園学雑. (J. Japan. Soc. Hort. Sci.) 69 (2) : 161 - 165. 2000.

Production of Commercial Bulbs of *Lilium rubellum* Baker: Changes in Carbohydrates in Bulblets and Sugars of Liquid Medium during their Culture

Yoshiji Niimi, Yutaka Misaki and Masaru Nakano
Faculty of Agriculture, Niigata University, 2 − 8050 Ikarashi, Niigata 950 − 2181

Summary

1. Bulblets of *Lilium rubellum* were cultured for 16 weeks in liquid MS medium containing 100, 150 and 250 mmol · liter^{-1} (mM) sucrose (referred to as 100, 150 and 250 mM sucrose medium, respectively). The 250 mM sucrose medium enhanced bulblet growth the most. The greatest gain in fresh weight occurred between 4 and 8 weeks after culture. The ratio of dry to fresh weights (DW/FW) of bulblets cultured in the 250 mM sucrose medium was always highest.

2. Three sugars (sucrose, glucose, and fructose) were detected in the autoclaved MS medium. When bulblets were cultured in the liquid sucrose medium, the concentration of glucose and fructose in the medium increased until week 4 as the sucrose concentration decreased. Sugars in the 150 mM sucrose medium were nearly depleted by week 8, whereas those in the 250 mM sucrose medium persisted for 12 weeks. The growth rate of the bulblets declined as sugar concentration in the medium decreased.

3. The sucrose content in bulblets, cultured in each medium, increased until week 4, then decreased. However, in bulblets, cultured in the 250 mM sucrose medium, the sugar content increased again after 12 weeks of culture, while starch content decreased. Hence, it appears that the starch accumulated in the bulblets was hydrolyzed to glucose.

These results indicate that the growth of bulblets of *L. rubellum* cultured in liquid medium can be promoted by subculturing the bulblets after 8 to 12 weeks of culture.

Key Words: bulblet culture, *Lilium rubellum*, liquid medium, sugars.

Introduction

We previously reported that the growth of in vitro cultured bulblets of *Lilium rubellum* Baker was better promoted in liquid medium than on a solid medium, both containing 5 % sucrose (Niimi and Saito, 1990), whereas bulblets of *L. auratum* developed best on a solid medium with 9 % sucrose (Takayama and Misawa, 1979), and those of *L. japonicum* grew best in liquid medium with 3 % glucose (Haruki et al., 1996). These results show that the type and concentration of carbohydrates suitable for bulblet growth depend on the type of *Lilium* spp. and/or culture conditions. Niimi et al. (1997) also reported that the renewal of liquid medium with 5 % sucrose at 4-week intervals enhanced growth of *L. rubellum* bulblets cultured for 16 weeks. Thus, the results indicate that the initial amount of sucrose added to the medium might be insufficient for the growth of bulblets. The purpose of this study was to examine the changes in carbohydrates of bulblets in sugars in the culture medium and to determine the most suitable time for subculturing the bulblets to improve their growth.

Received; May 12, 1999; Accepted; August 20, 1999.
The present study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan (No. 09460017). A part of this study was presented at the 1996 Autumn Meeting of the Japan. Soc. Hort. Sci.

Materials and Methods

1. Effect of sucrose concentration on bulblet growth

1) Preparation of bulblet explants

Bulblet explants of *L. rubellum* Baker were obtained as follows (Niimi and Saito, 1990): bulb scales were excised from bulbs, cultured on a basal medium (pH 5.7) consisting of MS salts, glycine, and vitamins (Murashige and Skoog, 1962) (MS medium), supplemented with 0.15 mol · liter^{-1} (M) sucrose, 7 g · liter^{-1} agar, 0.45 μmol · liter^{-1} (μM) NAA and 4.4 nmol · liter^{-1} (nM) BA at 24 ± 1℃ in the dark. The excised scales were cultured on a solid basal medium at 24 ± 1℃ in the dark for several months. The regenerated bulblets, weighing 30 to 60 mg, were isolated from the bulb scales and cultured in a liquid basal medium.

