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Simple protocol for plant regeneration of *Lilium ledebourii* using transverse thin cell layer

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Masoud Mirmasoumi[1]; Pejman Azadi[2]*; Ali Sharafi[3]; Valentine Otang Ntui[4] ; Masahiro Mii[4]

1- Department of Botany, School of Biology, Collage of Science, University of Tehran, Tehran, Iran; 2- Tissue Culture and Genetic Engineering Department, Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Iran 3- Novin Giti Gene (NGG) Co., Bio-Incubator Center NIGEB, Tehran, Iran 4- Laboratory of Plant Cell Technology, Graduate School of Horticulture, Chiba University, 648 Matsudo, Matsudo City, Chiba 271-8510, Japan

Abstract

Transverse thin cell layer sections excised from in vitro scales of *Lilium ledebourii* were cultured on Murashige and Skoog (MS) medium supplemented with various plant growth regulators (PGRs) at different concentrations. Although, bulblets were produced on PGR-free MS medium during organogenesis, addition of 0.25 mg l-1 6-benzyladenine or 5.0 mg l-1 indole-3-acetic acid to the medium increased the organogenesis response and produced 4.4 bulblets per explants. Two months later, the bulblets were transferred to MS PGRs-free medium. Bulblets were successfully transplanted to the soil after a cold treatment of 8 weeks, with a survival rate of 85%.

Key Words: *Lilium; Auxin*; Organogenesis; Regeneration.

* **Corresponding author:** azadip22@gmail.com

1. Introduction

Lilium ledebourii (Baker) Boiss (Liliaceae) is a perennial and endangered rare species endemic to Iran. This plant is at the risk of rapid eradication because of irregular grazing and poaching. It is under surveillance of Iranian Regional Environmental Protection Agency(1). *L. ledebourii* has a high ornamental value because of its beautiful large white flowers that can be used as a commercial lily. However, the main problem for the commercialization of this species is its sensitivity to moderate or high temperature conditions and the inability of the bulbs to produce mature plants in greenhouse. Molecular breeding could possibly overcome these challenges. In our previous study, a successful micropropagation system using scale culture to conserve this species, , was reported (2). Using this method, we produced an enormous number of plants for breeding program, but our attempts to transform the scale explants via Agrobacterium-mediated transformation were abortive. The scale explants were not suitable for *Agrobacterium*-mediated transformation because of low surface contact with both Agrobacterium and selection medium. Therefore, we considered use of the transverse thin cell layer (tTCL) method as it is known to be an excellent source of material for genetic transformation(3, 4). The transverse thin cell layer (TCL) method provides a large number of cells that are exposed to Agrobacterium infection and maximizes the exposure of putative transformed tissue to the selective medium, reducing the probability of escapes often associated with large explants (3).

TCLs have been used to study lily differentiation with the successful manipulation of all morphogenic programs. The effect of TCL explant source, sucrose concentration, explant position, activated charcoal (AC) and plant hormone regulators (PGRs) on the mass propagation of *Lilium* using tTCL explants were studied previously(4). Plant growth regulators are the most effective factors in tissue culture response using the tTCL method for lily plants(5, 3, 6–8, 4). In the present study, the effect of different PGRs on thin cell layer culture was considered for plant regeneration in *L. ledebourii*.

2. Materials and methods

Plant material, explant source and media culture

In vitro bulblets of *L. ledebourii* were obtained from the scales culture as described previously (2) and were used as explant source for the experiments. Transverse thin cell layers (tTCL) with 1.2 – 1.4 mm thickness were excised from the basal segment of scales of four-month-old bulblets. The tTCLs were cultured on MS medium (9) containing 30 g/l sucrose, 0.8% agar and supplemented with 6-benzyladenine (BA) alone at concentration of 0.0 and 0.25 mgl^{-1} or in combination with 0.0, 1.5, 3.0, 4.5, 6.0 and 7.5 mgl-1 of 2, 4-dichlorophenoxy acetic acid (2,4-D), dicamba or picloram, and 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mgl-1 of indole-3-acetic acid (IAA). The tTCLs in Petri dishes were cultured for two months with ubculturing in the same medium at two week intervals. In the third-month of culture, the explants were transferred to Petri dishes containing PGRs-free MS medium to allow enlargement of the bulblet. Then the enlarged bulblets were separated and individually cultured to the same medium in jars for rooting. Morphological characters were recorded from callus induction to plant regeneration.

Culture conditions

The pH of media was adjusted to 5.7 before autoclaving at 121ºC and 1.5 kg cm^{-1} pressure for 20 minutes. The cultures were incubated at 25 ± 1ºC in the dark for the first month and then transferred to a 16 hours photoperiod at a light intensity of 45 µ mol m^{-2} s^{-1} emitted by cool-white fluorescent tubes under the same temperature condition.

