# Studies on Clonal Mass-Propagation of *Calanthe sieboldii* by Using Tissue-Cultured Shoot Primordium Method.*

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# 苗条原基法によるキエビネのクローン大量増殖に関する研究*

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**Summary**

*Calanthe sieboldii*, a Japanese terrestrial orchid, is one of the most popular orchids for ornamental purposes in Japan. However, this species does not propagate vigorously in both natural and tissue culture conditions. Thus, tissue-cultured shoot primordium method (Tanaka and Ikeda, 1983) was applied for mass-propagation of this species.

By improving the sterilizing method, the meristems excised from November to May were less contaminated (contamination rate = 0～6%). Survival rate of meristems in B5 medium was better than that in MS and 1/2MS medium as the basal liquid medium. Modification of the B5 medium by reducing of the concentrations of micro elements, $CaCl_2$ and Fe-EDTA resulted in the increase of the survival rate. Shoot primordia were induced from the meristems in this modified B5 medium supplemented with 2.0 mg/ℓ BA. Then protocorm-like bodies (PLBs) were obtained from shoot primordia after transplanting onto agar medium with the same composition. These PLBs were massively and rapidly propagated by culturing in the same liquid medium. After two months of culture in the liquid medium, the number and fresh weight of PLBs became about 11 and 10 times, respectively. PLBs regenerated plantlets easily by plating onto agar medium. The plantlets were then acclimatized easily.

Successful method of clonal mass-propagation of *Calanthe sieboldii* established in the present experiment may be applied for other species of *Calanthe*.

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## Introduction

Since mass-propagation of orchid with meristem culture was successfully reported in *Cymbidium* by Morel (1960), many horticulturally important, epiphytic orchids such as *Cymbidium*, *Cattleya*, *Dendrobium* and *Oncidium* have become popular due to the application of this tissue culture technique. Whereas propagation by tissue culture of the terrestrial orchids such as *Paphiopedilum* (Huang, 1988), temperate *Cymbidium* (Wang, 1988), *Calanthe* (Tahara, 1977) was reported previously, but has not yet been applied practically.

Orchids have generally been propagated through protocorm-like-bodies(PLBs), but recently, it was shown that they can be propagated through tissue-cultured shoot primordium (Sato *et al.*,1987). "The tissue-cultured shoot primordium" has some specific characteristics for mass propagation. It proliferates vigorously and rapidly, and easily regenerates numerous plantlets of any plant species when it is placed onto agar medium with appropriate hormonal combination (Tanaka and Ikeda, 1983). However it has been difficult to induce such tissue-cultured shoot primordium line in the terrestrial species of orchids except for *Spiranthes sinensis* (Sato *et al.*,1987).

The spring-flowering species of the Japanese *Calanthe* and their hybrids are cultivated as useful ornamental orchids in Japan. In these species, beautiful hybrid and polyploid plants have been obtained by artificial inter-specific crosses (Tahara, 1986) and colchicine treatment, respectively (Tahara and Kato, 1987). On the other hand, the plants of Japanese *Calanthe* species are now rarely found in natural habitats because of the extensive exploitation of the growing area and plunder of the plants by men.

*Calanthe* species have low propagation rate in natural as well as cultivated conditions. Therefore, establishment of efficient tissue culture method of *Calanthe* has been seeked. In *Calanthe*, callus and shoot formation has been reported, but production and propagation of PLBs and shoot differentiation from PLBs were very difficult (Tahara, 1977; Shimasaki and Uemoto, 1987). Consequently, *Calanthe* orchids are still not so popular as ornamental plants.

In this study, therefore, the tissue-cultured shoot primordium method was applied for clonal mass propagation of *Calanthe sieboldii* (Fig.1).

## Materials and Methods

[Plant material and sterilization]

Shoot buds of *Calanthe sieboldii* Decne. were obtained from the plants cultivated in the Hiroshima Botanical Garden.

After removal of leaf sheathes, shoot buds were sterilized by shaking in 0.1%(v/v) benzalkonium chloride solution for 5 or 10 minutes, 1%(v/v) sodium hypochlorite solution for 5 or 10 minutes, and 70%(v/v) ethanol for 5 or 10 seconds, and finally rinsed with sterilized distilled water three times. Shoot meristems with one or two leaf primordia of 0.3-0.5 mm wide and 0.5-0.7 mm long were microsurgically taken out from the axillary buds on the shoot bud.

Fig.1. Flowering *Calanthe sieboldii*.

[Culture media]