2) Bulblet culture in a liquid medium containing sucrose at different concentrations

Isolated bulblets were cultured in a liquid basal medium, supplemented with 0.45 μM NAA, 4.4 nM BA, and 100, 150 or 250 mmol · liter^{-1} (mM) sucrose

(they are referred to as 100, 150, or 250 mM sucrose medium in this text). The osmotic pressures of the 100 and 150 mM sucrose media were adjusted to that of the 250 mM sucrose medium by the addition of 150 and 100 mmol · liter^{-1} (mM) mannitol, respectively, before autoclaving. Twenty ml of liquid medium was poured into a 100 ml Erlenmeyer flask and the flasks, capped with aluminum foil, were autoclaved for 10 minutes at 121 ℃ under a pressure of 1.2 kg · cm^{-2}.

Ten bulblets, weighing a total of 400 to 450 mg, were cultured in a 100 ml Erlenmeyer flask on a gyratory shaker (90 rpm) for 8 weeks at 24 ± 1 ℃ under continuous illumination (35 μmol · m^{-2} · s^{-1}) with white fluorescent lamps. After an 8-week culture, the original and newly-formed bulblets were collected from 6 culture-vessels and washed with tap water. The number and fresh weights of bulblets per flask were recorded after their roots were excised. Means were compared by Duncan's test.

2. Changes in carbohydrates of bulblets and in sugar concentration of liquid medium during bulblet culture

Ten bulblets, weighing a total of 400 to 450 mg, were cultured as above in a 150 mM or 250 mM sucrose medium for 16 weeks. Three flasks were collected randomly from among those in each medium at 4-week intervals. After newly-formed bulblets, roots, and scaly leaves were removed from each bulblet, the fresh and dry weights of bulblets per flask and the osmolality of each liquid medium were recorded. Data were subjected to analysis of variance. The number of scales per bulblet was also determined at 8 and 16 weeks after culture.

1) Analysis of carbohydrates in bulblets

Bulblets, cultured in each medium, were heated at 110 ℃ for 30 minutes, after which they were dried at 60 ℃ for 3 days in a forced-draught oven. The dry weight was then measured. The dried bulblets were pulverized and the powders were stored in a desiccator with silica gel at room temperature until they were analyzed.

Carbohydrate extraction and analysis were done according to methods described by Ohyama et al. (1986). A 200 mg sample of bulblets, with 10 mg sorbitol as an internal standard, was extracted with 80 % ethanol; the residues were washed twice with 80 % ethanol. The extract and the washings were then combined and brought to volume. A 5 ml aliquot was dried under vacuum at 60 ℃ , re-dissolved in 500 μl distilled water, and then filtered through a membrane filter (pore size, 0.2 μm). The sugar components were measured by a HPLC system (Hitachi L-6000), equipped with a refractive index detector (Shodex RI-71). A 5 μl sample was injected onto a Shodex SUGAR SP 0810 column, operated at a flow rate of 1 ml · min^{-1} at 80 ℃ with degassed distilled water as the mobile phase. The concentration of individual sugars was calculated from known peak areas of standard sugars.

The starch content of the residues was estimated according to Ohsaki (1989); starch was extracted by perchloric acid and diluted 100 times with distilled water, and the absorbance values of glucose at 630 nm were determined with the anthrone-sulphuric acid method.

2) Changes in sugar concentration and osmolality in liquid medium during culture

A test solution of 800 μl liquid culture medium was mixed in an Ependorf tube with a 200 μl sorbitol solution at a concentration of 25 g · l^{-1} as an internal standard and filtered through a membrane filter (pore size, 0.2 μm). The filtrate of 5 μl was injected into HPLC for analysis of sugars in the same way as described above.

The osmolality of the liquid medium was measured by a Vogel osmometer OM-801 (Asahi Life Science Corp.).

