Plant Tissue Cult. & Biotech. **35**(2): 417-426, 2025 (December) **ISSN 1817-3721, E-ISSN 1818-8745**
DOI: https://doi.org/10.3329/ptcb.v35i2.86683

PTC&B

Development of an Efficient *In vitro* Regeneration Protocol for Easter Lily (*Lilium longiflorum* Thunb.) Using Bulb Explants

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Key words: Regeneration, Lily, Bulb, Rooting, Acclimatization

Abstract

An efficient *in vitro* regeneration protocol was developed using bulb explants of Easter Lily (*Lilium longiflorum* Thunb.). MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA induced callus formation within 20-30 days imcluding 93% of success rate. The regenerative calli were then transferred to a shoot initiation medium containing 2.5 mg/l BAP and 0.5 mg/l NAA, leading to 95.56% of the calli initiating shoots within 20-25 days. The initiated shoots were subsequently transferred to a medium containing 3.0 mg/l BAP, which proved effective for shoot multiplication and proliferation, producing a mean number of shoots 35.0 ± 1.0 per explant. Healthy shoots were then placed in a root induction medium containing full-strength or half-strength MS medium supplemented with IAA at a concentration of 0.5 mg/l or 1.0 mg/l. This treatment induced efficient rooting, with an average root length of 7.2 ± 2.0 cm and a mean of 9.0 ± 1.0 roots per plantlet on full-strength MS medium supplemented with 0.5 mg/l IAA. The well-rooted plantlets were transplanted into pots containing different ratios of garden soil, cocopeat, and vermicompost. The acclimatized plantlets were successfully established under field conditions. Thus, the developed *in vitro* regeneration protocol serves as a robust and scalable method for producing high-quality *L. longiflorum* plantlets, expected to provide substantial benefits for both research and commercial floriculture industries in Bangladesh.

Introduction

Easter Lily is a widely cultivated herbaceous bulbous plant recognized for its significant commercial value as a cut flower. The genus *Lilium*, belonging to the family Liliaceae, comprises approximately 110 species that are distributed across temperate and subtropical regions of the Northern Hemisphere (Wang et al. 2025). To date, more than 8,000 varieties have been registered, commonly classified into three major hybrid groups:

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Longiflorum, Asiatic, and Oriental hybrids. Lilies are known for their large, attractive blooms with high ornamental value and their ability to adapt to diverse environmental conditions.

Globally, lilies rank fourth among the top ten cut flowers in international floriculture markets (Yang et al. 2014). *Lilium* cultivation is considered profitable due to its low labor and maintenance costs. The floriculture industry is rapidly expanding worldwide, particularly in developing countries that rely heavily on agricultural products (Kassa et al. 2025). Ornamental lilies are attractive for their long vase life, durability during transport, and high market price (Naing et al. 2022). In Bangladesh and other developing countries, commercial flower cultivation has markedly increased due to affordable labor and reduced production costs. This growth contributes to the national economy and enhances self-employment opportunities (Jain 2006). Cultivars of *L. longiflorum* are particularly popular in the United States, Japan, and several European and Asian countries (Nhut 1998). The Netherlands, Indonesia, and Malaysia are major suppliers of lilies in the European and Asian markets. Bulbous ornamentals, such as lily and tulip occupy leading positions in the global cut flower industry (Marasek-Ciolakowska et al. 2018).

Traditionally, *Lilium* has been propagated both vegetatively and generatively (Mir et al. 2012). Vegetative propagation typically involves the use of bulb or bulb-scales. While this method maintains genetic purity, it is labor-intensive, time-consuming, and often inefficient. Conventional breeding approaches are further constrained by self-incompatibility and heterozygosity among *Lilium* species (Naing et al. 2022). On the other hand, generative propagation through seed germination can produce large numbers of plantlets, but the results are highly variable and dependent on individual plant performance.

Consequently, neither traditional method is ideal for large-scale commercial production. In Bangladesh, traditional propagation of *Lilium* through bulbs or bulb scales faces some challenges such as low multiplication rate (approximately 3-4 bulbs per scale) and difficulties in producing disease-free plantlets, which inhibits the large-scale and economical production of *Lilium* (Mir et al. 2012). Clonal propagation using the *in vitro* propagation technique offers a reliable alternative for producing a large number of vigorous, uniform, and disease-free plantlets within a short period (Zaki et al. 2011). Micropropagation, the most successful branch of plant tissue culture, enables rapid, year-round multiplication of selected genotypes without seasonal limitations.

