Effect of low temperature on leaf emergence and rooting from regenerated bulblets of Lilium japonicum Thunb. Cultured in vitro.

Effect of low temperature on leaf emergence and rooting from regenerated bulblets of *Lilium japonicum* Thunb. cultured in vitro.

Masanori OHKAWA and Shigeru MIZUGUCHI

Faculty of Education, Kanazawa University, Kanazawa
920-1192, Japan

SUMMARY

Scales of *Lilium japonicum* Thunb. were cultured on MS-medium at 24℃ in a growth chamber *in vitro*. Bulblets formed on the scales placed in cold storage at 4℃ for 8 weeks. They were detached from the scales and then cultured on MS-medium for 10 weeks at 24℃ in a growth chamber. Leaf emergence and rooting from the bulblets were enhanced by the low temperature treatment, but root growth was not affected. Histological observations of the bulblets showed that the first leaf emerged on the 5th scale from the outermost scale of the bulblets and the secondary leaf on the 6th. Vasculature formation in the bulblets was not connected with the low temperature treatment. There is no correlation between the bulblet size and emergence of a scale leaf from the bulblets. The leaves that emerged from the bulblets could be classified into scale leaves (SL) and stem leaves (STL). Insufficient low temperature treatment reduced the rate of leaf emergence from the bulblets.

INTRODUCTION

Since flowers of *Lilium japonicum* Thunb. have beautiful pink color and sweet scent, this lily has horticultural value among the flower growers in Japan. Distribution of the lily is limited in the central region in Japan and it grows only in the bamboo or the bamboo grass bushes. Because the germination of its seeds are very difficult in nature, its propagation by seeds has been classified into a very difficult group among plants. Recently, researchers have tried to propagate this lily by tissue culture techniques (Fukui *et al*., 1989 ; Tanaka *et al*., 1991 ; Mizuguchi *et al*., 1994 ; Mizuguchi and Ohkawa, 1994), but they have not succeeded in production of the bulbs.

We demonstrate in this paper that leaf emergence and rooting from the bulblets, that were regenerated by scale culture *in vitro*, were enhanced by the low temperature treatment.

MATERIALS AND METHODS

1. Plant materials and cultural conditions for preparation of regenerated bulblets.

Bulbs of *Lilium japonicum* Thunb. were collected in late March, 1989, in the bushes of bamboo