Results

1. Growth of bulblets cultured in medium, containing sucrose at different concentrations

Of the three media, bulblet growth was enhanced most in the 250 mM sucrose medium. The ratio of dry to fresh weights (DW/FW) was also higher in the bulblets cultured in 250 mM sucrose medium. One to 2.4 bulblets per flask developed from the original bulblets cultured in each medium (Table 1).

2. Bulblet growth, changes in sucrose concentration in liquid medium and in carbohydrates in bulblets cultured in 150 mM and 250 mM sucrose media

1) Bulblet growth

The fresh weight of bulblets continuously increased until week 16, with the highest gain in fresh weight occurring between week 4 and 8. The DW/FW value in bulblets, cultured in 250 mM sucrose medium, was higher at all times than cultured in 150 mM medium. The highest value was observed at week 4, after which it gradually decreased (Fig. 1).

The number of scales per bulblet cultured in 150 mM and 250 mM sucrose media for 8 weeks was less than six. The number increased with time in those bulblets cultured in 250 mM sucrose medium, but it increased a little in those cultured in 150 mM sucrose medium (Fig. 2).

2) Sucrose concentration and osmolality in medium

Three sugars (sucrose, glucose, and fructose) were detected in autoclaved liquid medium. The concentrations of glucose and fructose initially increased for 4 weeks after the bulblets were cultured in the liquid medium, while sucrose concentration decreased. Almost all sugars in the 150 mM sucrose medium were depleted by week 8, and those in the 250 mM by week 12 (Fig. 3).

Table 1. Effect of sucrose concentration on growth of *Lilium rubellum* bulblets cultured for 8 weeks in liquid media[z].

Sucrose concentration (mM)	At inoculation		At the end of culture			
	Number of bulblets	Fresh weight ($g \cdot flask^{-1}$)	Number of bulblets	Fresh weight ($g \cdot flask^{-1}$)	Gain in fresh weight (%)[y]	DW/FW
100	10	0.4a	12.4a	1.78c	442c	0.29b
150	10	0.4a	11.0a	2.21b	557b	0.30b
250	10	0.4a	11.0a	3.12a	784a	0.35a

[z] Ten bulblets, which were isolated from bulb scales cultured on a solid basal medium for several months, were transferred into each flask containing a basal medium supplemented with 100, 150, and 250 mM sucrose. Values represent the mean for 6 flasks. Means in the same column followed by the same letter are not significantly different ($P < 0.05$; Duncan's test).
[y] (fresh weight at the end of culture/fresh weight at inoculation) × 100.

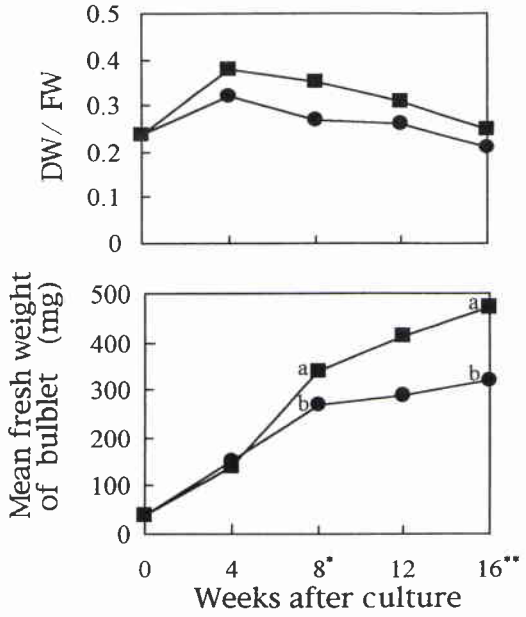

Fig. 1. Cumulative increase in fresh weight (lower) and the change in DW/FW ratio (upper) in bulblets cultured in a liquid basal medium containing 150 mM (●) or 250 mM (■) sucrose for 16 weeks. Different letters indicate significant differences by Duncan's test (*, ** indicate significance at $P < 0.05$ and 0.01, respectively).

