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Micropropagation of *Lilium formolongo* via Leaf Explants

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Abstract The effect of cultured media and plant growth regulator on micropropagation from the leaf of *Lilium formolongo* was cultured on three basic media; Murashige and Skoog (1962) (MS), 1/2MS and Nitsch and Nitsch (1969) (NN) media in the presence and absence of plant growth regulator 1.0 mg/l α-naphthaleneacetic acid (NAA) and 0.5mg/L Thiadiazuron (TDZ). The leaf explants that were cultured on the medium without plant growth regulator died within four weeks. Callus formation was obtained from the leaf explants cultured on the medium supplemented with plant growth regulator after culturing under dark condition for four weeks. Callus cultured on the MS medium with 1.0 mg/L NAA and 0.5 mg/L TDZ gives the best average size of 2.93 centimeters. The callus was then continuously cultured on the same medium but under light condition for 16 hours/day leading to shoot development. After 14 weeks incubation, 5.05 shoots and 36.5 leaves were formed

Keywords: tissue culture, lily, α-naphthaleneacetic acid (NAA), Thidiazuron (TDZ)

Introduction

Lilium sp. is one of most important flower species bulb corps in commercial market. About 8,000 cultivars have been registered. They are classified into three groups, Longiflorum, Asiatic and Oriental hybrids. The longiflorum cultivars possess down-ward white trumpet-shaped flowers with distinctive fragrance, and can be easily cultivated year-round (Zhou *et al*., 2008). Lilium have long been known as an amenable genus, which adapt easily to most tissue culture techniques. Plant regeneration via organogenesis is easily achieved from a vast array of explants ranging from flower organs to bulb scale (Gupta *et al.*, 1978). *L. formolongo* is one of *L. longiflorum* hybrids, a new lily variety with a white flower and high commercial value (Nguyen *et al.*, 2008)

L. longiflorum can be propagated using different explant such as bulb-scales, leaves, anthers, embryos cultured in liquid medium for high frequency of somatic embryogenesis and plant regeneration (Nhut *et al.*, 2006). Overy culture of *L. longiflorum* has been studied to compare different culture media

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and sucrose concentration for plant regeneration (Ramsay *et al*., 2003). Leaf explants have been cultured to induce *de novo* shoot formation of *L. davidii* var unicolor (Ling-Fei *et al*., 2009). There are no published accounts of *L. formolongo* showing successful micropropagation from leaf explants. Here we report *L. formolongo* leaf explants culturing conditions to induce callus and shoot regeneration.

Materials and methods

Plant materials

Seeds of *L. formolongo* were surface-sterilized in running tap water for 30 min, dipped in 70% ethanol for 1 min, 1% sodium hypochlorite with 1 drop tween-20 for 10 min, followed by three times rinses with sterile distilled water. Seeds were germinated on Murashige and Skoog (1962) basal medium (MS) supplemented with 30g sucrose and 8g/L agar (commercial grade).The medium was adjusted to pH 5.6 before it was autoclaved at 121^{o}C for 20 min. The cultures were incubated at 25 $\pm$ 2 ^{o}C under cool-white fluorescent lamps with 16 h photoperiod under an illumination of 20 $\mu molm^{-2}s^{-1}$ photosynthesis photon flux density. Leaves from 3 months-old seedling were used the explants.

Inducing callus and shoot induction

Leaf explants (approximately 5 mm) were placed on different media Nitsch and Nitsch (1969) (NN), 1/2MS and MS medium supplemented with 1 mg/L NAA and 0.5 mg/L TDZ or without plant growth regulators. All culture was maintained at 25$\pm$ 2 ^{o}C in dark room for 4 weeks for callus induction. The callus was cultured on the same media under cool-white fluorescent lamps with 16 h photoperiod for shoot regeneration. The explants were subcultured on the same medium every 4 weeks. The individual shoots were transferred to MS medium containing with 0.5 mg/L IBA for root formation.

Statistical analysis

In this experiment, a completely randomized design was conducted to test effect of medium and NAA and TDZ on callus and shoot regeneration. Six treatments were teast and each consisting of 20 explants and 4 replications. Data were analyzed for significances by analysis of variance with the mean separation by Duncan's multiple range tests.

Results

The results showed that seeds were germinated on MS medium (12.3%). Roots appeared within 3 weeks of culture and were followed leaves.

