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Production of Commercial *Lilium rubellum* Baker Bulbs: Effects of Volume and Renewal of Liquid Medium on In Vitro Growth, Bulb Rot Infection during Cold Treatment, and Post-in-vitro Growth of Bulblets

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Summary

Bulblets of *Lilium rubellum* Baker were cultured for 16 weeks in a 20-, 30- or 40-ml liquid medium renewed 0, 1, or 3 times. The bulblets were assessed for growth and rot infection.

1. Bulblets with fresh weight of more than 600 mg were obtained by using a 20- or 30-ml medium with 3 renewals, or 40 ml with one renewal.
2. Bulblets frequently turned brown when they were cultured in a 30- or 40-ml medium with 3 renewals; they frequently rotted both during a cold treatment and after transplantation.
3. Bulblets cultured in a 20-ml medium did not rot after a cold treatment irrespective of the number of medium renewals; they grew well after transplantation to the greenhouse.
4. Bulblets cultured in a 20-ml medium with 3 renewals most frequently developed into plantlets with elongated axes 15 weeks after transplantation.

These results indicate that the 20-ml medium with 3 renewals appears to be most suitable for bulblet culture of *L. rubellum* in a liquid medium.

Introduction

We examined ways to achieve an efficient production of commercial *Lilium rubellum* Baker bulbs in vitro (Niimi, 1984, 1985; Niimi et al., 1988; Niimi and Saito, 1990). In a previous paper (Niimi and Saito, 1990), we reported that bulblets cultured in a liquid medium grew better but tended to rot both during a cold treatment and after transplantation to the field, compared with those cultured on an agar-solidified medium. In this study, the optimum volume and frequency of renewal of the liquid medium in bulblet culture of *L. rubellum* were sought to enhance in vitro and post-in-vitro bulblet growth. Furthermore, means of preventing bulb rot infection during a cold treatment and after transplantation to the greenhouse were investigated.

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Materials and Methods

1. Preparation of bulblet explants

Bulblet explants of *L. rubellum* were obtained as follows (Niimi and Saito, 1990): bulb scales were excised from bulbs which had been cultured on a basal medium (pH 5.7) consisting of MS salts and vitamins (Murashige and Skoog, 1962), 0.1 mg·liter^{-1} NAA, 0.001 mg·liter^{-1} BA, 5% sucrose and 0.7% agar at 24±1 °C in the dark; the scales were cut into pieces and cultured on the basal medium at 24±1 °C in the dark for 4 months; regenerated bulblets, weighing 50 to 100 mg, were isolated from the bulb scales and used as explants for bulblet culture.

2. Bulblet culture in liquid medium

Ten bulblets (totally 600 to 750 mg) were cultured in a 100-ml Erlenmeyer flask containing a 20-, 30-, or 40-ml liquid basal medium. Cultures were maintained for 16 weeks at 24±1 °C in the

dark on a rotary shaker (90 rpm) with no medium renewal, with one renewal 8 weeks after the onset of culture or with 3 renewals every 4 weeks. Medium renewal was performed by removing the former medium with a culture pipette and then replenishing it with an equal volume of fresh basal medium. As a control, 10 bulblets were inoculated in a 100-ml Erlenmeyer flask containing a 40-ml basal medium solidified with 0.7% agar for 16 weeks.

After 16 weeks, the original and newly-formed bulblets were collected from culture vessels and washed with tap water. After roots were removed, the number and the fresh weight of bulblets and the percentage of browned bulblets per flask were recorded.

3. Cold treatment and transplantation of bulblets

Healthy and discolored bulblets collected from each of flasks were mixed with about 80 ml of moist vermiculite (tap water: vermiculite = 1: 4, v/v), placed in a polyethylene bag (100 × 70 × 0.04 mm), and stored at 4 °C for 12 weeks. After the cold treatment, the chilled bulblets were washed with tap water. The numbers of healthy and decayed bulblets were recorded for each flask, and the rotten bulblets were discarded.

A sample of 100 bulblets was planted 2 cm in depth to a plastic tray (33 × 48 × 10 cm) containing a synthetic soil mix of paddy soil: sand: leaf mold mix (1: 1: 2, v/v/v); each bulblet was spaced ca. 3 × 4 cm apart. The trays were transferred to a greenhouse kept at 10 to 20 °C (night) and 10 to 25 °C (day). The temperatures fluctuated, depending on the weather. Two to 3 weeks after transplantation, after most of the bulblets had sprouted, they were fertilized weekly with a solution of 500 mg·liter^{-1} Hyponex (N: P: K = 15: 30: 15). Bulblets were harvested 15 weeks after transplantation, washed with tap water, and their roots removed. The healthy bulblets were counted and weighed; the numbers of plantlets with elongated axes were recorded for each flask.

