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Regeneration of lily (*Lilium longiflorum* 'Easter lily') by callus derived from leaf explants cultured in vitro

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ABSTRACT: Bulb scales of *Lilium longiflorum* 'Easter lily' were cultured in vitro on Murashige and Skoog (MS) medium containing various combinations and concentrations of 6-benzyladenine (BA) and naphthaleneacetic acid (NAA). The MS medium supplemented with 15 μM BA and 0.5 μM NAA produced the largest numbers of multiple shoots (100%) after one month of culture. The MS medium containing 5 μM indole-3-butyric acid was effective for induction of roots. When individual isolated leaves from regenerated shoots were transferred to MS medium supplemented with 2,4-dichlorophenoxyacetate (2,4-D) and NAA, a yellow compact callus was obtained. The calluses differentiated into adventitious shoots and a high frequency of shoot organogenesis (70%) was found on MS medium containing 18 μM 2,4-D with 14 shoots.

KEYWORDS: bulb scales, micropropagation, multiple shoots, shoot organogenesis

INTRODUCTION

Lilies originated from East Asia (China, Korean peninsula, and Japan) and North America [1]. The genus *Lilium*, which belongs to the family Liliaceae, is one of the most globally important cut flowers. It has been used as ornamental plants for centuries mainly because of their large flowers. Lilies are usually propagated by scaling. For commercial purposes, however, this is an inefficient propagation method as the planted material has a very low multiplication rate over a short period. Recently, tissue culture techniques have been applied to generate lilies to rapidly obtain a large number of elite plants. Different sources of explants from lilies, including bulb scales [2], leaves [3], pedicel [4], and callus [5] have a potential for micropropagation, rapid multiplication, and commercialization. At present, gene transfer has become an important research tool that can yield novel plant cultivars. A plant regeneration protocol mediated from callus is a prerequisite to use genetic transformation to improve lily.

In this communication, we describe a method to produce regenerative callus from leaf explants and a protocol to regenerate *L. longiflorum* 'Easter lily' via organogenesis.

MATERIALS AND METHODS

Plant materials and surface sterilization

Bulb scales of *L. longiflorum* 'Easter lily' were collected from field-grown plants and washed thoroughly with running tap water. The explant surface was sterilized with 70% ethanol for 1 min, followed by immersion for 20 min in a sterilization solution of 20% (v/v) Clorox (5.25% NaOCl) containing 1–2 drops of Tween-20 emulsifier per 100 ml solution followed by 10% (v/v) Clorox for 10 min. After surface decontamination, the explants were rinsed 3 times with sterile distilled water. Following disinfection, the bulb scale explants were excised transversely and cut into 1-cm-long segments prior to transferring to Murashige and Skoog (MS) [6] basal medium for further study.

Medium preparation and culture conditions

After 2 weeks of initial culture on MS basal medium, the bulb scale explants were transferred to MS medium supplemented with 0, 5, 10, or 15 μM 6-benzyladenine (BA) and 0, 0.5, 1, or 1.5 μM naphthaleneacetic acid (NAA) either singly or in various combinations. To find suitable plant growth regulators for inducing callus from leaves, young leaves 1 cm in length were cut from regenerated plants and cultured

on MS medium supplemented with 0, 4.5, 9, 13.5, or 18 μM 2,4-dichlorophenoxyacetate (2,4-D) and 0, 5.3, 10.7, 16.1, or 21.4 μM NAA. Callus initiation from young leaves and the percentage of explants forming callus were recorded after 6 weeks of culture. All culture media consisted of MS salts and vitamins supplemented with 3% sucrose and 0.82% Mermaid agar. The pH of the media was adjusted to 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg/cm^2, 121 °C for 20 min. All explants were now transferred to a fresh medium and subcultured at 8-week intervals. Cultures were maintained at 25 ± 1 °C air temperature in a culture room with a 16-h light photoperiod under an illumination of 20 μmol m^{-2} s^{-1} photosynthetic photon flux intensity provided by cool white fluorescent light. Plant materials were stored in glass-capped culture jars (115 ml capacity) each containing 20 ml of medium.

Root induction and acclimatization

Regenerated shoots were transferred to MS medium containing 5 μM indole-3-butyric acid (IBA) for rooting. Well rooted plantlets were then transferred to plastic pots containing sterilized vermiculite for 1–2 weeks before being planted in a glasshouse.

Statistical analysis

One explant was implanted per culture and all experiments were conducted on two different days with 10 replicates per treatment. The percentage of callus induction was monitored as growth parameters. Shoot regeneration was examined for ANOVA. The differences among the mean values of different treatments were compared using Duncan's multiple range test at $P \leqslant 0.05$ using SPSS for Windows version 11.5.

RESULTS

After 2 weeks of culture on MS basal medium, each transverse section of bulb scales became swollen and began to change from a light yellowish to a green colour and subsequently shootlets developed. Table 1 shows the results of in vitro organogenesis in *L. longiflorum*. Supplementation of the media with both BA and NAA was essential for the development of adventitious buds and therefore a morphogenetic response in terms of shoot formation. The percentage of explants developing shoots was 100% and multiple shoots were formed at a high frequency of 4.1 ($P \leqslant 0.05$) on MS medium containing 15 μM BA and 0.5 μM NAA. Through successive subculture, masses of proliferating shootlets were established. The leaves of the adventitious shoots were healthy with a dark green colour and showed no sign of vitrification. Root formation occurred on plant growth regulator-free MS medium or with 5 μM IBA after 3 weeks of culture (Table 2). However, MS medium supplemented with IBA was more effective in terms of initiation of roots per shoot. With 5 μM IBA incorporated in the medium, numerous large and vigorous roots (6.0 ± 1.4) arising from the basal end of the shoots were obtained. The plants propagated from bulb scale did not show any morphological abnormality when compared with the original plants.

