Effects of Cold Treatment, BA and GA₃ on Enlargement and Leaf Emergence of In Vitro Cultured Bulblets of *Lilium japonicum* Thunb.

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Summary

While bulblets of *Lilium japonicum* Thunb. were being multiplied by tissue culture, the number of leaves emerging from the bulblets gradually decreased as the culture period was prolonged; finally, bulblets developed no leaves. Dormant bulblets which had been cultured in vitro for more than 15 months were exposed to 2°C or treated with BA or GA₃.

1. Bulblets incubated at 2°C to break dormancy and subsequently cultured at 20°C developed scale leaves and enlarged. Thus, dormancy prevents leaf emergence and reduces the rate of bulblet enlargement.

2. Bulblets cultured on medium with 2 mg·liter⁻¹ BA or 9.5 or 30 mg·liter⁻¹ GA₃ formed leaves. However, leaf emergence was suppressed when the bulblets were transferred to medium with low concentrations of NAA (0.01 mg·liter⁻¹) and BA (0.001 mg·liter⁻¹), indicating that BA and GA₃ stimulate leaf emergence directly and do not break dormancy.

Introduction

Lilium japonicum Thunb., a wild lily native to Japan, has been considered for utilization as a commercial ornamental plant. Hence, in vitro multiplication has been investigated extensively (Fukui et al., 1989; Kawarabayashi, 1993a, b; Niimi, 1988, 1990; Niimi and Onozawa, 1979). During in vitro propagation of *L. japonicum*, leaf emergence from bulblets gradually decreased as the culture period was prolonged (author, unpublished). The present paper shows that dormancy influenced leaf emergence and bulblet enlargement in vitro. Results of several culture conditions which enhance leaf formation and bulblet weight are also reported.

Materials and Methods

Plant materials

Plant materials were wild *Lilium japonicum*

Thunb. which were obtained from Ishikawa and Shizuoka Prefectures, Japan. In the results of each experiment, however, no difference in the behavior of bulblets from Shizuoka and Ishikawa Prefectures were observed and so, deviations of plants are not shown in each of results.

Induction of in vitro bulblets

A basic medium used in this study consisted of Linsmaier and Skoog's inorganic salts (1965), 100 mg·liter⁻¹ myo-inositol, 0.4 mg·liter⁻¹ thiamine-HCl, 30 g·liter⁻¹ sucrose, and 2 g·liter⁻¹ gelrite (pH 5.8). Stems and leaves of *L. japonicum* were surface-sterilized in 0.5% sodium hypochlorite solution for 5 min, cut into 5 mm transverse strips, and placed on the basic medium supplemented with 0.5 mg·liter⁻¹ NAA and 0.05 mg·liter⁻¹ BA (Niimi, 1990). Three to four months later, each stem and leaf explant generated an average of 1.1 and 1.7 bulblets, respectively. These bulblets were detached from the explants and transferred to the basic medium with 0.01 mg·liter⁻¹ NAA and 0.001 mg·liter⁻¹ BA (Niimi, 1988) for 15 to 27 months to promote growth. The enlarged bulblets of uniform size were

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selected and used in the following experiments. Bulblet size are represented as initial fresh weights in Tables 1, 2 and 3.

All cultures were kept at 20°C with 16 hr day-length. Four to six bulblets were cultured in a vessel, containing 40 ml of medium which was renewed every six weeks.

Experiment 1. Relation between leaf emergence and culture duration

Scales were dissected from three enlarged bulblets and placed on the basic medium containing 0.05 mg·liter⁻¹ NAA (Niimi, 1988). Four months later, 57 bulblets which were generated on the cut surfaces of the scales were detached, transferred onto the basic medium containing 0.01 mg·liter⁻¹ NAA and 0.001 mg·liter⁻¹ BA (Niimi, 1988) and cultured for a total of 249 days. The numbers of new, functional leaves on each of bulblets were counted.

Experiment 2. Effects of cold treatment

Bulblets were pre-cultured at 2°C (cold treatment) or at 20°C (control) for 70, 80, or 100 days on the basic medium with 0.01 mg·liter⁻¹ NAA and 0.001 mg·liter⁻¹ BA (Niimi, 1988), and subsequently cultured at 20°C on the same medium. The percentages of bulblets with visible, scaly leaves were recorded after the bulblets were cultured at 20°C for 28, 35, or 56 days. Fresh weights of bulblets pre-cultured for 70 or 80 days were taken on the initial and final days of culture at 20°C by the following method; the bulblets from which all scaly leaves and roots were detached were rinsed twice with sterilized, distilled water to remove medium components, centrifuged at 1,000rpm for 5 min to remove water, and then weighed.