Results

1. In vitro bulblet growth

1) Number of newly formed bulblets

New bulblets (1 to 4 bulblets·flask^{-1}) were formed during in vitro culture in a liquid medium irrespective of the volume and renewal of the medium, whereas no bulblets formed on a solid medium (Table 1). The number of bulblets tended to increase with the number of medium renewals.

2) Fresh weight

The rate of gain in fresh weight was enhanced by both the volume and renewal; thus, 20- and 30-ml liquid media with 3 renewals gave 863 and 831% gain in fresh weight, respectively; the highest gain of 889%, among all treatments, was obtained in 40 ml of the medium with 1 renewal (Table 1).

3) Bulblet browning

Bulblets, cultured for 16 weeks on the solid medium, did not brown, but those in the liquid medium browned to various extents (Table 1). The percentage of browned bulblets was relatively low when bulblets were cultured in 20 ml, irrespective of renewal of the medium (Fig. 1A) or in 30 ml without renewal; whereas bulblets cultured in the 30- or 40-ml medium with 3 renewals frequently browned (Fig. 1B). Cultures in which bulblet turned brown frequently had an alcoholic odor.

4) Weight classes of bulblets

Bulblets, weighing 300 to 599 mg, were most frequently obtained in all cultural treatments except for those on the solid medium (control); and in the 20-ml medium without renewal, bulblets weighed between 100 to 299 mg (Table 2).

In a 20- or 30-ml liquid medium, the number of bulblets weighing 600 mg or more increased with the number of renewals; especially with 3 renewals. In the 40-ml medium, bulblets weighing 600 mg or more were most frequently obtained with 2 renewals.

2. Bulb rot infection during cold treatment and post-in-vitro growth of bulblets

1) Bulb rot during cold treatment

No bulblets which had been cultured on the solid medium and in the 20-ml liquid medium rotted during a cold treatment (Table 3). However, bulblets cultured in a 30- or 40-ml medium with 1 or 3 renewals rotted frequently; of the bulblets derived from 30 and 40 ml of the medium with 3 renewals, 31 and 44% rotted, respectively.

2) Bulblet growth after transplantation

(1) Percent gain in fresh weight and bulblet rot

Bulblets which had been cultured on the solid medium all grew well after transplantation (Table

Table 1. Effects of medium volume and renewal frequency on growth of *Lilium rubellum* bulblets cultured for 16 weeks in liquid medium[z].

Medium		At inoculatin		At the end of culture			
volume (ml)	renewal[y] frequency	number of bulblets (flask^{-1})	fresh weight (mg·flask^{-1})	number of bulblets (flask^{-1})	fresh weight (mg·flask^{-1})	gain in fresh weight[x] (%)	bulblet browning[w] (%)
20	0	10	688±17[v]	10.9	2891	421	5
	8w-1	10	673±22	12.3	4296	642	2
	4w-3	10	673±12	13.9	5799	863	0
30	0	10	636±10	11.5	3670	578	1
	8w-1	10	643±15	11.9	5277	822	9
	4w-3	10	687±20	13.3	5674	831	50
40	0	10	658±18	11.0	4138	633	4
	8w-1	10	666±13	12.1	5892	889	15
	4w-3	10	684±22	11.3	4287	631	72
Solid medium[u]		10	663±17	10.0	1945	341	0
Significance							
Volume of the liquid medium (V)				NS	NS	*	**
Renewal of the liquid medium (R)				*	**	**	**
V×R				NS	**	**	**

[z] Values represent the mean of 8 flasks for liquid culture and 5 flasks for solid one.
[y] 0, no renewal; 8w-1, one renewal 8 weeks after the onset of culture; 4w-3, 3 renewals every 4 weeks.
[x] (fresh weight of bulblets at inoculation/fresh weight of bulblets at the end of culture) ×100.
[w] (number of browned bulblets at the end of culture/number of cultured bulblets) ×100.
[v] Mean±SE
[u] Bulblets were cultured on agar-solidified medium for 16 weeks without medium renewal.
NS, *, ** indicate nonsignificant and significant at $P=0.05$ and 0.01, respectively.