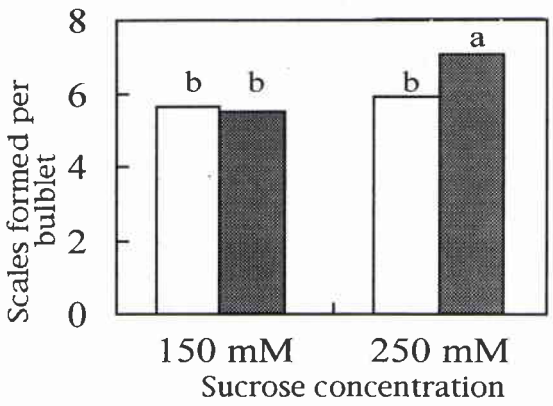

Fig. 2. Scales initiated per bulblet cultured in a liquid basal medium containing 150 mM or 250 mM sucrose. □, 8-week cultured bulblets. ■, 16-week cultured bulblets. Different letters indicate significant differences by Duncan's test at the $P < 0.05$ level.

The osmolality of the autoclaved medium was 290 $mOsm \cdot kg^{-1}$ in 150 mM sucrose medium and 425 $mOsm \cdot kg^{-1}$ in 250 mM sucrose medium. These values decreased rapidly in both media between 4 and 8 weeks after culture, and the depletion rate was particularly evident in the 250 mM sucrose medium (Fig. 3).

3) Carbohydrates in bulblets

Generally, the sucrose and starch contents of bulblets cultured in the 250 mM sucrose medium were higher than those of bulblets cultured in the 150 mM medium. The sucrose content in bulblets cultured in each medium increased until week 4, and then decreased. The bulblets cultured in the 250 mM sucrose medium, however, showed a statistically significant increase in sucrose content after 12 weeks of culture (Fig. 4). The concentrations of glucose and fructose in the bulblets were almost constant throughout the entire culture period. There were few statistically significant differences between the starch contents of bulblets in each medium at each measurement time. The starch content of bulblets cultured in 150 mM medium was nearly constant for 8 weeks and then gradually decreased, whereas that of bulblets cultured in 250 mM sucrose medium increased until week 4 and then gradually decreased.

Discussion

The aim of these experiments was to gain more information about the changes in sugars in liquid medium and in carbohydrates in bulblets of *L. rubellum* to enhance their growth. The bulblet growth was stimulated more in the medium containing 250 mM sucrose (ca. 9%) than in the medium containing 150 mM sucrose (ca. 5%) (Fig. 1). This is attributed to the disappearance of almost all sucrose in the 150 mM sucrose medium at week 8 after culture. The growth of bulblets of *L. auratum* (Takayama and Misawa, 1979) and potato tubers (Garner and Blake, 1989) was stimulated at a concentration of 9% sucrose. We previously reported that 5 % sucrose was optimum for the growth of bulblets *L. rubellum* (Niimi and Saito, 1990). The discrepancies

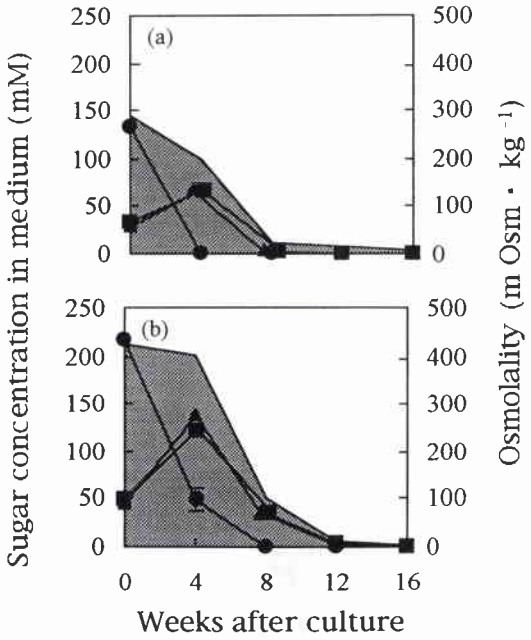

Fig. 3. Changes in sugar concentration and osmolality in (a) 150 mM and (b) 250 mM sucrose media in which bulblets were cultured for 16 weeks. ●, sucrose. ■, glucose. ▲, fructose. The shaded part indicates the values of osmolality. The bars represent SE.

