Enos and CL Cespedes-Acuna. *In vitro* micropropagation and alkaloids analysis by GC-MS of Chilean Amaryllidaceae plants: *Rhodophiala pratensis*. *Phytochem. Anal.* 2019; **31**, 46-56.
- [32] MYY Kam, LC Chai and CF Chin. The biology and *in vitro* propagation of the ornamental aquatic plant, *Aponogeton ulvaceus*. *SpringerPlus* 2016; **5**, 1657.
- [33] SM Haque and B Ghosh. A submerged culture system for rapid micropropagation of the commercially important aquarium plant, 'Amazon sword' (*Echinodorus* 'Indian Red'). *Vitro Cell. Dev. Biol. Plant* 2019; **55**, 81-7.
- [34] T Jabir, S George, A Raj, S Sree Lakshmi and A Joseph. Micropropagation and *in vitro* flowering of an ornamental aquarium plant *Lindernia antipoda* L. (Alston). *Int. J. Aquaculture* 2016; **6**, 8.
- [35] M Bolyard. *In vitro* regeneration of *Artemisia abrotanum* L. by means of somatic organogenesis. *Vitro Cell. Dev. Biol. Plant.* 2018; **54**, 127-30.
- [36] S George, A Joseph and A Korath. *In vitro* propagation of an ornamental aquatic plant, *Anubias barterii* Var. *nana* petite. *Int. J. Curr. Sci.* 2015; **18**, E1-E12.
- [37] DT Nhut. The control of *in vitro* direct main stem formation of *Lilium longiflorum* derived from receptacle culture and rapid propagation by using *in vitro* stem nodes. *Plant Growth Regul.* 2003; **40**, 179-84.
- [38] CB Fráguas, M Pasqual, LF Dutra and O Cazzeta. Micropropagation of fig (*Ficus carica* L.) 'Roxo de Valinhos' plants. *Vitro Cell. Dev. Biol. Plant* 2004; **40**, 471-4.
- [39] PR Poudel, I Kataoka and R Mochioka. Effect of plant growth regulators on *in vitro* propagation of *Vitis ficifolia* var. *ganeba* and its interspecific hybrid grape. *Asian J. Plant Sci.* 2005; **4**, 466-71.
- [40] DV Staden. *Plant growth regulators, II: cytokinins, their analogues and inhibitors*. In: EF George, MA Hall and GJD Klerk (Eds.). *Plant propagation by tissue culture*. 3rd ed. Springer, Dordrecht, Netherlands, 2008, p. 205-26.

- [41] S Priyadharshini, N Kannan, M Manokari and MS Shekhawat. *In vitro* regeneration using twin scales for restoration of critically endangered aquatic plant *Crinum malabaricum* Lekhak & Yadav: A promising source of galanthamine. *Plant Cell Tissue Organ Cult.* 2020; **141**, 593-604.
- [42] DH Abadi and B Kaviani. *In vitro* proliferation of an important medicinal plant Aloe - a method for rapid production. *Aust. J. Crop Sci.* 2010; **4**, 216-22.
- [43] J Gubis, Z Lajchova and J Farago. Effect of genotype and explants type on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.). *Czech J. Genet. Plant Breed.* 2003; **39**, 9-14.
- [44] S Ishag, MG Osman and MM Khalafalla. Effects of growth regulators, explants and genotype on shoot regeneration in tomato (*Lycopersicon esculentum* cv Omdurman). *Int. J. Sustain. Crop Prod.* 2009; **4**, 7-13.
- [45] S Mukhtar, N Ahmad, MI Khan, M Anis and IM Aref. Influencing micropropagation in *Clitoria ternatea* L. through the manipulation of TDZ levels and use of different explants types. *Physiol. Mol. Biol. Plants* 2012; **18**, 381-6.
- [46] SD Yancheva, S Golubowicz, E Fisher, S Lev-Yadun and MA Flaishman. Auxin type and timing of application determine the activation of the developmental program during *in vitro* organogenesis in apple. *Plant Sci.* 2003; **165**, 299-309.