平成12年10月2日受理

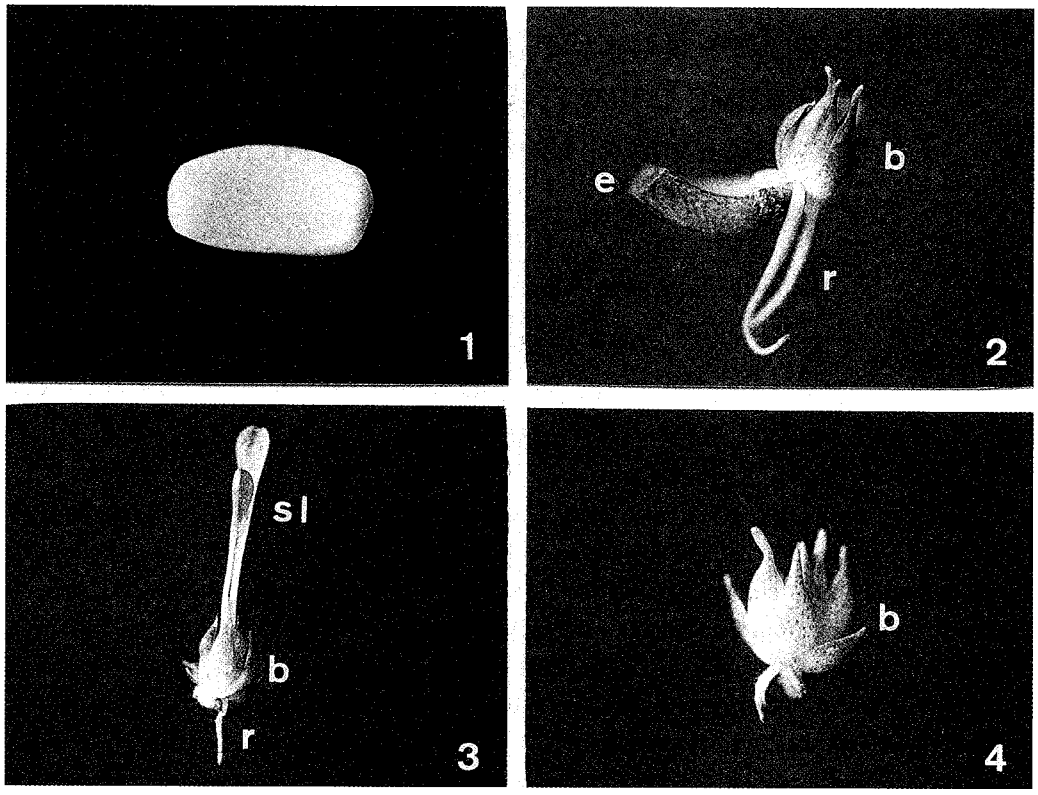

Fig. 1. Explant (1), regenerated bulblet from the explant (2), bulblet developing 2 scale leaves (3) and bulblet without scale leaf (4) of *Lilium japonicum*. b ; Bulblet, e ; explant, r ; root, sl ; scale leaf.

grass at Nanao City of Ishikawa Prefecture in Japan. After the bulbs were washed in tap water, bulb scales were detached from the bulbs. The bulb scales were surface sterilized for 30 seconds in 70% ethyl alcohol and for 15 minutes in 2% sodium hydrochlorite solution (0.1% as active chlorine). Then the scales were rinsed 3 times in sterile distilled water and farther treated with 0.2% thiabendazole solution (ON-147, SS Pharm. Co., Japan) for double sterilization (Ohkawa and Mizuguchi, 1994).

Scale, which was discarded the terminal part, was used as explant (Fig.1-1). The explants were placed on MS-medium (Murashige and Skoog, 1962) consisting of macro and micro salts, organic components, 3% sucrose and 0.8% agar. Plant hormones were not added to the medium. Medium pH was adjusted to 5.8 before autoclaving for 15 minutes at 121℃. The scales were cultured to prepare the regenerated bulblets in a growth chamber at 24℃ for 12 months with continuous light at 20 μmol $\cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent lamps *in vitro*.

2. Effect of low temperature on leaf emergence and rooting from the regenerated bulblets.

Regenerated bulblets (Fig.1-2) were transplanted on fresh MS-medium and, they were cultured to habituate themselves for 2 weeks in a growth chamber of the same conditions as the initial culture. After the habituation of the bulblets, they were treated at 4℃ for 2, 4, 6 and 8 weeks in a cold storage.

Then they were cultured at 24℃ for 10 weeks in a growth chamber under the same conditions as the initial culture. Leaf emergence from the bulblets was determined every second week. The rate of leaf emergence was indicated as the percentage of number of bulblets, that developed with scale leaves, to total number of bulblets. Contamination rate of each test was usually less than 10 percent.

The bulblets were separated into 2 groups : one with roots (Fig.1-3) and another without roots (Fig.1－4). Pre-existing roots of the former group were eliminated from the bulblets after the low temperature treatment, then all bulblets were cultured to habituate themselves at 24℃ for 2 weeks. After the habituation of the bulblets, they were treated at 4℃ for 2, 4, 6 and 8 weeks in a cold chamber. Then they were cultured at 24℃ in a growth chamber of the same conditions as the initial culture. Rooting from the bulblets was determined after 10 weeks in culture and expressed by the number of roots per bulblet.

3. Effect of low temperature on growth of the scale leaves and the roots developing from the bulblets.

After the bulblets (Fig.1-2) were detached from the explant, they were treated with low temperature and cultured in a growth chamber as experiment-2. The bulblets, which formed a leaf and a root, were selected, and the length of a leaf and a root of them were determined after 2 and 4 weeks in culture.