Tissue culture has been widely utilized in different *Lilium* species, including *L. longiflorum* (Bacchetta et al. 2003), Oriental hybrid lilies (Lian et al. 2002), and Asiatic hybrids (Taha et al. 2018). *In vitro* scale culture represents one of the most effective vegetative propagation methods for *Lilium* (Varshney et al. 2001, Bahr et al. 2004, and Han et al. 2005). Moreover, *L. longiflorum* has been propagated by using an *in vitro* technique using bulb scales (Kanchanapoom et al. 2011), leaves (Tang et al. 2010), and anthers (Li et al. 2025). The efficiency of adventitious bud regeneration from explants

depends on several factors, including plant growth regulator concentrations, sucrose levels, light conditions, and explant type (Varshney et al. 2000). Furthermore, *in vitro* culture plays an essential role in *Lilium* breeding and genetic enhancement by facilitating the rapid multiplication of genetically uniform plants (Rafiq et al. 2021).

However, an optimized mass-propagation protocol for *L. longiflorum* adapted to Bangladeshi environmental conditions is still lacking. The present study introduces a standard micropropagation protocol for *Lilium* using bulb explants. This method substantially improves multiplication efficiency and mitigates the limitations associated with conventional propagation techniques, such as low multiplication rates and extended production cycles. Thus, the aims of this study were to develop an cost-efficient *in vitro* regeneration protocol for *L. longiflorum* and optimize hormonal combinations to enable rapid, large-scale regeneration.

Materials and Methods

Bulb explants of *L. longiflorum* were collected from a commercial greenhouse in Godkhali, Jhikargacha, Jashore, Bangladesh. *Ex vitro* bulb scales were used as explants for callus induction and subsequent plantlet regeneration. The chemical agents used for surface sterilization included Savlon (Advanced Chemical Industries Ltd., Bangladesh), Tween-20 (Atlas Chemicals Industrials Inc., India), Propineb fungicide powder (Antracol, Bayer Crop Science Ltd., Bangladesh), and mercuric chloride ($HgCl_2$; Merck Specialties Private Ltd., India).

Freshly collected bulbs were washed under running tap water for 40 min. to remove soil particles and other contaminants. The explants were then placed in a conical flask and treated with Savlon and Clorox with constant shaking and rinsed several times. This process was repeated using 5-6 drops of Tween-20 in 100 ml distilled water for 10 min. For final sterilization, the explants were transferred to a laminar airflow cabinet and treated with 0.1% (w/v) $HgCl_2$.

Sterilized bulbs were placed in sterile petri dishes, and bulb scales were separated and dissected into pieces measuring 2-6 mm. Culture media were prepared using MS basal medium containing 4.40 g/l MS powder (Duchefa Biochemie, Netherlands), 6.50 g/l agar, and 30 g/l sucrose. The pH was adjusted to 5.70-5.80. Cytokinin and auxins were added according to experimental requirements.

Explants were inoculated onto callus-induction medium containing 0.5-2.5 mg/l 6-Benzylaminopurine (BAP) and 0.5 mg/l Naphthalene Acetic Acid (NAA). The cultures were incubated in a growth chamber at 23 ± 2°C with 55-60% relative humidity under dark conditions for 2-4 weeks. Calluses were sub-cultured three times before being transferred to the shoot regeneration medium. Regenerative calluses were placed on shoot-initiation medium supplemented with 2.0-3.0 mg/l BAP and 0.5 mg/l NAA and incubated under a 16/8 hrs light/dark photoperiod at 2,000-3,000 lux. Initiated shoots were separated from the callus–shoot complexes and transferred to shoot-multiplication

medium containing 2.0-4.0 mg/l BAP. After two subcultures, individual shoots were transferred to root-induction medium containing 0.5-1.0 mg/l Indole-3-acetic acid (IAA) and NAA. Well-rooted plantlets were then transplanted into plastic pots containing different ratios of autoclaved garden soil, cocopeat, and vermicompost. Acclimatization was carried out for one month, after which the hardened plantlets were transferred to greenhouse conditions.

All experiments were conducted using three independent biological replicates per treatment. Quantitative data were expressed as mean ± standard deviation (SD). The effects of different treatments were evaluated using one-way analysis of variance (ANOVA). Differences among treatment means were considered statistically significant at $P \leq 0.05$.