- [47] S Ignacimuthu, S Arockiasamy, M Antonysamy and P Ravichandran. Plant regeneration through somatic embryogenesis from mature leaf explants of *Eryngium foetidum*, a condiment. *Plant Cell Tissue Organ Cult.* 1999; **56**, 131-7.
- [48] W Xing, M Boa, H Qin and G Ning. Micropropagation of *Rosa rugosa* through axillary shoot proliferation. *Acta Biol. Cracov. S. Bot.* 2010; **52**, 69-75.
- [49] P Kumari, S Singh, S Yadav and LSP Tran. Pretreatment of seeds with thidiazuron delimits its negative effects on explants and promotes regeneration in chickpea (*Cicer arietinum* L.). *Plant Cell Tissue Organ Cult.* 2018; **133**, 103-14.
- [50] E Skala, R Grabkowska, P Sitarek, Ł Kuzma, A Błauz, H Wysokinska and H Wysokińska. *Rhaponticum carthamoides* regeneration through direct and indirect organogenesis, molecular profiles and secondary metabolite production. *Plant Cell Tissue Organ Cult.* 2015; **123**, 83-98.
- [51] WA Khateeb, E Hussein, L Qouta, M Alu'datt, B Al-Shara and A Abuzaiton. *In vitro* propagation and characterization of phenolic content along with antioxidant and antimicrobial activities of *Cichorium pumilum* Jacq. *Plant Cell Tissue Organ Cult.* 2012; **110**, 103-10.
- [52] S Barpete, M Aasim, KM Khawar, SF Özcan and S Özcan. Preconditioning effect of cytokinins on *in vitro* multiplication of embryonic node of grass pea (*Lathyrus sativus* L.). *Turk. J. Biol.* 2014; **38**, 485-92.
- [53] A Resetar, C Freytag, F Kalydi, S Gonda, M Hamvas, K Ajtay, L Papp and C Mathe. Production and antioxidant capacity of tissue cultures from four Amaryllidaceae species. *Acta Soc. Bot. Pol.* 2017; **86**, 3525.
- [54] UI Shehu, LA Sani and AB Ibrahim. Auxin induced rooting of cactus pear (*Opuntia ficus-indica* L. Miller) cladodes for rapid on-farm propagation. *Afr. J. Agr. Res.* 2016; **11**, 898-900.
- [55] P Overvoorde, H Fukaki and T Beeckman. Auxin control of root development. *Cold Spring Harb. Perspect. Biol.* 2010; **2**, a001537.
- [56] CM Fogaca and AG Fett-Neto. Role of auxin and its modulators in the adventitious rooting of *Eucalyptus* species differing in recalcitrance. *Plant Growth Regul.* 2005; **45**, 1-10.
- [57] TI Pop, D Pamfil and C Bellini. Auxin control in the formation of adventitious roots. *Not. Bot. Horti. Agrobo. Cluj-Napoca* 2011; **39**, 307-16.
- [58] SP Yan, RH Yang, F Wang, LN Sun and XS Song. Effect of auxins and associated metabolic changes on cuttings of hybrid aspen. *Forests* 2017; **8**, 117.
- [59] A Aygun and H Dumanoglu. *In vitro* shoot proliferation and *in vitro* and *ex vitro* root formation of *Pyrus elaeagnifolia* Pallas. *Front. Plant Sci.* 2015; **6**, 225.
- [60] N Fatima and M Anis. Role of growth regulators on *in vitro* regeneration and histological analysis in Indian ginseng (*Withania somnifera* L.) Dunal. *Physiol. Mol. Biol. Plants* 2012; **18**, 59-67.
- [61] A Pierret, CJ Moran and C Doussan. Conventional detection methodology is limiting our ability to understand the roles and functions of fine roots. *New Phytol.* 2005; **166**, 967-80.
- [62] S George, A Joseph and A Korath. *In vitro* propagation of an ornamental aquatic plant, *Anubias barteri* Var. Nana petite. *Int. J. Curr. Sci.* 2015; **18**, 1-12.
- [63] A Mathur, AK Mathur, P Verma, S Yadav, ML Gupta and MP Darokar. Biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum*. *Afr. J. Biotechnol.* 2008; **7**, 1046-53.