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Stimulation of *In Vitro* Bulblet Growth by the Addition of Liquid Medium in *Lilium* Oriental Hybrid 'Casablanca'

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Abstract

The bulb scales and shoot sections (7 mm × 15 mm) of *Lilium* oriental hybrid 'Casablanca' were cultured to compare bulblet growth *in vitro*. Shoots were induced from *in vitro* grown bulbscales on MS medium with 1.0 mg/L BA, 0.5 mg/L IAA, and 30 g/L sucrose. The regenerated shoots were cut into shoot sections, and cultured on MS medium with 2.0 mg/L BA, 0.5 mg/L IAA and 30 g/L sucrose for shoot proliferation. Culture of shoot sections stimulated bulblet growth significantly than the bulb scales on MS medium with 60 g/L sucrose. However, the bulblets from shoot sections did not reach ideal size to produce stems with several leaves. Therefore, liquid medium was added into the same vessels to stimulate bulblet growth further. After shoot sections were cultured on MS medium with 60 g/L sucrose and 2 g/L activated charcoal for two months in dark, 20 ml liquid media containing various concentrations of sucrose and MS salts were added. Two months later, the added liquid medium stimulated bulblet growth remarkably as compared to bulblets grown without added liquid medium. The added 25 ml liquid medium containing 120 g/L sucrose and double strength of MS salts were the most effective for growth of *in vitro* bulblets. More than 94% bulblets produced by this method sprouted stems with several leaves after cold treatment at 5℃ for three months.

Key words: Activated charcoal, shoot formation, sucrose

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Introduction

Lily is one of the most important horticultural crops because of their large and attractive flowers. Among all the lily cultivars, *Lilium* oriental hybrid 'Casablanca' has become very popular because of its white large flower and strong fragrance. Lilies are usually propagated by scaling, a technique which produces 3~5 bulbs from each bulb scale. Scale propagation makes it difficult to obtain large number of bulbs from a disease-free stock or new cultivars in a short period of time (Stimart and Ascher 1978). Therfore, *in vitro* micropropagation is essential to produce a large number of bulblets in lily. In micropropagation of *Lilium* species, it is desirable to produce bulblets that grow rapidly after their transfer to soil, and require a short time to flower. Fast growth of bulblets in *Lilium* spp occurs in large bulblets, and in bulblets that form a stem with several leaves instead of scales bearing one or two leaves. Stems are formed more frequently in large bulblets. Hence, bulblet growth *in vitro* is an important factor for rapid growth of lily bulblets after planting (Gerrits et al. 1997; Marinageli and Curvetto 1999).

Most of the micropropagated bulblets will not reach commercial size by the end of the second cultivation period due to their low weight and small diameter. Sprouting of bulblets follows different growth patterns, with or without stems. Bulblets producing stems in the first year of cultivation are desirable because they enlarge and reach commercial size faster when cultivated in soil (Gerrits et al. 1997; Marinangeli and Curvetto 1998; 1999). Therefore, appropriate cultural conditions should be optimized to stimulate bulblets to enlarge and produce flowers within a few years.

The factors influencing bulblet size are explant size and sucrose concentration in medium (Takayama and Misawa 1979; Gerrits et al. 1997). However, bulbscale size is different when the bulblets are produced *in vitro*. External bulbscales are larger than internal ones even in a bulblet. Therefore, it is very difficult to use similar size of bulbscales (explants) in an experiment or even in practice. On the other hand, external old bulbscales were not able to form more bulblets than internal young ones (Takayama and Misawa 1980). Thus, use of uniform and larger materials to regenerate large bulblets may be very difficult.

Han et al. (1999a; b) proliferated shoot clusters from abnormal bulblet sections in *Lilium* oriental hybrid 'Casablanc a' after inducing abnormal bulblets with swollen basal plate from bulbscales on the media containing plant growth regulators. These shoots produced normal bulblets on the media with high concentration of sucrose (Niimi 1984; Maesato et al. 1994).