Results and Discussion

In vitro micropropagation techniques provide an effective alternative for the mass production of healthy plants with uniform characteristics. Moreover, this method allows for the rapid generation of large numbers of plantlets within a short period (Abdalla et al. 2022). Various explants, such as bulb scales, leaves, and petioles have been used for the *in vitro* regeneration of *Lilium* species (Tang et al. 2010). In the present study, bulb scales were used as explants for callus induction and regeneration. The results are presented in Fig. 1.

Medium containing 1.5 mg/l BAP and 0.5 mg/l NAA induced callus within 20-30 days with a 93% response rate (Table 1). It appears that the quality of callus per bulb scale may be connected to the balance of auxin and cytokinin. The induced calluses were subcultured one to two times to increase callus biomass. Similarly, BAP combined with NAA has been reported as an optimal combination for callus induction in *Lilium* (Akter et al. 2022). Azad et al. (2012) reported that auxins promote the regeneration of bulblets. The combination of auxin and cytokinin is essential for callus induction in several species, including Rosa (Ali et al. 2020), Purple fleabane (Maheshwari et al. 2006), and sugarcane (Gopitha et al. 2010).

In this study, calluses were transferred to shoot-initiation medium containing 2.5 mg/l BAP and 0.5 mg/l NAA, which proved highly effective for shoot induction. Bulb-derived calluses exhibited a 95.56% response rate within 20-25 days with a mean number of shoots at 12.0 ± 1.0 (Table 2). After 25-35 days, most calluses had developed into shoots.

This investigation revealed that microbulbs induction and shoot regeneration responses were significantly affected by the concentrations and combination of cytokinin and auxin. The stimulating effect of BAP was also observed, as A'ida et al. (2025) found that BAP had a stronger physiological effect on shoot formation. The endogenous plant growth regulators (PGRs) balance within different explant types is a crucial factor in the response of tissues to different concentrations and types of exogenous PGRs (Nasution

and Nasution 2019). Han et al. (2004) reported successful shoot induction from bulb scales of *L. longiflorum* using MS medium with BA. According to Skoog and Miller (1957), an appropriate auxin-cytokinin balance is crucial for organogenesis, suggesting that the lower auxin concentration in the present study may have facilitated shoot regeneration.