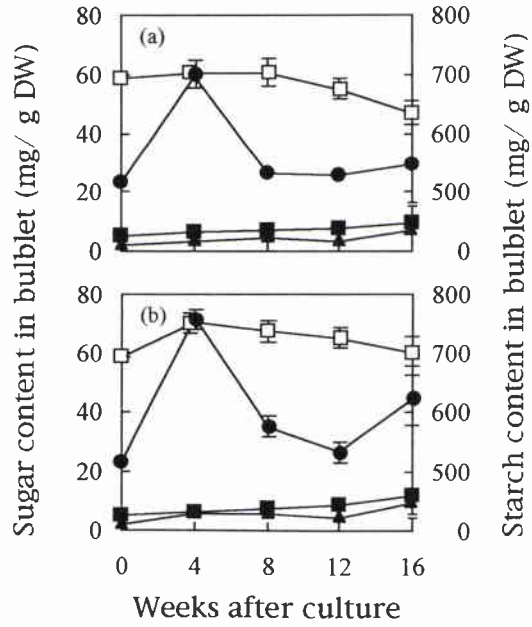

Fig. 4. Changes in carbohydrate content in bulblets cultured in (a) 150 mM and (b) 250 mM sucrose media for 16 weeks. ●, sucrose. ■, glucose. ▲, fructose. □, starch. The bars represent SE.

between these findings might be explained by differences in cultured plants, the concentrations of plant growth regulators, and cultural conditions, such as numbers and sizes of explants, culture vessels, light intensity, temperature, and rotation rate of the shaker. It appears that these factors affect the uptake and utilization of sugars by explants. Accordingly, it could be concluded that the sucrose concentration suitable for in vitro bulblet growth of *Lilium* spp. depends not only on plant species but also on various cultural conditions.

Autoclaved liquid basal medium with sucrose consisted of sucrose, glucose, and fructose (Fig. 3), indicating that the latter two sugars occurred during autoclaving, as indicated by Stehsel and Caplin (1969), whereas those which increased in the medium until week 4 might be attributed to the hydrolysis of sucrose. This hydrolysis via invertase and/or other enzymes occurred in tomato root culture (Weston and Street, 1969) and carrot cells (Kanabus et al., 1986). The latter showed that carrot cells use hexose, particularly glucose, released from sucrose by the action of extracellular invertase, as a source of carbon. We were unable to demonstrate whether the two hexoses are released by extracellular- or intracellular invertase.

When *L. rubellum* bulblets were cultured for 16 weeks, sugars almost disappeared from the 150 mM sucrose medium by week 8 and from the 250 mM sucrose medium by week 12 (Fig. 3), indicating that it is necessary for medium renewal to sustain the bulblet growth (Fig. 1 and 4). Sugars in the 250 mM sucrose medium were nearly depleted by week 12 after the beginning of culture (Fig. 3-b), after which the sucrose content in the bulblets increased again. The increase of the sucrose content might be attributed to starch hydrolysis in the bulblets (Fig. 4), which coincides with the decreases in the growth rate of bulblets and the value of DW/FW (Fig. 1).

These experimental results which revealed that the growth reduction of *L. rubellum* bulblets by the depletion of sugars in the liquid basal medium indicate that growth might be sustained by nutrient renewal (Niimi et al., 1997). Such a change would stimulate and maintain the growth of bulblets of *L. rubellum*, particularly if they are cultured for more than 8 weeks.

Literature Cited

Garner, N. and J. Blake. 1989. The induction and development of potato microtubers in vitro on media free of growth regulating substances. Ann. Bot. 63: 663-674.