Experiment 3. Effects of BA and GA₃

Bulblets were cultured for certain periods (Tables 2 and 3) on the basic medium supplemented with BA or GA₃ at several concentrations (Tables 2 and 3). Filter sterilized GA₃ solution was added to the autoclaved media. BA was added to the media before autoclaving. The percentages of bulblets with visible scaly leaves were recorded after the bulblets were cultured on the medium with BA or GA₃ for different culture duration. Bulblets cultured with or without BA or

GA₃ were weighed at the beginning and at the end of the experiment as in Exp. 2.

Experiment 4. Effects of removal of 2 mg·liter⁻¹ BA or 30 mg·liter⁻¹ GA₃ from medium on leaf formation

Bulblets which were cultured on the basic medium supplemented with 2 mg·liter⁻¹ BA or 30 mg·liter⁻¹ GA₃ for 63 days were transferred into a medium containing 0.01 mg·liter⁻¹ NAA and 0.001 mg·liter⁻¹ BA. Simultaneously, other bulblets were kept on the basic medium containing 2 mg·liter⁻¹ BA or 30 mg·liter⁻¹ GA₃. The number of leaves on these bulblets which were cultured for a total of 167 days, were counted.

Experiment 5. Effects of GA₃ on sprouting in soil

Eighteen or ten bulblets were cultured for 49 or 91 days, respectively, on the basic medium containing 30 mg·liter⁻¹ GA₃ followed by transplanting in soil without cold treatment. After 84 days in soil, the number of emerging leaves was counted.

Results

Experiment 1. Relation between scaly leaf emergence and culture duration

Bulblets developed many scaly leaves after their detachment from their explants (Fig. 1) at a rate of 0.05 bulblet⁻¹·day⁻¹ during the early stages of culture. However, the rate decreased as the culture was prolonged, and the bulblets developed a few leaves after 153 days in culture. The number of living leaves reached a maximum at 38 days of culture.

Experiment 2. Effects of cold treatment on leaf formation and bulblet enlargement

Eighty, 96 and 100% of the cold-treated bulblets formed new scaly leaves after 28, 35, and 56 days, respectively (Table 1). During the same period, 75, 19, and 5% of the control bulblets formed leaves. Seventy-five percent of the control bulblets in test 1 formed very small leaves (data not shown); thus, cold treatment is considered to promote leaf emergence and growth. Increases in mean fresh weight of cold-treated bulblets were larger than those of the control bulblets (Table 1).

Experiment 3. Effects of BA and GA₃ on leaf formation and bulblet enlargement

Two mg·liter⁻¹ BA (Table 2, Fig. 2) and 9.5 and 30 mg·liter⁻¹ GA₃ (Table 3) stimulated leaf emergence. Two mg·liter⁻¹ BA enhanced bulblet growth which is reflected by their mean fresh weight (Table 2), but GA₃ did not (Table 3).

Fig. 1. Relation between scaly leaf formation from bulblets of *L. japonicum* and duration of bulblet culture.

Newly formed bulblets were detached from explants and sub-cultured at 0 day. Rate of new leaf formation (▨), and the number of living (●) and total leaves (▲) were measured at 0, 38, 98, 153, 202 and 249 days of the culture.

Experiment 4. Effects of removal of 2 mg·liter⁻¹ BA or 30 mg·liter⁻¹ GA₃ on leaf formation

Bulblets on 2 mg·liter⁻¹ BA or 30 mg·liter⁻¹ GA₃ continuously formed new leaves, whereas those transferred to a medium containing low concentrations of NAA (0.01 mg·liter⁻¹) and BA (0.001 mg·liter⁻¹) failed to differentiate new leaves (Fig. 3).

Experiment 5. Effects of GA₃ on sprouting in soil

After bulblets cultured on medium containing 30 mg·liter⁻¹ GA₃ were transplanted into soil, no bulblets sprouted (data not shown).

Discussion

Bulblets of *Lilium* species become dormant during in vitro culture and do not sprout or do so slowly after transplanting into soil; cold treatment prior to transplanting into soil is one of the most effective methods for breaking dormancy (Aguettaz et al., 1990; Niimi et al., 1988; Stimart et al., 1982; Takayama et al., 1982). In vitro propagated bulblets of *L. japonicum* also remain dormant and do not sprout in soil; however dormancy was also broken by cold treatment (Yamagishi, 1993). From the previous reports, bulblets used in the Exp. 2, 3, 4 and 5 were considered to be in the dormant stage because they were cultured in vitro for more than 15 months.

Cold temperature exposure was achieved to break the dormancy of in vitro cultured bulblets of *L. japonicum*. The cold treatment restored leaf emergence of the bulblets (Table 1), indicating that

Table 1. Effects of cold-temperature treatment on leaf emergence and a weight increase of in vitro bulblets of *L. japonicum*.