4. Histology.

Three kinds of the bulblets (Fig.1-2) were subjected to histological examinations ; sample 1 : the bulblets without the low temperature treatment, sample 2 : the bulblets treated at 4℃ for 8 weeks, sample 3 : the bulblets cultured at 4℃ for 8 weeks followed by 1 week at cultural conditions that were the same as the initial culture. Specimens were fixed in formaldehyde-acetic acid-ethyl alcohol, dehydrated in a graded ethyl alcohol-tertiary butyl alcohol series, and embedded in paraffin. Twelve μm－thick sections were prepared by a microtome and stained with safranin and haematoxylin.

5. Relationship between bulblet weight and leaf emergence.

The bulblets (Fig.1-2) were transplanted on fresh MS-medium to habituate themselves at 24℃ for 2 weeks in a growth chamber. Then the bulblets were treated at 4℃ for 8 weeks in a cold storage. After the low temperature treatment, they were cultured for 10 weeks at 24℃ in a growth chamber of the same conditions as the initial culture. After this last culture period, they were taken out from the culture flasks and classified into 3 sizes by weight ; L (large)-size of 0.91±0.10 g, M (medium)-size of 0.35±0.03 g and S (small)-size of 0.10±0.01 g. Then they were planted in plastic boxes (130×270×150 mm) containing vermiculite and the soil of bamboo grass bushes (1 : 1, v/v). The plastic boxes were placed in a phytotron for 10 weeks at 24/15℃(day/night) cycle with 16-hr photoperiod under 50 μmol · m^{-2} · s^{-1}. The number of bulblets with the leaves and the type of leaf developed from the bulblets were determined every second week. The rate of leaf emergence was expressed as the percent of bulblets with the leaves to total bulblets. Eight to 10 bulblets of each size were used in the experiment.

RESULTS

1. Effect of low temperature on leaf emergence and rooting from the regenerated bulblets.

Emergence of scale leaves was found on 5th day after culture in all treatments. The rates of leaf emergence from the bulblets cold-treated for 2, 4, 6, 8 and 0 (control) weeks were 32, 41, 72, 85 and 5% after 6 weeks in culture, respectively (Fig.2). Though the rates of each treatment rapidly increased from 5 days to 2 weeks in culture, little change was observed during the succeeding 8 weeks. Leaf emergence occurred on the apical part of a scale in the bulblets, but not from a shoot. This leaf developed on the 5th or the 6th scale from the outer scale of the bulblets. This result showed that, for leaf emergence, the bulblets needed to be treated for 6 to 8 weeks at 4℃ and then cultured for 2 weeks at 24℃. It was recognized that there were 4 types in emergence of scale leaf development from one bulblet; 1-SL (1 scale leaf), 2-SL (2 scale leaves), 3-SL (3 scale leaves) and 0-SL (no scale leaves) types (Table 1). The rates of 2-and 3-SL increased by the low temperature treatment for 6 to 8 weeks.

Table 1 Effect of temperature on emergence of scale leaf from regenerated bulblets.

Treatment (Week)	Emergence of scale leaf(%)			
	0-SL[z]	1-SL[z]	2-SL[z]	3-SL[z]
0(Cont.)	89.0	11.0	0.0	0.0
2	66.6	26.0	7.4	0.0
4	59.3	33.3	7.4	0.0
6	25.9	37.0	29.7	7.4
8	14.9	40.7	40.7	3.7

z 0-SL, 1-SL, 2-SL and 3-SL mean zero, one, two and three scale leaves emerged from one bulblet, individualy. The bulblets were cultured at 24℃ for 10 weeks *in vitro*.