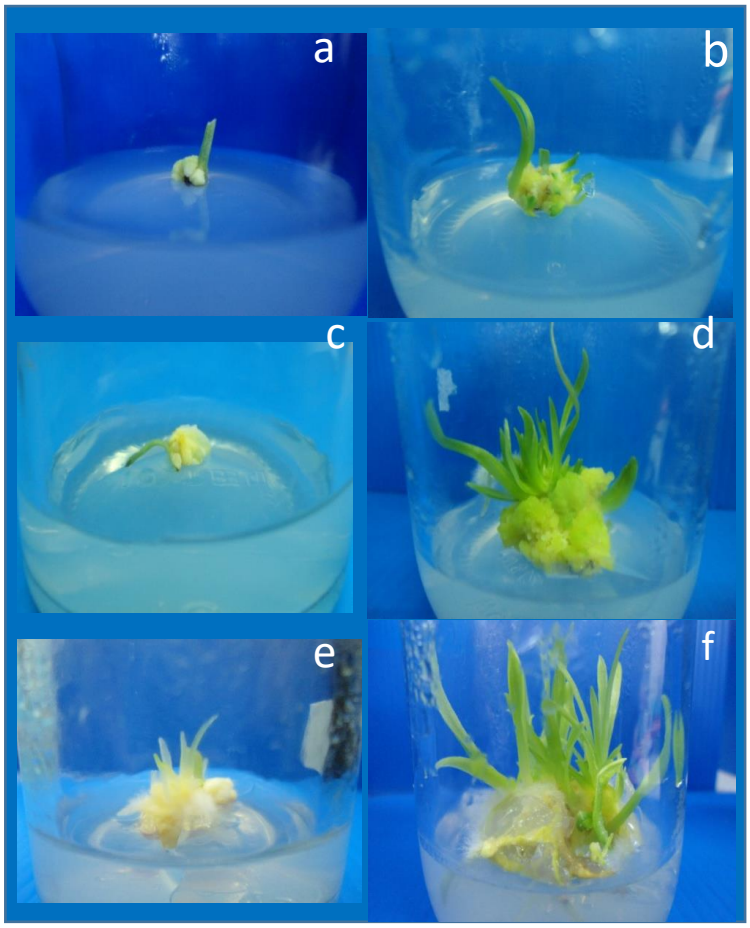

Figure 1. Plant regeneration from leaf explants of lily. (a, b) Regenerated callus from leaf explants grown on NN medium with 0.1 mg/L NAA and 0.5 mg/L TDZ (c, d) ½ MS medium wit 1.0 mg/L and 0.5 mg/L TDZ (e, f) MS medium with 1.0 mg/L NAA and 0.5 mg/L TDZ in 4 weeks and 14 weeks.

The effect of 1 mg/L NAA and 0.5 mg/L TDZ on lilium leaf culture was prominent and callus was inducted after 4 weeks in the dark condition (Fig 1a, c, e). Leaf explants culture on medium without plant growth regulator showed no callus. The callus transferred to light conditions changed after 1 weeks form white to yellow. The effect of NAA and TDZ on different medium, NN, MS and ½ MS medium was shown different size and weight of callus (Fig. 1b, d, f). Highest callus, size (2.93 cm) and

weight (5.78 g), was obtained with 1 mg/L NAA and 0.5 mg/L TDZ. Continued incubation in the light produced on average 5 shoots and 36.5 leaves. In comparison, leaf explants cultured on ½ MS contained 1 mg/L NAA and 0.5 mg/L TDZ showed callus of 1.97 cm and 3.5 g on average and these produced on average 2.25 shoots and 22.1 leaves. The leaf explants cultured on NN medium contained 1 mg/L NAA and 0.5 mg/L TDZ produced callus of 0.4 cm and 0.37 g, generating on average 0.9 shoots and 5.2 leaves (Table 1). The produced shoots were subcultured on the same medium during 4 weeks. (Table 1 and Fig.2)

Table 1. Effect of plant growth regulators and media on callus and shoot induction of leaf culture of *Lilium formolongo* within 14 weeks

Medium	PGR (mg/L)		Callus		Shoot	Leaf
	NAA	TDZ	size (cm)	weight (g)	number	number
NN	0	0	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00c
	1	0.5	0.40±0.22c	0.37±0.22c	0.90±0.51c	5.20±3.05c
1/2MS	0	0	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00c
	1	0.5	1.97±0.23b	3.50±0.68b	2.27±0.41b	22.10±2.94b
MS	0	0	0.00±0.00c	0.00±0.00c	0.00±0.00c	0.00±0.00c
	1	0.5	2.93±0.30a	5.78±0.93a	5.00±0.43a	36.50±4.85a

Values represent the mean ± S.E. Means followed by different letters are significantly different at P=0.05 according to the least significant test.