Bulblets were pre-cultured at 2 °C (cold treated) or at 20 °C (control) and then cultured at 20 °C. Leaf emergence was determined after bulblets were cultured at 20 °C for different culture periods.

Test	Temperature of pre-culture (°C)	Duration of pre-culture (days)	No. of bulblets tested	Duration of culture at 20 °C (days)	% of bulblets with leaves	Fresh weight of bulblets (mg) ^z	
						On initial day of culture at 20 °C	On final day of culture at 20 °C
1	2	70	20	28	80	985 ± 20	1,434 ± 85
	20	70	20	28	75	985 ± 19	1,184 ± 25
2	2	80	45	35	96	1,680 ± 83	1,978 ± 89
	20	80	36	35	19	1,540 ± 74	1,748 ± 79
3	2	100	20	56	100	nt ^y	nt ^y
	20	100	20	56	5	nt	nt

^z Mean ± standard error. ^y Not tested.

Table 2. Effects of BA in culture medium on leaf emergence and a weight increase of in vitro bulblets of *L. japonicum*. Leaf emergence was determined after the bulblets were cultured on the medium with or without BA for different culture duration.

Plant growth regulator (mg · liter ⁻¹)	No. of bulblets tested	Culture duration (days)	% of bulblets with leaves	Fresh weight of bulblets (mg) ^w	
				On initial day of culture	On final day of culture
BA (0.002)	16	28	6	nt ^y	nt ^y
BA (0.02)	19	28	5	nt	nt
BA (0.2)	20	28	15	nt	nt
BA (2)	19	28	84	nt	nt
BA (20)	8	28	0 ^x	nt	nt
Control ^z	20	28	5	nt	nt
BA (2)	44	70	86	1,113 ± 13	1,650 ± 32
Control ^y	40	70	20	1,098 ± 13	1,439 ± 27
BA (2)	9	63	89	2,248 ± 100	2,864 ± 79
Control ^y	9	63	0	2,228 ± 57	2,593 ± 110

^z Plant growth regulator free. ^y 0.01 mg · liter⁻¹ NAA plus 0.001 mg · liter⁻¹ BA. ^x Masses of very small bulblets were produced on the enlarged basal plates of bulblets. ^w Mean ± standard error.

^v Not tested.

Fig. 2. Scaly leaves emerging from bulblets of *L. japonicum*. The medium in each of four culture vessels contained 2 mg · liter⁻¹ BA.

Table 3. Effects of GA₃ in culture medium on leaf emergence and a weight increase of in vitro bulblets of *L. japonicum*. Leaf emergence was determined after the bulblets were cultured on the medium with or without GA₃ for different culture duration.

Plant growth regulator (mg · liter ⁻¹)	No. of bulblets tested	Culture duration (days)	% of bulblets with leaves	Fresh weight of bulblets (mg) ^y	
				On initial day of culture	On final day of culture
GA ₃ (0.03)	20	70	25	1,074 ± 23	1,418 ± 36
GA ₃ (0.3)	20	70	15	1,023 ± 16	1,409 ± 31
GA ₃ (3)	20	70	60	1,105 ± 17	1,507 ± 30
Control ^z	40	70	20	1,098 ± 13	1,439 ± 27
GA ₃ (3)	20	56	35	nt ^x	nt ^x
GA ₃ (9.5)	20	56	75	nt	nt
GA ₃ (30)	20	56	95	nt	nt
Control ^z	20	56	20	nt	nt
GA ₃ (3)	8	63	38	2,179 ± 88	2,714 ± 85
GA ₃ (30)	9	63	100	2,299 ± 75	2,754 ± 118
Control ^z	9	63	0	2,228 ± 57	2,593 ± 110
GA ₃ (3)	9	91	0	2,122 ± 21	2,848 ± 59
GA ₃ (9.5)	9	91	44	2,109 ± 26	2,963 ± 58
GA ₃ (30)	8	91	75	2,096 ± 29	2,986 ± 64
Control ^z	8	91	0	2,092 ± 20	2,968 ± 31

^z 0.01 mg · liter⁻¹ NAA plus 0.001 mg · liter⁻¹ BA. ^y Mean ± standard error. ^x Not tested.

Fig. 3. Effects of removal of 2 mg·liter⁻¹ BA or 30 mg·liter⁻¹ GA₃ from medium on leaf formation from bulblets of *L. japonicum*.