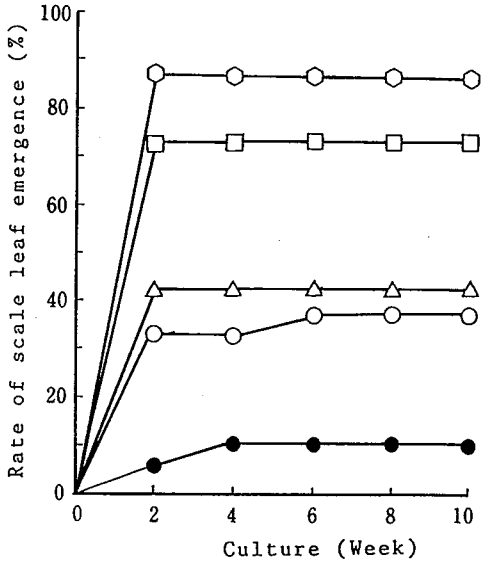

Fig. 2. Effect of low temperature on leaf emergence from bulblets. The bulblets were treated at 4℃ for 2-week ; ○, 4-week ; △, 6-week ; □, 8-week ;⬡ and 0-week (control) ; ●, and then they were cultured at 24℃ for 10 weeks. Rate of leaf emergence showed as percentage of number of bulblets with scale leaf to total bulblets.

The bulblets with roots gave greater number of newly grown roots than those without roots, and the low temperature treatment for 6 weeks was the most effective on rooting from the bulblets after 10 weeks in culture (Fig.3).

2. Effect of low temperature on growth of the scale leaves and the roots developing from the bulblets.

In proportion, as the period of low temperature treatment increased, the growth of scale leaves was enhanced. But no differences were found in root growth after 4 weeks in culture (Fig.4).

3. Histology.

The branching of scale and the beginning of leaf formation could be found on the 5th scale in sample 2(Fig.5, 2-a and 3-b, arrow). The scale leaf on the apical part of the 5th scale was already grown in sample 3(Fig.5, 3-a and 3-b). The 5th

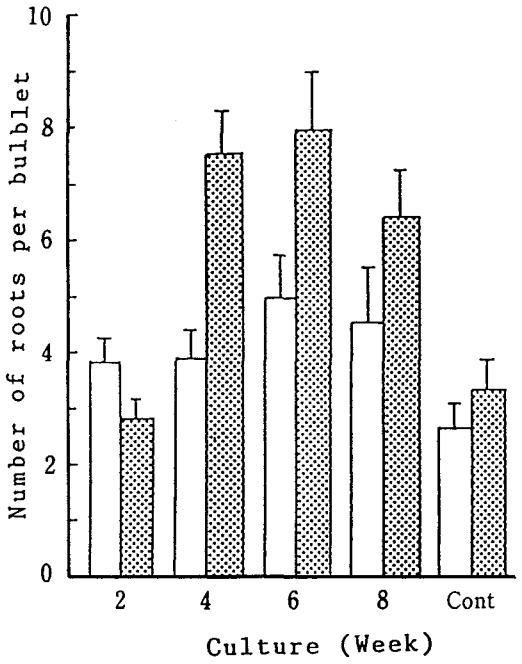

Fig. 3. Effect of low temperature on rooting from the regenerated bulblets. The bulblets were treated at 4℃ for 2, 4, 6, 8 and 0 (control) weeks, and then they were cultured at 24℃ for 10 weeks. Number of roots per bulblet was expressed. Bulblet with roots ; ▦, bulblet without roots ; □. Vertical bars expresent mean±SE.

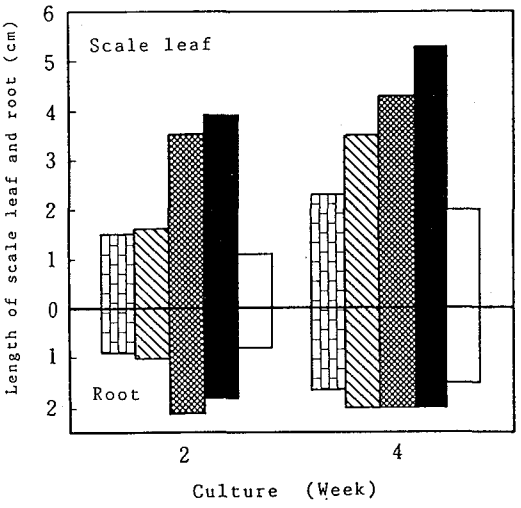

Fig. 4. Effect of low temperature on growth of scale leaves and roots. The bulblet were treated at 4℃ for 2 weeks ; □, 4 weeks ; □, 6 weeks ; ▨, 8 weeks ; ■ and 0 weeks (control) ; □, and then they were cultured at 24℃ for 4 weeks. Length of scale leaf and root were determined on 2 and 4 weeks after culture.