Maene and Debergh (1985) added liquid media in same vessels after shoots proliferated, instead of transplanting the tissues to a fresh medium that stimulated elongation and rooting of shoots without transplanting. The positive effects of such practice were also demonstrated by De Riek et al. (1997) in *Rosa multiflora*.

This paper describes an easy method to produce larger bulblets in *Lilium* oriental hybrid 'Casablanca' by culture of shoot sections, and by addition of liquid medium.

Materials and Methods

Plant material

Shoots were regenerated from *in vitro* grown bulbscales of *Lilium* oriental hybrid 'Casablanca' on agar-solidified MS medium (Murashge and Skoog 1962) with 1.0 mg/L BA, 0.5 mg/L IAA and 30 g/L sucrose. The regenerated shoots were cut into 7 mm × 15 mm sections after removing the leaves from the upper part of shoots. There were then proliferated on an agar-solidified MS medium with 2.0 mg/L BA, 0.5 mg/L IAA and 30 g/L sucrose after every two months interval (Han et al. 1999a).

The shoot sections from the proliferated shoot clusters and the bulb scales from *in vitro* bulblets were used as explants for further experiments. Liquid medium was added to stimulate bulblet growth further in the same vessels.

Bulblet formation and growth, and addition of liquid medium

The shoot sections (7 mm × 15 mm) were excised from shoot clusters and cultured on 2.0 g/L phytogel-solidified MS medium with 60 g/L sucrose and 2.0 g/L activated charcoal for two months (Han et al. 1999b). To the same vessels, 20 ml liquid media was added further growth of bulblets to grow further. To each test tube, 20 ml of liuid medium was added and autoclaved at 121℃ for 15 min. This medium was poured in each vessel aseptically. The effects of sucrose concentrations (30～150 g/L), the strength of MS salts, (1/2～2 x), and different MS salts (MS macro-, MS macro- and micro-, and MS full salts) were examined on bulblet growth for two months after adding the liquid medium. The quantity of liquid medium was also tested.

Culture conditions for bulblet formation and growth

All experiments were performed in polyester vessels (Ψ 10 cm × 5 cm), and each vessel contained 100 ml medium. Media were adjusted to 5.8 pH before autoclaving at 121℃ for 15 min. Shoots were induced and proliferated under light with 16 h photoperiod per day at quantum flux density of 50 μmol m^{-2} s^{-1} from fluorescent lamps, but bulblet growth was performed in dark. All cultures were incubated at 25±2℃. For each treatment, nine explants per vessel were cultured and the experiment was repeated three times with three vessels. Data were recorded two months after the liquid medium was added.

Statistical analysis

For each treatment, 7 explants were cultured on pholyester vessels (10 cm diameter × 5 cm height) and three vessels were used for each treatment. Each vessel was regarded as individual replicate and used for statistical analysis. For all experiments, data were collected on number of bulblets and bulblet size two months after the addition of liquid medium. For statistical analysis, means were subjected to Duncan's multiple range test using SAS statistical package (SAS Institute Inc. 1985).

Results

Bulb scales from *in vitro* grown bulblets and shoot sections (7 mm × 15 mm) from regenerated shoot clusters were cultured on MS medium with 60 g/L sucrose for three months to compare bulblet formation and growth (Table 1). There was no difference in the number of bulblets produced from the two materials. However, significant differences were observed for in fresh weight, bulblet weight, and diameter of bulblets (Table 1)(Figure 2). Cultured shoot sections were more effective for bulblet growth than that of

Table 1. Comparison of bulblet growth in *Lilium* oriental hybrid 'Casablanca' affected by cultural materials on MS medium with 60 g/L sucrose and 2 g/L activated charcoal after three months in culture.