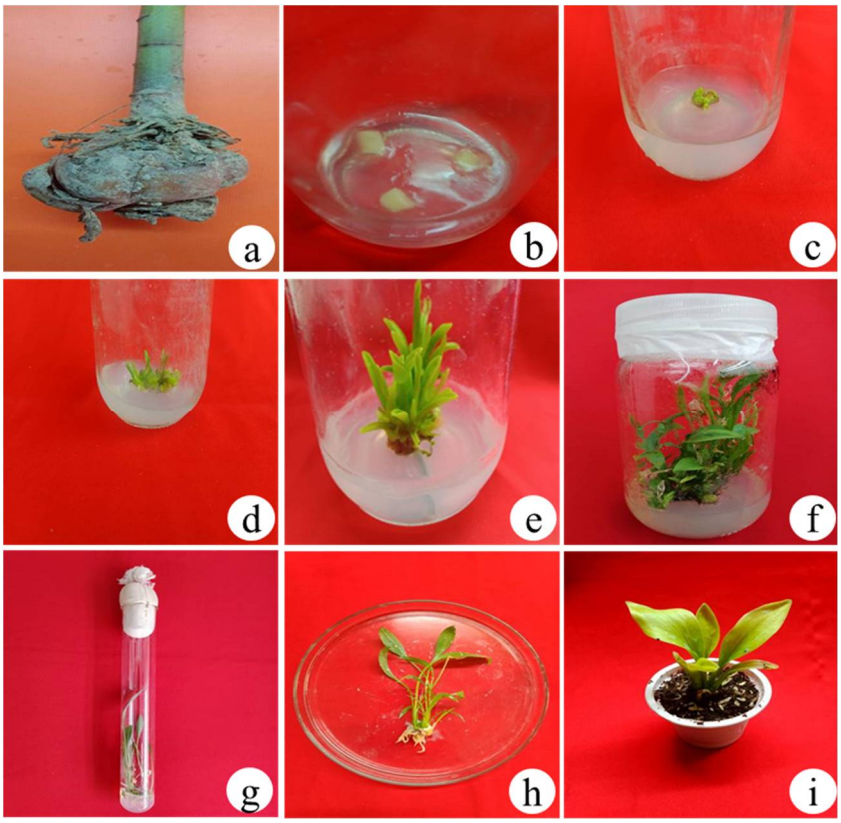

Fig. 1. Different stages of micropropagation of Easter lily (*L. longiflorum*): (a) bulb used as explants for *in vitro* regeneration, (b) segmentation of bulbs, (c) callus induction from explants in dark conditions on MS supplemented with 1.50 mg/l BAP with 0.5 mg/l NAA, (d) sub-culturing callus on shoot initiation medium supplemented with 2.5 mg/l BAP with 0.5 mg/l NAA, (e) shoot elongation from callus following sub-culturing on MS supplemented with the same medium, (f) proliferation of shoots after sub-culturing in BAP hormone-containing medium, (g) root formation from shoots within 3-4 weeks in half and full-strength hormone-free MS medium, (h) showing the well-rooted plantlets, and (i) acclimatization of regenerated plantlets in soil conditions.

To enhance shoot proliferation, regenerated shoots were transferred to medium containing only cytokinin (BAP) without auxin (Table 3). Medium containing 3.0 mg/l BAP produced the highest number of shoots and promoted vigorous shoot growth, consistent with findings from previous studies demonstrating the superior effectiveness of BAP over other cytokinins for shoot multiplication (Arab et al. 2014). Moreover BAP supports the maximum shoot multiplication of Banana (Jafari et al. 2011).

Table 1. Effects of different hormonal combinations in MS for callus formation from bulbs.

Hormonal supplements (mg/l)		No of inoculated explants	Days of callus induction	No of responsive explants	% of responsive explants (Mean ± SD)
BAP	NAA				
0.5	0.5	45	50-60	20	44.3 ± 2.1[d]
1.0	0.5	45	30-40	33	73.7 ± 1.5[b]
1.5	**0.5**	**45**	**20-30**	**42**	**93.0 ± 1.0[a]**
2	0.5	45	15-30	40	88.7 ± 1.5[b]
2.5	0.5	45	25-35	35	77.7 ± 1.5[c]

*Values represent mean ± SD (n = 3). Means followed by different superscript letters (a-d) are significantly different at $p \leq 0.05$ according to one-way ANOVA.

Table 2. Response of bulbs derived callus towards shoot regeneration in the MS containing BAP and NAA.

Hormonal supplements (mg/l)		No of subculturing callus	Days to shoot initiation	No of responsive callus	% of responsive explants	Mean no of shoots (Mean ± SD)
BAP	NAA					
2.0	1.0	45	30-40	34	75.56	7.0 ± 1.0[c]
2.5	**0.5**	**45**	**20-25**	**43**	**95.56**	**12.0 ± 1.0[a]**
3.0	0.5	45	25-30	38	84.44	10.0 ± 1.0[b]

*Values represent mean ± SD (n = 3). Means followed by different superscript letters (a-c) are significantly different at $p \leq 0.05$ according to one-way ANOVA.

Table 3. Effects of sub-culturing with different concentrations of BAP hormone in MS on multiple shoot formation.

Hormonal supplements (mg/l)	No of subculturing callus	% of responsive clumps	Mean no of shoots/ subculturing clumps (Mean ± SD)	Mean length of shoot (cm) after 100 days (Mean ± SD)
BAP				
2.0	45	80	25.0 ± 1.0[c]	5.0 ± 0.2[b]
3.0	**45**	**95**	**35.0 ± 1.0[a]**	**7.0 ± 0.2[a]**
4.0	45	75	30.0 ± 1.0[b]	4.5 ± 0.2[c]

*Values represent mean ± SD (n = 3). Means followed by different superscript letters (a-c) are significantly different at $p \leq 0.05$ according to one-way ANOVA.

Healthy shoots were then transferred to root-induction medium consisting of full- and half-strength of MS medium supplemented with IAA and NAA at concentrations of 0.5 and 1.0 mg/l (Table 4). All treatments resulted in 100% rooting and the highest number of root was observed in full MS medium with 0.5 mg/l IAA without callusing. Callus formation during rooting is undesirable because it can hinder root function and reduce survival during acclimatization. Although the numbers of root were relatively low, the roots were well-developed, reaching up to 7.2 ± 0.2 cm in average in length with branching. Observations indicated that MS strength mainly influenced root length, whereas auxin concentration affected root number and branching, which may influence

overall acclimatization success. Several *in vitro* regeneration studies have demonstrated similar high rooting responses using IAA, where IAA effectively enhanced adventitious root initiation without excessive callus formation. (George et al. 2008).