There was a significant difference in callus formation and regeneration frequency ($P \leqslant 0.05$, Table 3) on different media. With 2,4-D and NAA incorporated in the media, a yellow compact callus was formed. In the beginning, a light yellow compact callus started from the cut end of the cultured leaves and growth was rather slow. But after a 4-week subculture period, the speed of callus proliferation

Table 1 Effect of different combinations of BA and NAA on shoot multiplication in *L. longiflorum* bulb scale explants cultured on MS medium.

BA (μM)	NAA (μM)	Shoot induction (%)	No. of shoots/explant (Mean ± SE)
0	0	0	0^{d}
0	0.5	100	2.5 ± 0.1^{c}
0	1	100	1.5 ± 0.1^{c}
0	1.5	100	1.6 ± 0.1^{c}
5	0	100	2.1 ± 1.6^{c}
5	0.5	100	3.0 ± 0.8^{b}
5	1	100	3.1 ± 0.1^{b}
5	1.5	100	3.2 ± 0.1^{b}
10	0	100	3.2 ± 1.3^{b}
10	0.5	100	3.8 ± 0.9^{a}
10	1	100	1.5 ± 0.0^{c}
10	1.5	100	3.3 ± 0.4^{b}
15	0	100	3.1 ± 1.2^{b}
15	0.5	100	4.1 ± 0.0^{a}
15	1	100	3.0 ± 0.0^{b}
15	1.5	100	4.0 ± 0.0^{a}

The different letters within column show significant differences by ANOVA and DMRT at $P \leqslant 0.05$.

Table 2 Effect of IBA on root induction in *L. longiflorum*.

IBA (μM)	No. of roots/explant (Mean ± SE)	Root length (cm) (Mean ± SE)
0	3.4 ± 0.6^{b}	4.1 ± 0.4^{b}
5	6.0 ± 1.4^{a}	5.7 ± 0.1^{a}

The different letters within column show significant differences by ANOVA and DMRT at $P \leqslant 0.05$.

Table 3 Effect of 2,4-D and NAA on callus formation and shoot regeneration in *L. longiflorum* leaf explants cultured on MS medium.

2,4-D (μM)	NAA (μM)	Callus formation (%)	No. of shoots/explant (Mean ± SE)
0	0	0	0 ± 0.0^{d}
4.5	0	5	1 ± 0.2^{c}
9.0	0	5	1 ± 0.2^{c}
13.5	0	40	8 ± 0.3^{b}
18.0	0	70	14 ± 0.7^{a}
0	5.3	60	12 ± 0.6^{a}
0	10.7	45	9 ± 0.6^{b}
0	16.1	35	7 ± 0.5^{b}
0	21.4	15	3 ± 0.3^{c}

The different letters within column show significant differences by ANOVA and DMRT at $P \leqslant 0.05$.

was much faster and this resulted in a callus clump that covered the entire explant surface. After completion of the callusing phase, the compact calluses were directly capable of forming shoots via organogenesis with the presence of green and opaque structures. Differentiation of these structures was examined to see that they further developed into normal plants. The average number of regenerated shoots per callus was approximately 14 shoots when cultured on MS medium supplemented with 18 μM 2,4-D (Table 3). There was a considerable increase in shoot number with higher concentrations of 2,4-D and lower NAA. Regenerated shoots obtained from the callus induction media did not produce any roots on this medium. Clumps of shootlets from callus culture subcultured to MS medium supplemented with 5 μM IBA for 4 weeks gave complete plantlets with roots.

DISCUSSION

Bulb scales of lily have a high regeneration potential hence they are most commonly used as explants for vegetative propagation. In this study, an efficient protocol for shoot regeneration from bulb scales in *L. longiflorum* has been developed. The shoots occurred directly via organogenesis and without forming callus on MS medium containing BA and NAA. This result was in agreement with results from other *Lilium* spp[7,8]. BA and NAA showed both individual and synergistic effects and the results indicated that differences in the regeneration response were found on organ formation. Such a synergistic effect of BA and NAA agrees with the results with other ornamental plants such as *Dianthus*[9] or *Tagetes*[10,11]. The induction of roots is an important procedure to form the complete plantlets. In this study, regenerated shoots have been rooted and successfully acclimatized to greenhouse conditions and also demonstrated that IBA has a powerful ability to stimulate root formation.

Leaves have a weaker regeneration capacity than other tissues[3]. This indicates that the leaf segments could be an alternative to the bulb scales as a source of material for propagation. In this study 2,4-D seemed to be more effective for inducing callus than NAA and this agrees with the finding of Priyadarshi and Sen[12] who showed 2,4-D was more effective than other auxins in causing dedifferentiation and callus formation in 'Easter lily'.

In conclusion, our results have demonstrated a practical procedure by which large numbers of true to type plantlets can be propagated from bulb scales of *L. longiflorum*. The induction of callus with a high regenerative capacity from in vitro grown leaves has also been established and this could be useful for establishing a genetic transformation system for the Easter lily.

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