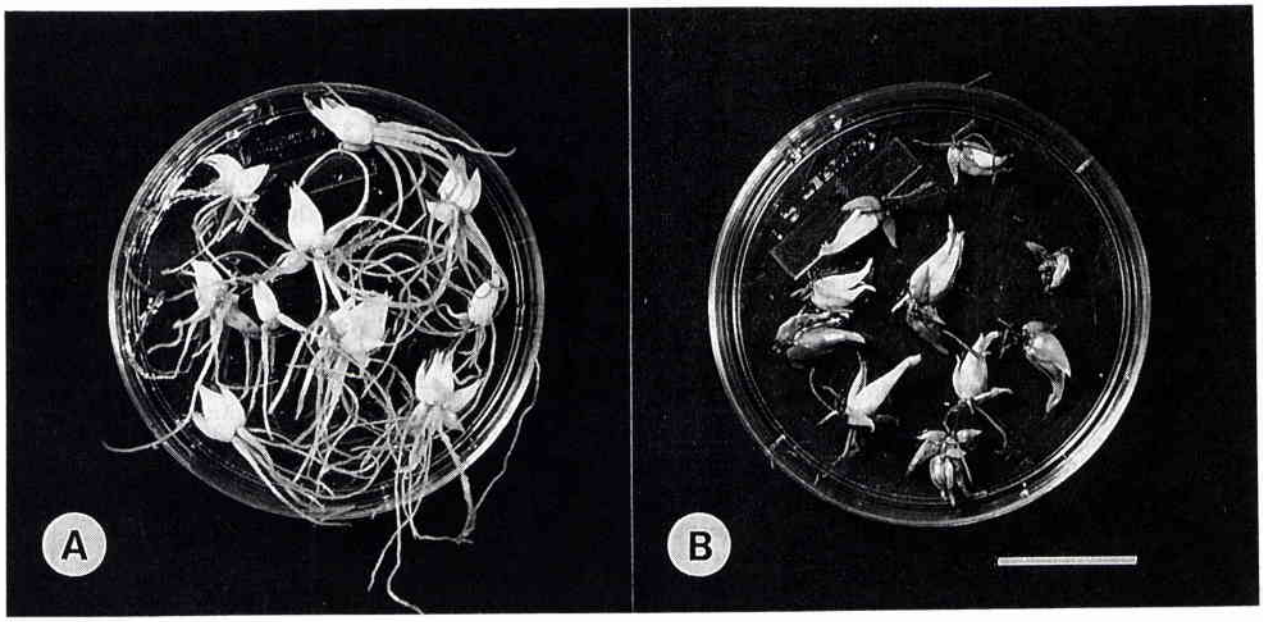

Fig. 1. Bulblets cultured for 16 weeks in 20 ml of liquid medium with 3 renewals-(A) and in 40 ml with 3 renewals-(B). Bar=3 cm.

Table 2. Effects of medium volume and renewal frequency on the number of bulblets belonging to each weight class 16 weeks after the onset of bulblet culture of *Lilium rubellum* in liquid medium.

Medium		Total number of bulblets analyzed	Weight classes of bulblets[y]				
volume (ml)	renewal[z] frequency		~99 mg	100~ 299 mg	300~ 599 mg	600~ 999 mg	1,000 mg~
20	0	94	7 (7)	52 (55)	25 (27)	10 (11)	0 (0)
	8w-1	109	9 (8)	34 (31)	48 (44)	18 (17)	0 (0)
	4w-3	124	7 (6)	28 (23)	50 (40)	35 (28)	4 (3)
30	0	92	5 (5)	32 (35)	48 (52)	7 (8)	0 (0)
	8w-1	95	3 (3)	25 (26)	42 (48)	11 (13)	5 (3)
	4w-3	96	7 (7)	21 (22)	40 (42)	24 (25)	4 (4)
40	0	88	8 (9)	25 (28)	42 (48)	11 (13)	2 (2)
	8w-1	87	4 (5)	20 (23)	31 (36)	29 (33)	3 (3)
	4w-3	90	4 (5)	35 (39)	35 (39)	13 (14)	3 (3)
Solid medium[x]		50	10 (20)	34 (68)	6 (12)	0 (0)	0 (0)

[z] Abbreviations are the same as in Table 1.
[y] Parenthesized figures represent the percentage of bulblets belonging to each class of total bulblets analyzed.
[x] Bulblets were cultured on agar-solidified medium for 16 weeks without medium renewal.

Table 3. Effects of medium volume and renewal frequency in bulblet culture on rot infection during cold treatment and cultivation in soil[z].