Haruki, K., K. Yamada, T. Hosoki and K. Ohta. 1996. Effect of sugars and temperature on the growth of miniature bulbs of *Lilium japonicum* Thunb. cultured on a rotary shaker. J. Japan. Soc. Hort. Sci. 65: 363-371 (In Japanese with English summary).

Kanabus, J., R. A. Bressan and N. C. Carpita. 1986. Carbon assimilation in carrot cells in liquid culture. Plant Physiol. 82: 363-368.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Niimi, Y. and I. Saito. 1990. Production of bulbs of *Lilium rubellum* Baker. An attempt to improve in vitro growth of bulblets regenerated from cultured bulb scales. J.

Japan. Soc. Hort. Sci. 59: 635－640 (In Japanese with English summary).
Niimi, Y., M. Nakano and S. Saito. 1997. Production of commercial *Lilium rubellum* Baker bulbs: Effects of volume and renewals of liquid medium on in vitro growth, bulb rot infection during cold treatment, and post－in vitro growth of bulblets. J. Japan. Soc. Hort. Sci. 66: 113－119.
Ohsaki, M. 1989. Analysis of Starch. p. 204－208. In: Editorial Committee of Experimental Methods of Plant Nutrition (eds.). Experimental methods of plant nutrition. Hakuyusha, Tokyo (In Japanese).
Ohyama, K., T. Ikarashi and A. Baba. 1986. Analysis of the reserve carbohydrates in bulb scales of autumn planting bulb plant. Japan. J. Soil Sci. Plant Nutr. 57: 119－125 (In Japanese).
Stehsel, M. L. and S. M. Caplin. 1969. Sugars: Autoclaving vs sterile filtration on the growth of carrot tissue in culture. Life Sci. 8, Part Ⅱ: 1255－1259.
Takayama, S. and M. Misawa. 1979. Differentiation in *Lilium* bulbscales grown in vitro. Effect of various cultural conditions. Physiol. Plant. 46: 184－190.
Weston, G. D. and H. E. Street. 1969. Sugar absorption and sucrose inversion by excised tomato roots. Ann. Bot. 32: 521－529.

ヒメサユリの成球生産に関する研究
培養中の子球の糖・デンプン含量および液体培地の糖の種類・含量の経時的変化と子球生長との関係

新美芳二・三崎　裕・中野　優

新潟大学農学部　950－2181　新潟市五十嵐２の町 8050

摘　要

ヒメサユリ (*Lilium rubellum* Baker) 子球 (培養開始時の新鮮重 30~60 mg) の生長に適した液体培地のショ糖濃度を決定するため，培地および培養中の子球の糖変化を調査した．

1. 100，150および250 mMのショ糖を添加したMS培地 (それぞれ，100，150および250 mMショ糖培地とよぶ) で子球を８週間培養した結果，その新鮮重増加率は250 mMショ糖培地で高く，その増加は培養開始４から８週間後で最大であった．

2. 250 mMショ糖培地をオートクレーブで滅菌したあと培地中の糖を調査したところ，217.5 mMショ糖，32.5 mMブドウ糖と32.5 mM果糖が検出された．

3. 150 mMおよび250 mMショ糖培地に含まれる３種類の糖 (ショ糖，ブドウ糖および果糖) は，150 mMショ糖培地で培養開始８週間後，250 mMショ糖培地では12週間後にほとんど消失した．

4. 上述の２種類の培地で培養した子球中の糖 (ショ糖，ブドウ糖，果糖) およびデンプン含量の変化を調査した．ショ糖は培養４週間まで増加し，そのあと減少した．一方，ブドウ糖および果糖は培養期間中ほぼ一定であった．デンプンは150 mMショ糖培地より250 mMショ糖培地で培養した子球で高かった．そして，培養後半には子球内デンプン含量は減少し，その減少率は前者の子球で大きかった．

培養期間中の培地の糖および子球の炭水化物含量の変化に関する結果から，液体振盪培養したヒメサユリ子球の生長の促進・維持は，培養開始後8~12週間後に培地を更新することにより可能であることが明らかとなった．