Cold treatment and transplantation

After the scaly leaves had wilted, rooted plantlets were removed from jars, washed to remove all adhering gelling materials and then transferred to jars containing autoclaved peat. The jars were then closed slightly with lids and maintained for 8 weeks at 4ºC. After cold treatment, plantlets were transplanted into pots containing perlite, leaf-mold and peat moss (1:1:1 v/v/v) and placed in a greenhouse with 22 ± 2ºC mean temperature, 70 ± 5% relative humidity and natural light conditions.

Data collection and statistical analysis:

A completely randomized design was used with 3 replicates per treatment. All collected data were subjected to the analysis of variance, and mean separation was performed using Duncan's multiple range tests. All percent data were subjected to arc sine (√x) transformation before statistical analysis.

3. Results

All media containing auxin (picloram, 2,4-D, IAA or dicamba) alone or in combination with 0.25 mg l -1BA showed callus induction except media containing high concentrations of 2,4-D. Increasing the 2,4-D concentration dramatically decreased callus production, and only the medium containing 1.5 mg l^{-1} 2,4-D and 0.25 mg l^{-1} BA produced calli, with a low number of bulblets (Table 1; data for dicamba not shown). The highest rate of callus induction 44.4% and 40.0% was occurred in medium containing 1.5 mg l^{-1} or

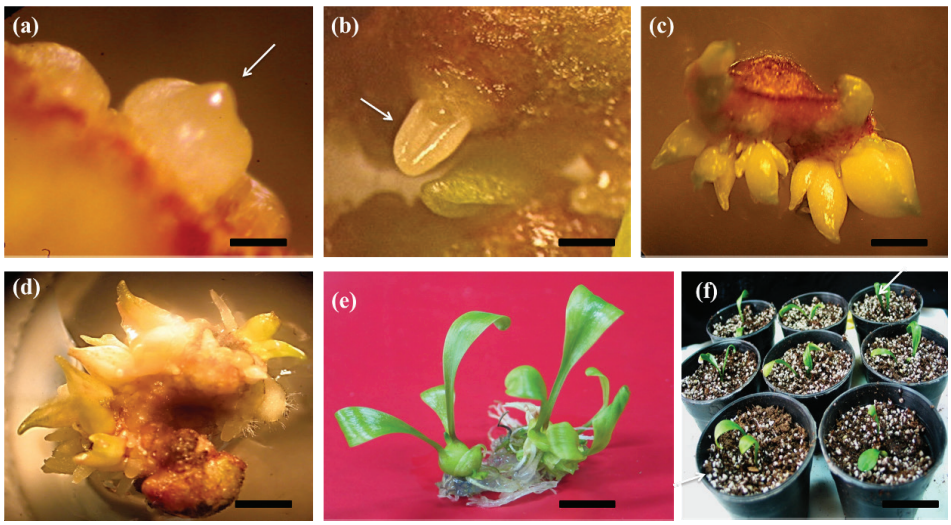

Figure 1. Plant regeneration using transverse thin cell layers of *Lilium ledebourii*. Early stages of shoot formation (arrows) after 25 (a) and 35 days (b) of culture (bar = 1 mm) on MS medium supplemented with 0.25 mg l^{-1} BA in combination with 2 mg l^{-1} IAA and (c,d) regenerated bulblets after 60 days on Petri dishes containing PGRs-free medium(bar = 3mm). (e) Enlargement of bulblets after three weeks of culture in jars containing MS PGRs-free medium (bar = 1 cm). (f) Adaption of plantlets in the greenhouse (bar = 4 cm).

Table 1. Effect of plant growth regulators on callus formation and regeneration of *Lilium ledebourii* transverse thin cell layer.