Medium		Number of cold-treated bulblets (flask^{-1})	At the end of cold treatment		After 15 weeks of transplantation		
volume (ml)	renewal[y] frequency		number of healthy bulblets (flask^{-1})	rotten bulblets[x] (%)	number of healthy bulblets (flask^{-1})	gain in fresh weight[w] (%)	rotten bulblets[v] (%)
20	0	10.9±0.4[u]	10.9±0.4	0	10.9±0.4	260	0
	8w-1	12.3±0.5	12.3±0.5	0	12.3±0.5	287	0
	4w-3	13.9±0.8	13.9±0.8	0	13.4±0.7	286	2
30	0	11.5±0.3	11.1±0.3	3	10.9±0.3	282	2
	8w-1	11.9±0.7	10.8±0.9	10	10.0±0.9	273	8
	4w-3	13.3±1.2	9.5±1.5	31	5.9±1.5	161	34
40	0	11.0±0.3	10.0±0.6	10	9.0±1.3	227	13
	8w-1	12.1±0.7	10.4±1.1	15	9.1±0.9	239	11
	4w-3	11.3±0.4	6.3±0.7	44	2.0±0.6	78	76
Solid medium[t]		10.0±0	10.0±0	0	10.0±0	279	0
Significance							
Volume of the liquid medium (V)				**		**	**
Renewal of the liquid medium (R)				**		**	**
V×R				**		**	**

[z] Ten bulblets were initially inoculated to each flask and cultured for 16 weeks in liquid medium. After cold treatment for 12 weeks, only healthy bulblets were transferred to pots and grown in the greenhouse. Values respresent the mean of 8 and 5 flasks for cultures using liquid and solid media, respectively.
[y] Abbreviations are the same as in Table 1.
[x] (number of healthy bulblets at the end of cold treatment/number of cold-treated bulblets) ×100.
[w] (fresh weight of healthy bulblets at transplantation/fresh weight of healthy bulblets 15 weeks after transplantation) ×100.
[v] (number of healthy bulblets 15 weeks after transplantation/number of healthy bulblets at the end of cold treatment) ×100.
[u] Mean±SE
[t] Bulblets cultured for 16 weeks on agar-solidified medium without medium renewal were used.
NS, *, ** indicate nonsignificant and significant at $P=0.05$ and 0.01, respectively.

Table 4. Effects of medium volume and renewal frequency in bulblet culture on the number of healthy bulblets belonging to each weight class and of those with elongated axes 15 weeks after transplantation in *Lilium rubellum*[z].

Medium		Total number of healthy bulblets analyzed	Weight classes of bulblets[x]					Bulblets with elongated axes
volume (ml)	renewal[y] frequency		~99 mg	100~ 299 mg	300~ 599 mg	600~ 999 mg	1,000 mg~	
20	0	94	3 (3)	15 (16)	28 (30)	32 (34)	16 (17)	3 (3)
	8w-1	109	2 (2)	10 (9)	8 (7)	30 (28)	59 (54)	11 (10)
	4w-3	120	2 (2)	7 (6)	16 (13)	15 (12)	80 (67)	37 (31)
30	0	88	0	4 (5)	17 (19)	24 (27)	43 (49)	2 (2)
	8w-1	85	1 (1)	5 (6)	11 (13)	8 (9)	60 (71)	10 (12)
	4w-3	66	0	7 (11)	11 (17)	12 (18)	36 (55)	14 (22)
40	0	76	0	6 (8)	9 (12)	19 (25)	42 (55)	8 (11)
	8w-1	76	0	9 (12)	9 (12)	11 (14)	58 (76)	13 (17)
	4w-3	25	0	2 (8)	5 (20)	2 (8)	16 (64)	0
Solid medium[w]		50	2 (4)	13 (26)	21 (42)	9 (18)	5 (10)	1 (2)

[z] Ten bulblets were initially inoculated to each flask and cultured in liquid medium. After cold treatment, healthy bulblets were transferred to pots and grown in the greenhouse.
[y] Abbreviations are the same as in Table 1.
[x] Parenthesized figures represent the percentage of bulblets belonging to each class or those with elongated axes of total bulblets.
[w] Bulblets cultured on agar-solidified medium without medium renewal were used.

3). Similarly, bulblets which had been cultured in a 20-ml liquid medium with or without renewals scarcely rotted during cultivation in soil. Bulblets gained 287 and 286% fresh weight in the 20-ml liquid medium with 1 and 3 renewals, respectively. Bulblets which had been cultured in a 30- or 40-ml liquid medium with 3 renewals rotted with high frequencies; in particular, 76% of bulblets, cultured in the 40-ml liquid medium with 3 renewals, rotted.

(2) Weight classes of bulblets and plant development

In the 20-ml medium treatment, the percentage of bulblets weighing 1,000 mg or more increased in a 20-ml liquid medium as the number of medium renewals increased; 31% of bulblets, cultured in the 20- ml medium with 3 renewals developed into the plantlets with elongated axes (Table 4).

