

Rapid Production of *Lilium auratum* Bulbs from Zygotic Embryos

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Received 12 February 2004 / Accepted 21 November 2004

Abstract. An *in vitro* culture system for *Lilium auratum* Lindl. zygotic embryos was established to accelerate the development of bulblets. High rates of germination were observed on 50 days after pollination (DAP) or later, while the rate of normal growth increased after 70 DAP. Peeling of the seed coats was necessary for rapid *in vitro* germination, indicating inhibition by the seed coats. We found that embryos obtained from 90 DAP capsules were the best materials for suppressing dormancy. Further development of bulblets was accelerated by culturing them in the presence of 9 % sucrose. In this culture system, the period for development of mature bulblets was reduced from a couple of years to one year after pollination.

Keywords. *Lilium auratum* Lindl, ovule culture, embryo culture, propagation, genetic diversity

INTRODUCTION

Lilium auratum Lindl. is an important genetic resource as an ornamental plant. Since its flower has a notable fragrance and is the largest among lilies, wild *L. auratum* has been cultivated and also used to breed many oriental hybrid lilies. Unfortunately, the population of wild *L. auratum* is decreasing, and it is necessary to conserve them.

In horticulture, *L. auratum* is usually reproduced from scales and bulbs. *In vitro* propagation methods have also been established in lilies (Sheridan 1968; Novak and Petru 1981; Takayama and Misawa 1983; Niimi 1995; Maesato *et al.* 1991). Usually, *in vitro* methods are used for clonal propagation of plants with superior characteristics. Cloning is essential for establishing a cultivar in allogamous plants. On the other hand, only certain genotypes are selected by clonal propagation. For conservation of wild or native populations as genetic resources, we think propagation through zygotic embryos or seeds and also important.

Propagation through seeds is not efficient in *L. auratum*, since it is late maturing. Seeds that mature in autumn germinate in the soil in the next autumn, and show thickening of the bulb without any development of green leaves. It takes more than one year to produce leaves, and another three or more years for inducing flowers (Takayama and Ohkawa 1990). If the period from seeding to flowering is reduced, *L. auratum* will be more popular in the conservation and the breeding of lilies. Thus it is necessary to establish methods for reducing the period to flower.

There have been reports on the acceleration of germination in lilies by ovule and embryo culture (Fukui *et al.*, 1989). *In vitro* cultures of ovules and embryos are possible methods

for eliminating dormancy and shortening the period to flower. In this report, we investigated the appropriate stage of the plant material to ensure high rates of *in vitro* germination and normal growth of embryos. We established a method of getting mature bulblets of *L. auratum* within two years after pollination.

MATERIALS AND METHODS

The bulbs of *L. auratum* Lindl. were planted in plant boxes. Magamp K (Hyponex corp. Ltd) was given 50 g per 15 l of culture soil. The lily bulbs planted in the plant boxes were kept in an open space in University of Tsukuba. Flowers produced from July to August were self-pollinated on the day of anthesis. Capsules were harvested every 10 days from 40 days to 110 days after pollination (DAP).

The capsules were sterilized with 70% (v/v) ethanol for 1 min. After ethanol has evaporated, the capsules were longitudinally dissected, and the ovules were aseptically cut. Seed coats were peeled off from the ovules using forceps under a stereomicroscope. To pick up embryos, intact ovules were cut transversely at the position adjacent to the embryo. Ovules, peeled ovules and embryos (Figure 1A-C) were used as the culture material. These explants were placed on a MS medium (Murashige and Skoog 1962) supplemented with 5×10^{-9} M

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Table 1. The rate of germination of ovules, peeled ovules and embryos during culture.

Organ*	Culture periods		
	30 days	60 days	90days
Ovules	0**	10	45
Peeled ovules	70	95	100
Embryos	90	100	100

*Ovules and embryos were obtained from capsules on 80 DAP.

**Values are based on percentage.

NAA and 0.3% (W/V) Gellan Gum, and adjusted at pH 5.0. Ten ml of the medium was poured into a test tube (25 x 120 mm), and an ovule or an embryo was placed on the medium. Cultures were maintained at 24°C under white fluorescent light of 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with the photoperiod of 12 hrs light and 12hrs dark at 24°C. The experiment was conducted twice using 10 explants for each condition.

The day of germination was determined when radicle grew to 1 mm in length. We defined normal growth of ovules and embryos by increases in the size of bulb scales.