Cultural Material	No.of bulblets /explant	Fresh wt. /explant (mg)	Diameter of bulblet (mm)	Fresh wt. /bulblet (mg)	No.of roots /explant
Bulb scale[a]	1.4 a[c]	505.2 b	7.3 b	364.5 b	4.8 a
Shoot section[b]	1.2 a	944.7 a	10.2 a	718.6 a	3.4 a

[a]Bulb scales were excised from bulblets grown on MS medium with 90 g/L sucrose for 3 months.
[b]Shoots were cultured on MS medium with 30 g/L sucrose, 2.0 mg/L BA, and 0.5 mg/L IAA.
[c]Mean separation by Duncan's multiple range test at $P \leq 0.05$.

Table 2. Effect of sucrose in liquid medium on bulblet formation and growth from shoot sections of *Lilium* oriental hybrid 'Casablanca' at two months after 20 ml liquid medium was added.

Sucrose (g/L)	No. of bulblets /explant	Fresh wt. /bulble t(mg)	Diameter of bulblet (mm)	Dry wt. /Fresh wt. (%)
0	1.6 b[a]	543 b	6.9 b	18.4 ab
30	2.3 a	853 ab	7.1 b	17.7 b
60	1.6 b	801 ab	8.0 b	19.0 ab
90	2.5 a	1,144 a	8.2 b	19.9 ab
120	2.2 a	1,228 a	11.0 a	19.5 ab
150	2.5 a	1,185 a	9.1 b	20.7 a

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$.

bulb scales because they already had bulblet form morphologically (Figure 1) (Matsuo et al. 1986; Maesato et al. 1994). To generate stems from *in vitro* grown bulblets in the first year, heavier bulblets of more than 1.1 g should be produced (Yae et al. 2001; Lian et al. 2002). In this experiment, the bulblets from shoot sections did not attain enough weight to produce stems in three months culture on MS medium with 60 g/L sucrose and 2 g/L activated charcoal. Therefore, liquid medium was added to the same vessels to stimulate bulblet growth.

After shoot sections were cultured on medium with 60 g/L sucrose and 2 g/L activated charcoal for two months, a 20 ml liquid medium containing various concentrations of sucrose was added (Table 2). Two months later, bulblet formation and growth were improved with increased sucrose concentrations. Bulblet weight and the ratio of dry and fresh weight (DW/FW) increased remarkably on media added with 90~150 g/L external sucrose, and diameter of bulblet was maximum on the medium added with 120 g/L external sucrose. Therefore, adding liquid medium with 120 g/L sucrose promoted further bulblet growth.

Sucrose is one of the important factors influencing bulblet growth in *Lilium* species (Takayama and Misawa 1979; Van Aartrijk and Blom-Barnhoorn 1980; Nhut et al. 2001). In this report, bulblet growth was stimulated by external sucrose addition. This might be due to increased carbohydrate and starch contents in bulblets with increasing sucrose concentration in the medium (Van Aartrijk and Blom-Barnhoorn 1980; Gerrits et al. 1997; Lian et al. 2002).

The effects of MS salts, when added to the liquid medium, were also examined. After two months, bulblets were formed and grew remarkably well on the medium added with MS full salts. Number of bulblets, their fresh weight, and rate of DW/FW increased significantly after MS full salts were added (Table 3).

The MS medium strength has not influenced bulblet formation even as the MS salt concentration was increased. With increased strength of MS medium, however, the growth of bulblets was stimulated. Hence, bulblet weight, its diameter, and the ratio of DW/FW also increased (Table 4).

Based on previous results, the volumes of liquid medium

Figure 1. Shoot cluster proliferated from shoot section (7 mm × 15 mm) on MS medium containing 2.0 mg/L BA and 0.5 mg/L IAA.

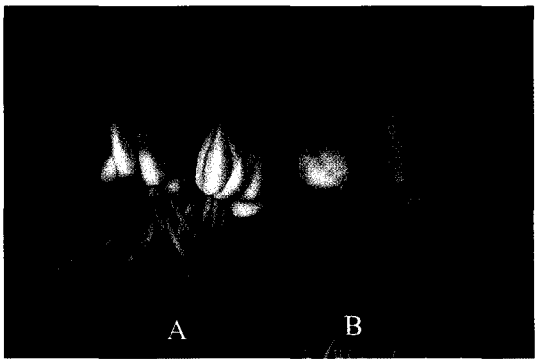

Figure 2. Comparison of bulblet growth from bulb scales (A) and bulblet sections (B) on MS medium with 60 g/L sucrose and 2 g/L activated charcoal after three months in culture.