scale in sample 1 (Fig.5, 1-a and 1-b) exhibited only a covering shape in the apical part and the branching of it could not be found. Root primordia were detected on sample 3 (Fig.5, 3-a). Many vasculatures were detected in the parenchyma cells in sample 1, 2 and 3 (Fig.5, 1-b, 2-b and 3-b). The root primordia were recognized in the base of scale and stained with haematoxylin.

4. Relationship between bulblet size and leaf emergence.

Leaf emergence from the bulblets started from 10 to 14 days in culture in a phytotron (Fig.6). The rates of leaf emergence of L-, M-and S-size were 33, 33 and 36% after 2 weeks in culture, respectively, and there were no differences among the three sizes. Leaf emergence from these bulblets rapidly increased in the rates after 4 weeks in culture, but after this period, the rates were almost unchanged. And the rates of leaf emergence of them were 89, 83 and 76% for 10 weeks, respectively. The leaves that emerged from either a scale or a stem have been named a scale leaf or a stem leaf, respectively. The rates of emergence of stem leaf from L- and M-size were 11 and 8%, respectively. But any stem leaf from S-size was not detected (Fig.7).

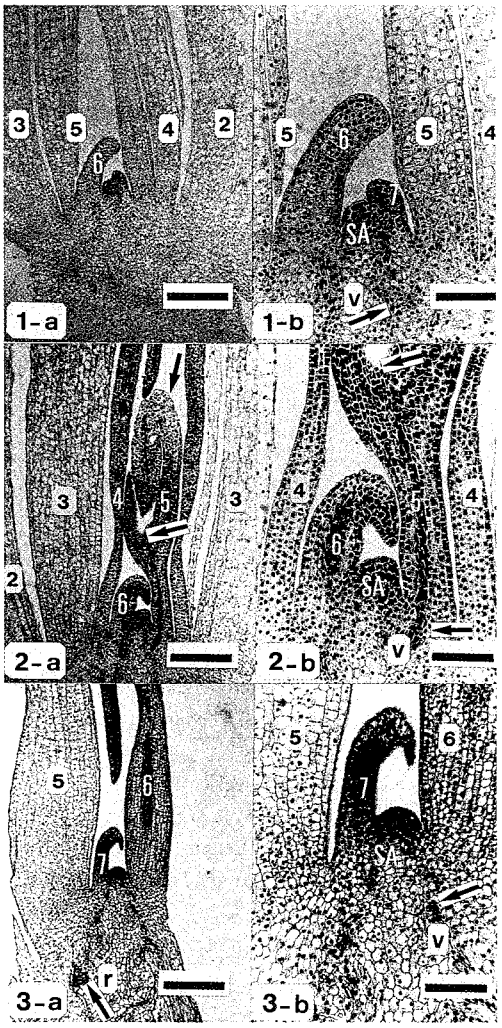

Fig. 5. Optical micrographs of longitudinal section of re-generated bulblets. Formations of scale leaf and root primodium were promoted with low temperature. Photograph 1 ; the bulblet separated from initial explant of bulb, 2 ; the bulblet treated at 4℃ or 8 weeks, 3 ; the bulblet treated at 4℃ for 8 weeks and then cultured at 24℃ for 1 week. Scales are 500 μm(1 a, 2 a and 3 a) and 200 μm (1 b, 2 b and 3 b). Numbers in the photographs show the order of scale leaf from outer of bulblet. Symbols of r, SA and v are root primodium, shoot apex and vasculature.