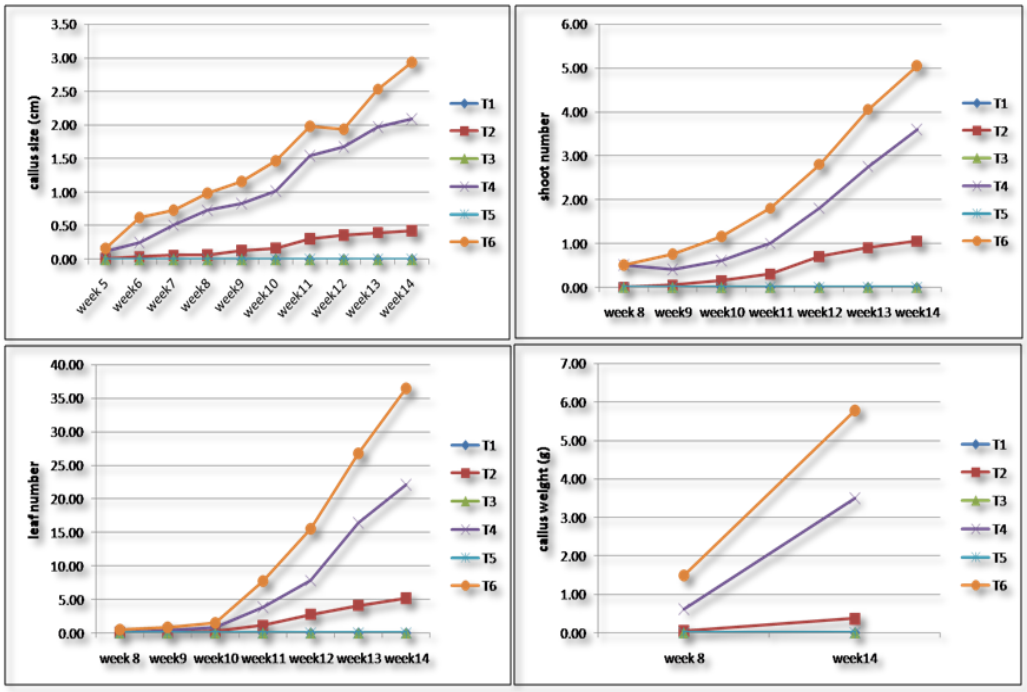

Figure 2. Effect of plant growth regulator and media on callus size, shoot number, leaf number and callus size of leaf culture of *Lilium formolongo*. T1- NN medium, T2- NN medium with 1 mg/L NAA and 0.5 mg/L TDZ, T3- 1/2MS medium, T4- 1/2MS medium with 1 mg/L NAA and 0.5 mg/L TDZ, T5- MS medium and T6- MS medium with 1 mg/L NAA and 0.5 mg/L TDZ.

Figure 3. Plantlets in greenhouse non-cover and covering with plastic sheets

Figure 4. *L. formolongo* growth (a) A rooted shoot (b) plants grown in pot kept outdoors for 4 months (c, d) tissue culture plants grown for more than 1 year

Shoots regeneration from leaf explants grown on medium with NAA and TDZ was transferred to MS medium contained 0.5 mg/L IBA for root regeneration. The rooted shoots were transplanted on 3 different substates soil, soil : coconut fiber (1 : 1), and perite in 6 inch plastic pots. The plants were covered with clear plastic (Fig. 3). It was found that the highest servivial 98% was achieved from soil substate and cover with clear plastic for 5 weeks (data not showed). The plants flowered within a year after the start of tissue cultureing. The micropropagated plants were grown to full maturity and bulbs were collected (Fig.4).

Discussion

The lilium *in vitro* leave explant culture has been described for *L. davidii* var. unicolor. The shoots occurred directly from the leaves without forming callus (Ling-Fei *et al*., 2009). In this experiment, after 4 weeks of incubation leave explants of *L. formolongo* in dark and transfer to light condition, most leave explants were cultured on media with NAA and TDZ showed callus initiation. The callus was regenerated to form shoots when incubated in light. Kedra and Bach (2005) reported that hormone-free medium stimulated the formation of callus on the bud and seeding explants, albeit less frequently than what we find for leaf explants. Plant growth regulators play an important role in organogenesis. The type, ratio and concentration of cytokinin and auxin regulate the direction of organogenesis (Xi *et al*., 2013). The plant growth regulator stimulated various type of callus, 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (Picoram) induced a yellow friable callus, whereas benzyladenine (BA) alone induced cream-white, compact callus of *L. martagon* (Kedra and Bach, 2005). Callus germination of Gymea lily (*Doryanthes excels* Corrrea) was observed at the highest frequencies when grown on MS medium containing 50 µM NAA and 0.5 µM TDZ. Adventitious shoot organogenesis was observed at the

highest frequency when grown on medium containing 0.5 µM NAA and 50 µM TDZ (Dimech *et al.*, 2007). Several part of *L. longiflorum* flower bud cultured on one half MS medium with NAA or IBA was shown shoot regeneration without passing through a callus phase (Nhut *et al*., 2006). However, have been report BA and NAA had not significant effect on bulblets formation in *L. rubellum* (Nimi, 1985) and *L. ledebourii* (Azadi, 2007). The effect of medium in this experiment showed high efficiency callus induction and shoot induction when using explants culture on MS medium containing 1mg/L NAA and 0.5 mg/L TDZ, ½ MS medium with 1 mg/L NAA and 0.5 mg/L TDZ and NN medium with 1 mg/L NAA and 0.5 mg/L TDZ respectively. The difference between the culture media is the concentration and type of mineral nitrogen. MS medium contains a high concentration of ammonium and this may be beneficial to regeneration of Lilium. The total ionic concentration of macronutrients is also higher in MS medium than in NN medium. Ramsay *et al*. (2003) reported ovary culture of Easter lily on MS medium containing 5% sucrose was best for callus formation. Shoot differentiation was also highes on MS medium. Root induction was obtained by applying, 0.5 mg/L IBA similar to the results reporeted by Ling-Fei *et al.* (2009). At 2 mg/L IBA the highest number of roots was reported (Saifullah *et al*., 2010).

Plants transferred to soil showed the best survival under plastic tunnels. The high humidity in plastic was important for survival.

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