Germinated ovules and embryos were transferred to fresh MS media containing 0.3% (w/v) Gellan Gum, and 0, 3, 6, 9, 12 and 18 % (w/v) sucrose. The fresh weights of bulblets were measured every 90 days.

RESULTS AND DISCUSSION

For the establishment of a rapid and efficient propagation system of *L. auratum* from zygotic embryos, it is necessary to suppress dormancy. Dormancy was observed both in mature seeds and in plantlets soon after germination. *In vitro* cultures of immature ovules and embryos are ideal methods to suppress dormancy. From preliminary experiments, the rate of germination was likely depending on the developmental stages of ovules. We used ovules of 40 to 110 DAP to reveal the best stage for *in vitro* culture. Seed coats and endosperm were the other factors that possibly affect the rates of germination. We also compared the culture efficiency between ovules, ovules with their seed coats peeled off (peeled ovules).

Ovules and embryos did not synchronously germinate even in the culture condition. Table 1 shows the rates of germination of ovules, peeled ovules, and embryos (80 DAP) after 30, 60 and 90 days of culture. All of the embryos germinated within 60 days, while some of the peeled ovules needed more than 60 days for germination. Same patterns were observed in 40 to 110 DAP materials (data not shown). To avoid elimination of genetic diversities of zygotes, we decided to culture all specimens for at least 90 days.

Figure 2 shows the rate of germination from ovules,

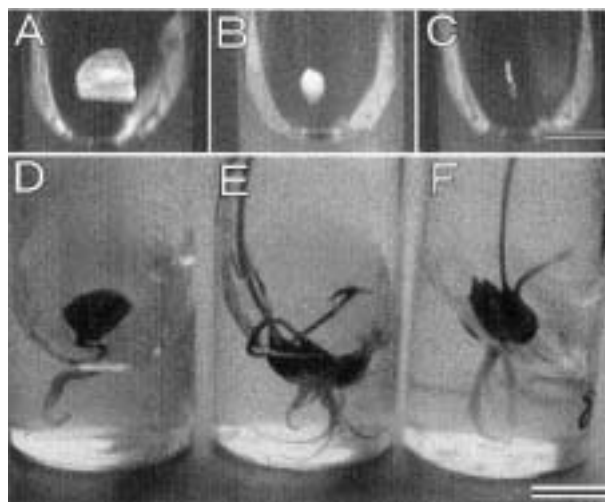


Figure 1. *In vitro* germination of *Lilium auratum* Lindl. An ovule (A), an ovule detached its seed coats (peeled ovules) (B), and an embryo (C) were placed on MS medium containing 5 x 10⁻⁹M NAA. D to F show typical fates of the ovule (D), the peeled ovule (E), and the embryo (F) after 3 months of culture. The bar indicates 10 mm.

peeled ovules, and embryos in different developmental stages. Germination rates were low in 40 DAP materials. Embryos of *L. auratum* that were obtained in this experiment were smaller than 1 mm in length on 40 DAP. The low germination rate observed in 40 DAP samples was likely to be attributable to the immaturity of embryos. The rates of germination increased after 50 DAP, and were quite different between ovules and peeled ovules after 60 DAP. The highest rate in intact ovules was 85%, which was recorded from 50 DAP samples. After this period, the rates of germination in intact ovules decreased along with maturation. On the other hand, peeled ovules and embryos showed high rates after 50 DAP, with an exception of a transient decrease in peeled ovules on 90 DAP. Inhibition of germination by seed coats is a well known phenomenon (King 1976; Perata *et al.*, 1990). In *L. auratum*, the seed coats were likely to inhibit the germination in later stages of ovule maturation.

Typical examples of ovules, peeled ovules, and embryos after 3 months of culture are shown in Figure 1D-F. More than 50% of peeled ovules and embryos showed normal morphological development, however, the majority of the germinated intact ovules showed abnormalities such as swelling of leaves, callus formation, and necrosis. Thus, it was necessary to evaluate the initial materials for culture not only by the rate of germination but also by the rate of normal development in culture. In this experiment, ovules and embryos that developed into bulblets of diameters of at least 5 mm were regarded as normal plantlets.