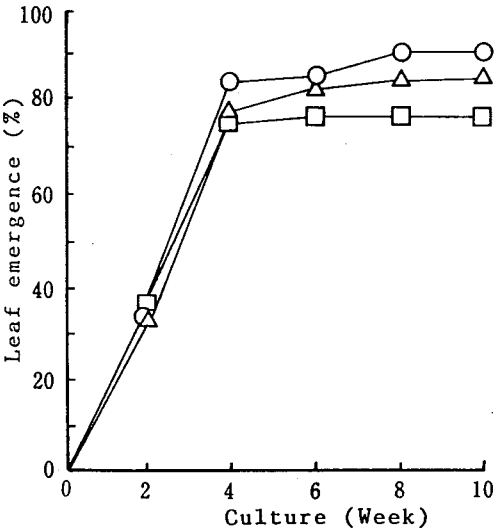

Fig. 6. Effect of size of the regenerated bulblets on leaf emergence. L-size ; ○, M-size ; △, S-size ; □.

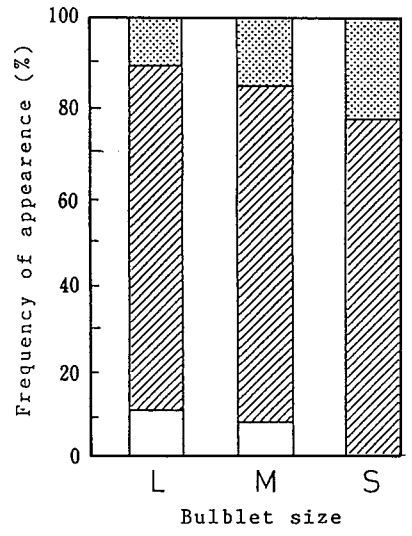

Fig. 7. The frequency of emergence of scale and stem leaves from regenerated bulblets. Stem leaf ; □, scale leaf ; ▨, no leaf ; ⁘.

DISCUSSION

When the regenerated bulblets of *Lilium japonicum* Thunb. had been cultured at 24℃ about 10 months *in vitro*, neither the leaf emergence nor rooting from the bulblets were observed. The bulblets might have been in the dormant stage. Takayama *et al*. (1982) and Niimi (1984) reported that breaking of bulblet dormance of *L. longiflorum* and *L. rubellum* needed low temperature of 3 or 5℃ for 60 to 70 days. Also Syoyama *et al*. (1987) reported that all bulbs sprouted to become plants when the rooted bulbs were stored at 4℃ for 1 month and then transplanted in vermiculite. We found the dormancy of *L. japonicum* could be broken at 4℃ for 6 to 8 weeks in this study (Fig.2, 3 and 4). After the bulblets were treated with low temperature, leaf emergence could be observed after 5 days in culture at 24℃ and the rate of leaf emergence was 85% on 2 weeks. From these results, it was considered that the degree of dormancy of *L. japonicum* is light among the lilies.

From the observations of growth processes of the bulbs in nature, storage substances in the outer scales (4 to 5 pieces) of a bulb were consumed during the bolting of a new shoot and the flowering time, and the outer scales completely wilted in August. On the other hand, a daughter bulb formed on the dwarf stem before August and it consisted of 6 to 7 pieces of the scales. As the results, 3 to 4 pieces of the scale increase in a bulb every year. We found in this study that scale leaves emerged on the 5th or the 6th scale from the outermost scale of the bulblets and did not emerged from more outer scales (Fig.5). This fact suggested the outer scales may have a role as storage organs and nutrition would be transported to inner ones.

Histological studies showed that the scale leaf was not formed on the bulblets which were not treated with low temperature. On the contrary, the first scale leaf emerged from the 5th scale of the bulblets which were treated at 4℃ for 8 weeks, and a secondary one emerged on the 6th scale. The vasculature was formed on both bulblets treated with and without low temperature. It was confirmed that the formation of a scale leaf and a root from the bulblets was needed the low temperature treatment from 6 to 8 weeks, but the formation of vasculature was not need the low temperature treatment (Fig.5). The rates of leaf emergence of S-, M- snd L-sized bulblets were 76, 83 and 89% after 10 weeks in culture, respectively (Fig.6). Thus there is no correlation between the bulblet size and emergence of a scale leaf from the bulblets.