Table 3. Effect of MS salts in liquid medium on bulblet formation and growth from shoot sections of *Lilium* oriental hybrid 'Casablanca' at two months after 20 ml liquid medium containing 120 g/L sucrose was added.

MS salts	No. of bulblets /explant	Fresh wt. /bulblet (mg)	Diameter of bulblet (mm)	Dry wt. /Fresh wt. (%)
Distilled water	1.5 b[a]	912 b	8.6 b	21.0 c
MS macro elements	1.6 b	1,149 ab	9.6 ab	21.7 bc
MS macro and Micro elements	2.8 a	1,238 a	11.4 a	22.8 ab
MS macro, micro and vitamines	2.4 ab	1,323 a	12.3 a	24.0 a

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$.

Table 4. Effects of MS salt strength of liquid medium on bulblet formation and growth from shoot sections of *Lilium* oriental hybrid 'Casablanca' at two months after 20 ml liquid medium containing 120 g/L sucrose was added.

MS salt strength	No. of bulblets /explant	Fresh wt. /bulblet (mg)	Diameter of bulblet (mm)	Dry wt. /Fresh wt. (%)
Distilled water	2.8 a[a]	711 c	7.0 b	18.6 b
1/2 x	2.2 a	943 b	7.8 ab	19.9 ab
1 x	1.8 a	923 bc	9.3 a	20.6 a
2 x	2.6 a	1,187 a	11.5 a	21.5 a

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$.

Table 5. Effects of the added volume of liquid medium with 120 g/L sucrose and MS double strength salts on bulblet formation and growth from shoot sections of *Lilium* oriental hybrid 'Casablanca' at two months after the addition of liquid medium.

Volume of liquid medium (ml/vessel)	No. of bulblets /explant	Fresh wt. /bulblet (mg)	Diameter of bulblet (mm)	Dry wt. /Fresh wt. (%)
0	2.6 a[a]	639 d	7.3 c	16.9 b
10	2.7 a	993 c	8.7 bc	17.7 b
15	1.7 b	1,024 c	9.8 bc	19.3 ab
20	3.1 a	1,276 ab	11.4 a	19.2 ab
25	3.1 a	1,440 a	11.3 a	20.5 a
30	2.9 a	1,370 a	10.9 ab	19.8 ab

[a]Mean separation by Duncan's multiple range test at $P \leq 0.05$.

were determined (Table 5). The formation of bulblets did not show differences in volumes of liquid medium added except the addition of 15 ml liquid medium. The weight and diameter of bulblets increased after adding 20~30 ml liquid medium. Rate of DW/FW was the highest on medium added with 25 ml liquid medium. Thus, addition of 25 ml liquid medium was generally favorable for bulblet growth (Figure 3). The bulblets grew to enough size to generate stems after adding 25 ml liquid medium containing MS double strength medium and 120 g/L (Table 5).

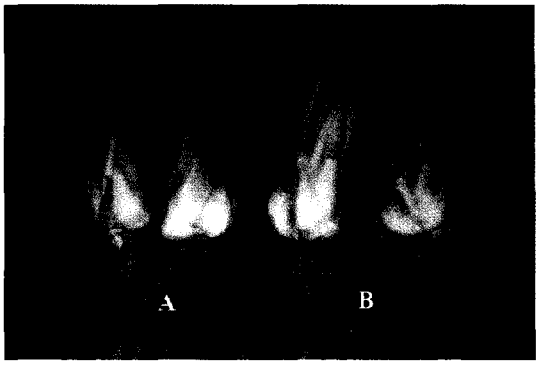

Figure 3. Comparison of bulblet grown on MS medium with 60 g/L sucrose and 2 g/L activated charcoal with (B) and without (A) the added 25 ml of liquid medium containing MS salts and 120 g/L sucrose.