Figure 3 shows the rate of normal development of ovules, peeled ovules and embryos from different days after

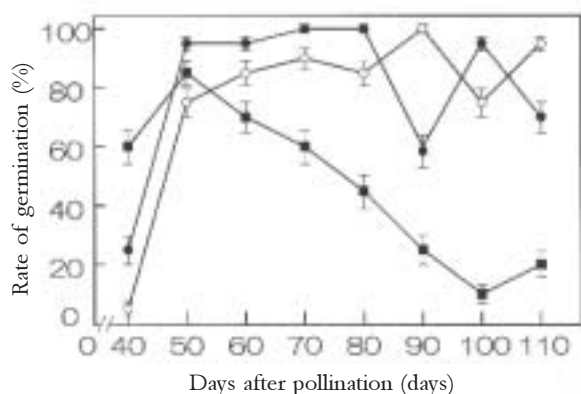


Figure 2. Influence of days after pollination to germination. Ovules (■), peeled ovules (●), and embryos (□) were obtained every 10 days and cultured for 3 months. Values are means ± SE (n=20).

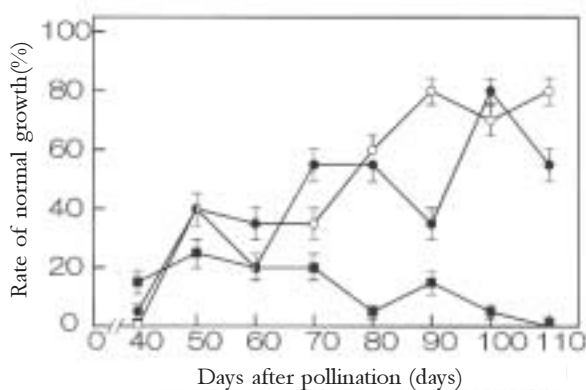


Figure 3. Influence of days after pollination to normal development. Ovules (■), peeled ovules (●), and embryos (□) were obtained every 10 days and cultured for 3 months. Values are means ± SE (n=20).

pollination. Only 25 % or less ovules developed normally. On the other hand, peeled ovules and embryos showed high rates of normal development even after 70 DAP. Differences in the rates between ovules and peeled ovules clearly showed the inhibitory effects of seed coats on normal development (Fig. 3). The rates of germination increased after 50 DAP (Fig. 2), while those of normal growth rose after 70 DAP in peeled ovules and embryos (Fig. 3). This difference in time indicates that immature embryos ceased radicle development and gave rise to abnormal structures, suggesting that it was necessary to wait maturation of embryos for efficient *in vitro* culture to preserve genetic diversities. The inhibitory effect of the seed coats was also not simple. Normal development was completely inhibited by seed coats in ovules after 80 DAP (Fig. 3), although 45% of those ovules were still able to germinate. Seed coats may have several different mechanisms of inhibiting germination and early development. The endosperm gradually became jelly like after 60 DAP, and turned hard in those 80

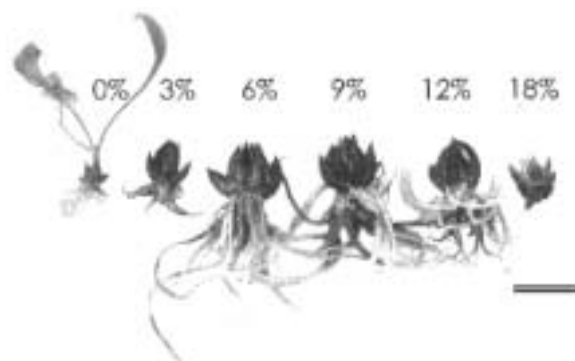


Figure 4. Typical bulblets developed on media containing different concentration of sucrose. The germinated ovules were transferred onto the media, and cultured for 180 days. Media were changed every 90 days in the total culture period. Percentage presented in the figure show the concentration of sucrose. The bar indicates 10 mm.

DAP seeds. On the other hand, seed coats lost water after 80 DAP, became thin and dry, and stuck to the endosperm. In this seed condition, it was difficult to remove the seed coats. The long process of removing the seed coats may have caused the peeled ovules to be dehydrated. It seemed that the decrease in germination rate in 90 DAP samples was caused by this dehydration process. While it is difficult to obtain peeled ovules in a good condition, embryos obtained from 90 DAP ovules developed to normal seedlings at a high rate. We concluded that embryos at 90 DAP were the best material for the propagation of *L. auratum*.