Nineteen and 20 bulblets were cultured on the basic medium supplemented with 2 mg·liter⁻¹ BA (---) and 30 mg·liter⁻¹ GA₃ (·····), respectively, for 63 days followed by subculture in the basic medium containing 0.01 mg·liter⁻¹ NAA and 0.001 mg·liter⁻¹ BA. Simultaneously, 19 and 15 bulblets were cultured on the basic medium constantly containing 2 mg·liter⁻¹ BA (— · —) and 30 mg·liter⁻¹ GA₃ (—) for all culture duration.

the decrease in new leaf emergence with increasing culture period (Fig. 1) was caused by dormancy in the bulblets. This result supports the proposition of Delvallée et al. (1990) that dormancy of in vitro cultured bulblets of *L. speciosum* became deeper and the rate of scale leaf emergence slowed down with prolonged culture. Fresh weight of non chilled i.e., dormant bulblets increased during culture (Table 1), as reported by Delvallée et al. (1990) but their rate of increase was slower than that of non-dormant bulblets. This weight increase is attributed to the breaking of dormancy which activates the absorption of sugars and other nutrients from medium and enhances the accumulation of storage carbohydrates in the bulblets.

Two mg·liter⁻¹ BA enhanced scaly leaf formation and weight increase on bulblets (Table 2). This might be caused by the enlargement of basal plates of the bulblets as they enlarged abnormally when nine bulblets were cultured with 2 mg·liter⁻¹ BA for 160 days (data not shown). GA₃ promoted scaly leaf formation but had no effects

on a weight increase of bulblets (Table 3).

The enhancement of leaf formation occurred only when bulblets were cultured on media with 2 mg·liter⁻¹ BA or 30 mg·liter⁻¹ GA₃. When bulblets cultured with 2 mg·liter⁻¹ BA or 30 mg·liter⁻¹ GA₃ were transferred and recultured on a medium with low concentrations of NAA and BA, leaf formation ceased (Fig. 3). In addition, bulblets cultured on medium containing 30 mg·liter⁻¹ GA₃ failed to sprout when transplanted into soil (Exp. 5). These results showed that BA or GA₃ directly promoted leaf emergence, but did not break dormancy of bulblets although BA and GA₃ are plant growth regulators which effectively break dormancy on in vitro plants (Hussey, 1977; Jonard et al., 1983; Pruski et al., 1990; Sutter, 1986). Therefore, effects of BA and GA₃ were different from that of a cold treatment which broke dormancy followed by stimulation of leaf emergence. Niimi et al. (1988) found that GA₃ application to in vitro cultured bulblets of *L. rubellum* stimulated leaf formation when transplanted to pots and that GA₃ treatment, however, was less effective for breaking dormancy compared to a cold treatment. Contrarily, bulblets of *L. japonicum* cultured with 30 mg·liter⁻¹ GA₃ did not sprout when transplanted into soil (Exp. 5). The difference might be attributed to the experimental procedures; the bulblets of *L. rubellum* were incubated in a solution containing more than 250 mg·liter⁻¹ GA₃ for 24 hr (Niimi et al., 1988), whereas, in my preliminary experiment, eight bulblets cultured on a medium containing 300 mg·liter⁻¹ GA₃ for 21 days turned brown and died, indicating that such a high concentration of GA₃ in culture medium was toxic.

In contrast to the present results, 0.1 or 1 mg·liter⁻¹ GA₃ reduced the level of dormancy in the bulblets of *L. speciosum* (Aguettaz et al., 1990). The discrepancies might be due to the age of bulblets used in each experiment; bulblets used in this experiment were older than those used by Aguettaz et al. Sensitivity to GA₃ might change according to bulblet age.

In conclusion, the rate of leaf emergence from bulblets of *L. japonicum* gradually decelerated as the culture period was prolonged; few leaves developed during later stage of culture (Fig. 1). Breaking dormancy by the cold treatment restored new leaf formation and stimulated the growth of in

vitro cultured bulblets; hence, chilling to bulblets shortened the culture period while enhancing their growth.

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ササユリ子球の *in vitro* における肥大と出葉に及ぼす低温処理と BA, GA₃ の影響

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摘 要

ササユリ子球を組織培養で増殖した。外植体である母球の鱗片より子球をとりはずした直後は、子球は鱗片葉を盛んに分化したが、培養期間が長くなるにつれて出葉の頻度は低下した。次に、*in vitro* で 15 カ月間以上培養し、完全に休眠している子球に、低温処理、または BA および GA₃ の処理を行った。子球の休眠を低温処理により破り、その後再び 20 °C で培養したところ、子球は盛んに出葉し、同時に子球の肥大も促進された。出葉の低下は休眠が原因であることと、休

眠打破は子球の肥大促進に有効であることが分かった。BA または GA₃ を加えた培地で子球を培養したところ、2 mg・liter⁻¹ BA または 9.5, 30 mg・liter⁻¹ GA₃ は、鱗片葉の分化を促進した。この出葉は、これらの生長調節物質を培地に加えたときのみ起こり、除くと停止することより、休眠が破れた結果出葉したのではなく、これらの生長調節物質が直接出葉を促進したと考えられた。また、培地中の BA や GA₃ は、休眠を破る効果はなく、子球の肥大を促進しなかった。