Figure 4. Bulblets formed a stem with several leaves by the addition of liquid medium after cold treatment at 5℃ for three months.

After the cold treatment of *in vitro* formed bulblets at 5℃ for three months, the cultures were incubated at room temperature in dim light for one week. Most of the bulblets (about 94%) produced stems with several leaves (Figure 4).

Discussion

Shoots were induced from *in vitro* grown bulbscales of *Lilium* oriental hybrid 'Casablanca' on MS medium with 1.0 mg/L BA, 0.5 mg/L IAA, and 30 g/L sucrose. The induced

shoots were cut into shoot sections, and cultured on MS medium with 2.0 mg/L BA, 0.5 mg/L IAA and 30 g/L sucrose for shoot proliferation. The shoot sections and bulb scales were cultured to compare bulblet growth.

The culture of shoot sections stimulated bulblet growth significantly than did bulb scales because bulblet formed morphologically already (Matsuo et al. 1986; Maesato et al. 1994; Han et al. 1999a). This result also supports the report of Han et al. (1999b). They obtained heavier bulblets from shoot sections than did bulbscales. Larger bulblets mean higher survival rates and more frequent stems.

In *L.* oriental hybrid 'Casablanca', most of bulblets grown *in vitro* have failed to produce stems with several leaves during the first year of cultivation because of their smaller weight and diameter. The bulblets from shoot sections did not reach enough size to produce stems with several leaves in this experiment (Table 1). To generate stems from *in vitro* grown bulblets in the first year, heavier bulblets of more than 1.1 g should be produced *in vitro* (Yae et al. 2001; Lian et al. 2002). Therefore, liquid medium was added to the same vessels to stimulate bulblet growth. After shoot sections were cultured on medium with 60 g/L sucrose and 2 g/L activated charcoal for two months, liquid media containing various concentrations of sucrose and MS salts were added.

Two months later, bulblet formation was not influenced by adding concentrations of external sucrose, but bulblets grew remarkably after adding 25 ml liquid medium containing 120 g/L external sucrose and double strength of MS medium.

Sucrose is one of the important factors influencing bulblet growth in *Lililum* species (Takayama and Misawa 1979; Van Aartrijk and Blom-Barnhoorn 1980; Nhut et al. 2001). Takayama and Misawa (1979) found that in full strength MS medium, a linear increase in bulblet weight was observed at increasing sucrose concentration in *Lilium aurantum* and *L. speciosum*. Maximum growth of bulblets was obtained on full strength of MS medium containing 90~120 g/L sucrose.

This report revealed that bulblet growth was stimulated by external sucrose addition, possibly because of increased carbohydrate and starch content in bulblets with increasing sucrose concentration in the medium (Van Aartrijk and Blom-Barnhoorn 1980; Gerrits et al. 1997; Lian et al. 2002). In *Lilium* species, the increase of bulblet growth was maximum in the first month and gradually declined thereafter because of non-optimal and unbalanced nutrient level, water stress, and limited space (Marinangeli and Curvetto1999). In *Lilium* species, more nutrients are required for bulblet growth. Takayama and Misawa (1979) observed that bulblet growth was enhanced with increasing MS medium strength from 1/2x to 2x in *L. aurantum* and *L. speciosum*. Table 3 shows that bulblets probably required full MS salts for their growth. Han et al. (1999c) reported similar result that bulblet growth was more effective on MS medium rather than those of changing MS salt concentrations. In many studies of *Lilium* species, low concentration of MS medium and sucrose generally stimulated formation of bulblets, but the bulblet growth required high concentrations of MS medium and sucrose (Takayama and Misawa 1979; Van Aartrijk and Blom-Barnhoorn 1980; Gerrits et al. 1997). This leads to the production of large bulblets with stem. As such, this can produce bulblets that grow rapidly in soil, and increase the frequency of large commercial bulbs in a short time.

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