Normal growing plantlets from ovules and embryos were transferred onto a fresh media containing 0, 3, 6, 9, 12 and 18 % (w/v) sucrose. We transferred them every 90 days onto the same media, and continued the culture for 180 days. The typical bulblets in each medium are shown in Figure 4. Development of bulblets was strongly affected by their individual characteristics. Therefore, statistical evaluation of optimum concentration of sucrose was difficult. However, some bulblets cultured on medium containing 9 % sucrose grew up to 9 g in fresh weight. From our experience, bulblets of about 10 g in fresh weight are possible to develop flowers within a year. Leaves appeared from bulblets cultured on a medium containing no sugars (Fig. 4). Uptake of sugars from the media was likely to suppress the development of leaves in lilies.

Our purpose was to accelerate the development of mature bulbs of *L. auratum* by *in vitro* ovule culture. After 70 DAP, peeled ovules and embryos developed normally in culture. The highest germination rate and growth rate were recorded in 90 DAP or older embryos. For propagation and conservation of *L. auratum*, *in vitro* culture, 90 DAP embryos most effectively suppressed dormancy. However, there was a slight problem to use 90 DAP embryos in certain situations,

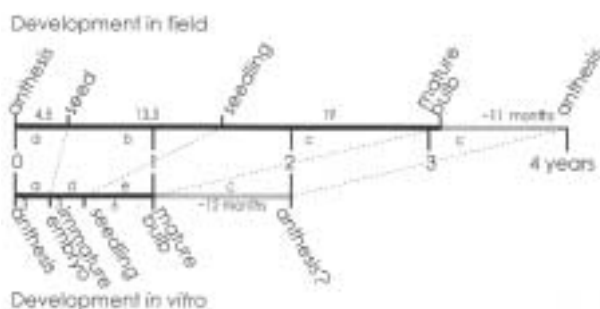


Figure 5. Development of *L. auratum* both in field and *in vitro*. Stages of plants are indicated with necessary periods. The characters a to e indicate methods or states; a) maturation of seeds in natural condition, b) germination, c) development in natural condition, d) *in vitro* embryo or ovule culture, e) *in vitro* culture of bulblets. The time of anthesis of the *in vitro* growing progeny is a prospect in an appropriate condition.

for example, when non-professional personnel propagates lilies. Alternatively, peeled ovules on the 70 DAP can be used, although the theoretical genetic diversity should somehow drop since the rate of normal growth of 70 DAP peeled ovules was only 55 %. After germination, rapid development of bulblets was achieved by culture in the presence of high concentration of sucrose.

In natural condition, *L. auratum* takes 22 months from pollination to develop leaves, and two or three years more for the bulbs to mature. We found that the culture of embryos was an effective method to induce rapid germination and growth of *L. auratum*. In our culture system, it took 6 months from pollination to develop leaves, and another 6 months to produce bulblets of about 10 g in fresh weight (Figure 5) depending on individual characteristics. This method is useful to propagate *L. auratum* without theoretically decreasing its genetic diversities. Since the method needs no professional skills, it is possible to apply in practical courses in schools as an educational activity.

ACKNOWLEDGEMENTS

The authors are grateful to Professor H. Gemma of the Institute of Agriculture and Forestry, University of Tsukuba for his encouragement throughout the course of this study. TF thanks his colleagues in Sashima Senior High School for giving an opportunity to pursue this study.

REFERENCES

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

King, R.W. 1976. Abscisic acid in developing wheat grains and its relationship to grain growth and maturation. *Planta* 132:43-51.

Maesato, K., Sarma, K.S., Fukui, H. and Hara, T. 1991. *In vitro* bulblet induction from shoot apices of *Lilium japonicum*. *HortScience* 26: 211.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

Niimi, Y. 1995. *In vitro* propagation and post-*in vitro* establishment of bulblets of *Lilium japonicum* Thunb. *Journal of the Japanese Society for Horticultural Science* 63: 843-852.

Novak, F.J. and Petru, E. 1981. Tissue culture propagation of *Lilium* hybrids. *Scientia Horticulturae* 14: 191-199.

Perata, P., Picciarelli, P. and Alpi, A. 1990. Pattern of variation in abscisic acid content in suspensors, embryo, and integuments of developing *Phaseolus coccineus* seeds. *Plant Physiology* 94:1776-1780.

Sheridan, W.F. 1968. Tissue culture of the monocot *Lilium*. *Planta* 82: 189-192.

Takayama, S. and Misawa, M. 1983. A scheme for mass propagation of *Lilium* *in vitro*. *Scientia Horticulturae* 18: 353-362.