Table 4. Effects of IAA and NAA containing full and half MS medium on root induction from regenerated shoots.

Plant growth regulators (mg/l)		% of root formation	Days to root initiation	Average no. of roots (Mean ± SD)	Average length of roots (cm) (Mean ± SD)	Intensity of callus formation
Half MS	IAA					
	0.5	100	30-40	5.7 ± 0.6^{c}	6.2 ± 0.2^{c}	-
	1.0	**100**	**25-30**	**8.3 ± 0.6^{a}**	**6.8 ± 0.2^{b}**	-
	NAA					
	0.5	100	16-25	3.7 ± 0.6^{e}	2.4 ± 0.2^{e}	**+**
	1.0	100	15-20	4.3 ± 0.6^{d}	3.8 ± 0.2^{d}	**++**
Full MS	IAA					
	0.5	**100**	**12-18**	**9.0 ± 1.0^{a}**	**7.2 ± 0.2^{a}**	**-**
	1.0	100	15-20	6.7 ± 0.6^{b}	8.2 ± 0.2^{a}	**-**
	NAA					
	0.5	100	15- 25	5.3 ± 0.6^{c}	4.0 ± 0.2^{c}	**+**
	1.0	100	14-22	6.7 ± 0.6^{b}	5.0 ± 0.2^{b}	++

*Intensity of callusing: (-) No callusing, (+) Slight callusing and (++) Considerable callusing.

*Values represent mean ± SD (n = 3). Means followed by different superscript letters (a-e) are significantly different at $p \leq 0.05$ according to one-way ANOVA.

Table 5. Effects of different soil combinations on acclimatization of the regenerated plantlets.

Soil components (Ratio)			No of plantlets	% of survived plantlets	Mean no of shoots per plantlet	Mean no of shoot length (cm) (Mean ± SD)
Garden soil	cocopeat	Vermi-compost				
1.00	1.00	1.00	20.00	98	4	5 ± 0.2^{b}
2.00	**1.00**	**1.00**	**20.00**	**100**	**5**	**7 ± 0.2^{a}**
3.00	1.00	0.00	20.00	97	4	4 ± 0.2^{c}
3.00	0.00	1.00	20.00	95	3	4 ± 0.2^{c}

*Values represent mean ± SD (n = 3). Means followed by different superscript letters (a–c) are significantly different at $p \leq 0.05$ according to one-way ANOVA.

Well-rooted plantlets were transferred to acclimatization, where four substrate combinations were tested using different ratios of garden soil, compost, and vermicompost (Table 5). Measurements were taken at 10-day intervals over 40 days showed that plantlet survival was highest (100%) in garden soil : cocopeat : vermicompost at 2 : 1 : 1, and shoot number, shoot length were also highest in the same combination. Similar findings regarding acclimatization and hardening have been

reported in other bulbous species, such as *L. candidum* (Preece and Sutter 1991, Khawar 2005). Vermicompost provides beneficial microorganisms, plant growth regulators, and necessary macronutrients that can promote vegetative growth during the hardening stage (Atiyeh et al. 2000). Using vermicompost up to 30%, is considered optimum to producing *Lilium* Asiatic hybrid var. Navona (Moghadam et al. 2012). The application of vermicompost has been associated with better nutrient uptake and superior vegetative development in bananas (Hassan et al. 2022), whereas the use of cocopeat-enriched substrates has been demonstrated to promote successful acclimatization in pomegranates (Desai et al. 2018). The importance of substrate selection has also been emphasized by Petric et al. (2014), who found that mixtures containing peat moss, vermiculite, sand, and perlite (2 : 1 : 1 : 1) support effective acclimatization.

The present study successfully developed an efficient and reproducible protocol for the *in vitro* regeneration and mass propagation of *Lilium* plantlets, and confirmed the effectiveness of *in vitro*-derived plant material. Furthermore, this study will provide valuable insights into the various micropropagation stages of *Lilium* and may contribute significantly to its commercial cultivation and the broader floriculture industry.

Acknowledgments

The authors are highly grateful and acknowledge the University Grants Commission of Bangladesh for providing the research grant no. 37.01.0000.073.01.114.23.2880 (Fiscal Year 2022-2023) to carry out the research, and also thanks to the Laboratory of Functional Genomics and Proteomics, Department of Genetic Engineering and Biotechnology, Jashore University of Science and Technology, Jashore 7408, Bangladesh for providing the laboratory facilities for this work.

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(Manuscript received on 7 December, 2025; revised on 18 December, 2025)