Matsuo *et al*. (1982) reported that the leaf emergence from the bulbs of *L. longiflorum* showed 3 types ; hypogenous type plant (HTP), hypo-epigenous type plant (HETP) and epigenous type plant (ETP). In our study, the bulblets could be separated into 2 groups ; ones with a scale leaf (SL) and the others with a stem leaf (STL), and further the SL groups could be separated into 4 types ; no scale leaf (0-SL), 1 scale leaf (1-SL), 2 scale leaves (2-SL) and 3 scale leaves (3-SL). ETP and HTP of *L. longiflorum* just correspond to the bulblets with the scale leaf (SL) and the stem leaf (STL) in our study. But we did not find HETP in *L. japonicum.* Matsuo and Arisumi (1979) reported that cold treatments promoted shoot emergence from Easter lily bulbs, but this treatments retarded leaf emergence from the bulblets developed from scales detached from the intact bulb. They discussed that leaf emergence from a bulblet on the scales might shows different responses with shooting from a

intact bulb. In our experiments, the bulblets detached from a mother scale were used. Leaf emergence from the bulblets was promoted with low temperature and insufficient treatment reduced leaf emergence.

LITERATURE CITED

Fukui, H., Adachi, N., Hara, T., Nakamura, M.1989. In vitro growth and rapid multiplication of *Lilium japonicum* Thunb. Plant Tissue Culture Letters. **6** : 119-124.

Matsuo, E., Arisumi, K. 1979. Differences in chilling effects on shoot emergence from the bulb and on leaf emergence from the scale bulblet in *Lilium longiflorum* Thunb. HortScience. **14** : 68-69.

Matsuo, E., Arisumi, K., Kawashima, H.1982.Cultural practices influencing premature daughter leaf and/or shoot emergence in scale-propagated Easter lily. HortScience. **17** : 196-198.

Mizuguchi, S., Ohkawa, M., Ikekawa, T. 1994.Effects of naphthaleneacetic acid and benzyladenine on the growth of white callus and formation of bulblet from callus induced from mother-scale of *Lilium japonicum* Thunb. J. Japan. Soc. Hort. Sci. **63** : 131－137.

Mizuguchi, S., Ohkawa, M.1994.Effects of naphthaleneacetic acid and benzyladenine on growth of bulblets regenerated from white callus of mother scale of *Lilium japonicum* Thunb. J. Japan. Soc. Hort. Sci. **63** : 429-437.

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

Niimi, Y.1984.Effcts of α-naphthaleneacetic acid and 6-benzyl aminopurine on the development of excised-bulbs (*Lilium japonicum* Baker) cultured *in vitro* both in diffused light and in continuous darkness, and the leaf emergence from the bulbs *in vitro*. J. Japan. Soc. Hort. Sci. **53** : 59-65.

Ohkawa, M., Mizuguchi, S. 1994. Effect of thiabendazole on prevention of contamination in scale culture on mother bulb of *Lilium japonicum* Thunb. *in vitro*. Bull. Fac. Edu. Kanazwa Univ., Natl. Sci. **43** : 25-28.

Shoyama, Y., Hasegawa, N., Nishioka, I. 1987. *In vitro* propagation of *Lilium japonicum* by culture of bulblet. Shoyakugaku Zasshi. **41** : 352-355.

Takayama, S., Misawa, M., Takashige, Y., Tsumori, H., Ohkawa, K. 1982. Cultivation of in vitro-propagated Lilium bulbs in soil. J. Amer. Soc. Hort. Sci. **107** : 830-834.

Tanaka, A., Hoshi, Y., Kondo, K., Taniguchi, K. 1991. Induction and rapid propagation of shoot primordia from shoot apices of *Lilium japonicum*. Plant Tissue Culture Letters. **8** : 206-208.