Plant growth regulators (mg l^{-1})*				No. of explants producing calli** (%)	Number of bulblets/ explant**
BA	Picloram	2,4-D	IAA		
0.0	0.0	0.0	0.0	30.0±4.2 ^{b}	1.1±0.2^{d}
0.0	1.5	0.0	0.0	44.4±5.1^{a}	0.0^{e}
0.0	3.0	0.0	0.0	37.3±2.4^{ab}	0.0 ^{e}
0.0	4.5	0.0	0.0	38.1±3.2^{ab}	0.0 ^{e}
0.0	6.0	0.0	0.0	40.0±7.5^{a}	0.0 ^{e}
0.0	7.5	0.0	0.0	33.3±6.4^{b}	0.0 ^{e}
0.0	0.0	1.5	0.0	20.8±3.5^{c}	0.0 ^{e}
0.0	0.0	3.0	0.0	25.0±6.8 ^{bc}	0.0 ^{e}
0.0	0.0	4.5	0.0	0.0 ^{f}	0.0 ^{e}
0.0	0.0	6.0	0.0	0.0 ^{f}	0.0 ^{e}
0.0	0.0	7.5	0.0	0.0 ^{f}	0.0 ^{e}
0.0	0.0	0.0	1.0	25.0±6.0 ^{bc}	1.0±0.3^{d}
0.0	0.0	0.0	2.0	25.0±6.0 ^{bc}	1.5±0.3^{c}
0.0	0.0	0.0	3.0	22.0±2.5 ^{c}	1.4±0.2^{c}
0.0	0.0	0.0	4.0	25.0±3.0 ^{bc}	2.1±0.5^{bc}
0.0	0.0	0.0	5.0	25.0±5.0 ^{bc}	4.4±0.3^{a}
0.25	0.0	0.0	0.0	35.4±7.4 ^{bc}	4.4±0.7^{a}
0.25	1.5	0.0	0.0	25.9±4.5 ^{bc}	1.0±0.2^{d}
0.25	3.0	0.0	0.0	29.5±5.5 ^{bc}	0.0 ^{e}
0.25	4.5	0.0	0.0	33.3±2.8^{b}	0.0 ^{e}
0.25	6.0	0.0	0.0	33.3±2.8 ^{b}	0.0 ^{e}
0.25	7.5	0.0	0.0	30.0±4.0 ^{b}	0.0 ^{e}
0.25	0.0	1.5	0.0	12.5±4.4^{d}	0.7±0.4^{d}
0.25	0.0	3.0	0.0	8.3±2.5^{e}	0.0 ^{e}
0.25	0.0	4.5	0.0	12.5±3.1 ^{e}	0.0 ^{e}
0.25	0.0	6.0	0.0	0.0 ^{f}	0.0 ^{e}
0.25	0.0	7.5	0.0	0.0 ^{f}	0.0 ^{e}
0.25	0.0	0.0	1	10.0±2.0^{e}	3.2±1.0^{b}
0.25	0.0	0.0	2.0	25.0±3.8^{bc}	4.2±0.6^{a}
0.25	0.0	0.0	3.0	16.6±2.5^{d}	1.8±0.3^{c}
0.25	0.0	0.0	4.0	23.3±3.4^{c}	1.9±0.3 ^{c}
0.25	0.0	0.0	5.0	8.3±1.8^{e}	2.0±0.2 ^{c}

6 mg l^{-1} picloram respectively; no bulblets were produced under these conditions. The media containing 0.25 mg l^{-1} BA alone or in combination with 2 mg l^{-1} IAA produced the highest number (4.4 and 4.2 respectively) of bulblets per explant (Fig. 1a-d). The tTCL explants on MS free hormone (1 per explant) or containing 0.25 mg l^{-1} BA alone (4.4 per explant) produced bulblets (Table 1).

Bulblets appeared in all other concentrations of IAA either alone or in combination with BA. Although the media supplemented with picloram produced calli, bulblets were obtained only at a low concentration of picloram (1.5

mg l^{-1}) in combination with 0.25 mg l^{-1} BA. In this study, all media containing dicamba alone or in combination with BA produced calli, but only medium with 1.5 mg l^{-1} dicamba and 0.25 mg l^{-1} BA showed low regeneration frequency (data not shown).

The produced bulblets were cultured in jars containing PGRs-free MS medium to enlarge the bulblets (Fig. 1e). After cold treatment for eight weeks, the bulblets were successfully transplanted to soil with a survival rate of 85%. The plantlets were uniform with emerging new leaves and no significant differences were observed between plantlets (Fig. 1f).

4. Discussion

In plants, the composition of plant growth regulators in the culture medium directs the callus morphotype(10, 11). In this study, effect of different combinations of plant growth regulators on plant regeneration of *L. ledebourii* using tTCL as explants was studied. Interestingly, in MS medium lacking any plant growth regulators (PGRs), 30.0% of explants formed calli with 1.1 bulblets per explants via organogenesis. Production of calli and a high number of bulblets were observed on the medium containing 0.25 mg l^{-1} BA alone. In contrast, Nhut et al. (2001b) reported necrosis when tTCLs of *Lilium longiflorum* were cultured on media lacking PGRs, and enlargement of explants when they were cultured on MS medium supplemented with BA alone in. Production of calli and bulblets on PGRs-free medium suggests a high level of endogenous hormones in the scales of bulblet of *L. ledebourii* .

Callus formation has been reported to occur using picloram in *L. formolongi*(12, 13). There is no report considering the effect of the above mentioned auxins in regeneration of *Lilium* using tCTL explants. In our study, the media supplemented with picloram alone or in combination with BA showed higher percentage of callus production compared to media supplemented with the other auxins. However, it failed to regenerate plantlets except at low concentration in combination with BA (Table 1). Media containing dicamba could not produce any bulblets, probably because of the toxicity of dicamba on the bulb scales(14).

The effectiveness of IAA on regeneration of *Chrysanthemum*, using tTCLs has been shown(4). Our results showed that the highest number of bulblets was achieved in media supplemented with IAA. This suggests that IAA could be a suitable PGR for plant regeneration using tTCL as an explant in *L. ledebourii*. The media supplemented with high concentration of 2,4-D could not produce bulblets. Nhut et al. (2001b), also reported a significant decrease in bulblet regeneration from tTCL using high concentration of 2,4-D in *L. longiflorum*.

Here, we successfully developed a simple plant regeneration system using tTCL as explants. In our previous studies, a high efficiency transformation protocol for *Lilium* using *Agrobacterium*-mediated system was developed(15–17). Experiments in which these two methods are combined are in progress for *L. ledebourii*.

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