Bulblets weighing 1,000 mg or more were also obtained in the treatment of 30- or 40-ml medium with 1 or 3 renewals, but the numbers were low because many bulblets rotted after transplantation in soil (Table 4).

Discussion

That growth of bulblets is stimulated by culturing them in a liquid medium was reported for *L.* × 'Oriental hybrids'(Ishiba et al., 1992), *L. auratum* (Takayama and Misawa, 1983a, b), *L. japonicum* (Haruki and Yamada, 1992; Kawarabayashi, 1993 a, b), *L. longiflorum* (Takayama and Misawa, 1983 b; Takayama and Takizawa, 1994) and *L. speciosum* (Takayama and Misawa, 1983b). Niimi and Saito (1990) confirmed not only that the liquid culture system is superior to the solid culture system in enhancing bulblet growth in *L. rubellum*, but they also found that bulblets cultured in 40 ml of the liquid medium were prone to rot during the cold treatment and subsequent cultivation in soil. This study indicates that the rot problem can be overcome by a careful modification of medium volume and renewal of the liquid culture system. Thus when 10 bulblets, each weighing 50 to 100 mg, were cultured for 16 weeks in an Erlenmeyer flask, containing a 20-ml medium with 3 renewals every 4 weeks, bulblet rot was minimized and in vitro growth of bulblets was enhanced. However, it should be noted that this optimum condition may depend on the initial number and weight of bulblets, as Kato et al. (1994) has already reported that an initial inoculum concentration affects subsequent bulblet growth in a liquid culture system of *L.* × Oriental hybrids.

Our study revealed that there is a correlation between in vitro browning of bulblets and their rotting during the cold treatment and cultivation

in soil. Bulblets in treatments which browning occurred, rotted with high frequencies (Tables 1 and 3). This indicates that in vitro browning may account for bulblet rotting and that prevention of browning appears to be one of the critical challenges for establishing an efficient bulblet culture system using a liquid medium in *L. rubellum*. We can conclude that the culture system, using a 30- or 40- ml liquid medium with renewal, induced browning and therefore should be avoided for in vitro bulblet production of *L. rubellum*. Because these bulblets are often submerged, the browning may be attributed to the lack of oxygen. An alcoholic order, emitted by cultures in which bulblets frequently browned, appears to be related to this condition.

Kawarabayashi (1993a) reported that growth of *L. japonicum* bulblets in a liquid medium was best when it contained a 3/4-strength MS inorganic salts; the frequency of bulblet browning increased with the strength of inorganic salts in the medium. That sucrose concentration of liquid media affects bulblet growth in several *Lilium* species has been reported by Niimi and Saito (1990), Ishiba et al. (1992) and Kato et al. (1994). Niimi and Saito (1990) reported that 5% sucrose stimulated in vitro growth of bulblets in *L. rubellum* more than 7% sucrose did. Therefore, growth and browning of bulblets seem to be related to total amounts of available inorganic salts or sucrose in the liquid medium. That bulblets cultured in the 30- or 40-ml liquid medium with 3 renewals frequently browned is attributed to the excess of inorganic salts or sucrose. Further studies are needed to fully support our postulation that the lack of oxygen or the excess of inorganic salts or sucrose or both leads to the browning and subsequent rotting of *L. rubellum* bulblets.

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ヒメサユリの成球生産に関する研究

液体培地の量と更新が子球の試験管内での生長，低温処理中の腐敗および移植後の生育に及ぼす影響

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摘　要

ヒメサユリ（*Lilium rubellum* Baker）子球の培養における液体培地の量（20，30，40 ml）および更新（0，1，2回）が子球の生長および腐敗に及ぼす影響を調査した．

1．子球の新鮮重増加率は培地の量と更新の両方に影響され，培地量が20および30 mlの場合は更新を3回行った際に増加率が最も高かったが，培地量40 mlの場合は1回の更新で最も高かった．新鮮重が600 mg以上の子球数は，培地量が20あるいは30 mlで3回の更新を行った場合に最も多く得られた．

2．培養中の子球の褐変は培地量が30および40 ml，培地3回の更新の場合に多く観察された．

3．20 mlの液体培地中で培養された子球では，培地の更新に関係なく低温処理中の腐敗は観察されず，温室内での生長も良好であった．一方，子球を30あるいは40 mlの培地で培養し3回の更新を行った場合には，低温処理中および温室に移植した後に子球の腐敗が高頻度で観察された．

4．移植15週間後の地上型植物は，20 mlの液体培地を3回更新した培養区で最も多く得られた．

以上の結果から，ヒメサユリの子球培養においては，液体培地量を20 mlとし，培地更新を3回行う方法が適していると考えられた．