Liquid media of Gamborg's B5(B5)(Gamborg *et al.*, 1968), Murashige and Skoog(MS) (Murashige and Skoog, 1962) and 1/2 MS media (half strength of macro, micro and organic elements) were used as the basal media. Each medium was supplemented with benzyladenine (BA) and $\alpha$ -naphthalene acetic acid (NAA) at different concentrations and adjusted at pH 5.7～5.8 with KOH or HCl (Table 4,5,6,7). Sucrose concentrations added were 20g / ℓ for B5 medium, and 30g / ℓ for MS and 1/2MS media. Among the 3 basal media, B5 medium was selected for testing the effect of the components of the media on the survival rate of shoot meristem. The constituents were divided into three groups; macro ($NaH_2PO_4 \cdot 2H_2O$, $KNO_3$, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$), micro ($CaCl_2$, Fe-EDTA, $MnSO_4 \cdot 4H_2O$, $H_3BO_3$, $ZnSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, KI), and organic elements (Nicotinic acid, Thiamine · HCl, Pyridoxine · HCl, myo-Inositol). $CaCl_2$ is usually considered as a macro element, but in this experiment it is treated as a micro element in convenience. Then, each of three elementary groups was varied at 3 different strengths, 1 , 1/2 and 1/4, respectively, and combined with each other to make 27 kinds of the media (Table 7). All of these modified B5 medium were supplemented with 0.02mg / ℓ BA and 2.0mg / ℓ NAA (B5-9) or with 2.0mg/ ℓ BA alone (B5-16). Induced PLBs were plated onto No.5 agar medium (see Table 7) supplemented with BA and NAA each at a concentration of 0.0 or 0.02mg/ ℓ , sucrose at 10 or 20g/ ℓ , and 9g/ ℓ agar. Test tubes and Erlenmeyer flasks used for these experiments were sealed with aluminium foil before autoclaving for 15 minutes at 121 ℃.

[Culture conditions]

Shoot meristems of *Calanthe sieboldii* were placed in test tubes(30mm X 200mm) containing 25mℓ liquid medium and cultured on a rotary culture equipment (100cm diameter) at 2 cycles/minute. The cultures were maintained at 22℃ under continuous illumination of 3,000 to 5,000 lux by fluorescent lamps. Plantlets were induced by transferring the cultures onto agar medium at 25 ℃ under 16-hour photoperiod of 1,500 lux fluorescent lamps.

[Sections of cultures]

The materials cultured were cut into five or six portions, and fixed with FAA (70% ethanol: formalin: glacial acetic acid=90: 5: 5). The fixed materials were embedded in paraffin after dehydration with a series of n-BuOH. They were cut into 10-15 $\mu$m sections in thickness and stained with Delafield's hematoxylin.

Other experimental methods were described in results and discussion.

## Results and discussion

1) Contamination of microbes

Since the *in vivo* shoot bud culture systems of *Calanthe* previously reported were highly contaminated with bacteria, fungi and so on, it has been considered that elimination of microbial contamination from shoot meristem culture is rather difficult (Tahara, 1977; Shimasaki and Uemoto, 1987). In contrast to the high contamination rate (25-48%) in sterilization method I, very low rates of microbial contamination (0.0-5.9%) were obtained using the method II (Table 1).

By using the different sterilization method for shoot bud culture of *Calanthe*, Tahara (1977) previously reported that microbial contamination rate was low in March and high in May. Similar result was also obtained in the present experiment. It seems

Table 1. Difference in contamination rate of shoot meristems of *Calanthe sieboldii* due to different sterilization methods and plating season

| | Sterilizing method* | Plating season | Contamination of microbes (%) |
|---|---|---|---|
| I | 0.1% benzalkonium chloride for 5 minutes → 1% sodium hypochlorite for 5 minutes → 70% ethanol for 5 seconds → sterilized distilled water 3 times | April 1988 | 8/ 32 ** 25.0% |
| | | May | 42/168 25.0 |
| | | June | 35/ 79 44.3 |
| | | July | 14/ 29 48.3 |
| | Total | | 99/308 32.1 |
| II | 0.1% benzalkonium chloride for 10 minutes → 1% sodium hypochlorite for 10 minutes → 70% ethanol for 10 seconds → sterilized distilled water 3 times shoot buds were vigorously shaken in each step | November 1988 | 4/137 2.9 |
| | | December | 1/ 85 1.2 |
| | | March 1989 | 0/ 31 0.0 |
| | | April | 0/ 32 0.0 |
| | | May | 9/153 5.9 |
| | Total | | 14/438 3.2 |

* After removing leaf sheath, lateral buds were sterilized for 5 or 10 minutes in 0.1%(v/v) benzalkonium chloride solution, for 5 or 10 minutes in 1%(v/v) sodium hypochlorite solution, 5 or 10 seconds in 70%(v/v) ethanol, and finaly rinsed three times with sterilized distilled water. After these procedures, apical meristems with one or two leaf primordia were excised.

** Number of shoot meristems contaminated / Number of shoot meristems plated.

that shoot buds are wrapped firmly with leaf sheathes in late autumn through spring, during which microbes may not propagate vigorously. However, in May, leaf sheathes become loose as leaves sprout, and consequently new axillary buds on shoot buds can be exposed to active microbes. Also, micro surgical excision of shoot meristems is very difficult in May through October, because growing axillary buds become to have hard surface.

Our sterilization method for *Calanthe* is only applicable for the meristem culture during cool season, but it is easier and better than other sterilizing methods. For example, the time of sterilization by this method is 25 minutes which is shorter than that of Tahara's method (60 minutes), and contamination rate by our method was 5.9% even in May and that of Tahara's method was 52%.

The size of shoot meristem used for the previous experiment was 1.5-2.0㎜ in width and length (Tahara, 1977). In our previous experiment using the same size of explants, survival rate was very high but meristem tissues rapidly and vigorously developed into single shoots. In the present experiment, we obtained PLBs and mass of shoot primordia by using 0.3-0.5㎜ size of shoot meristems. Therefore, the size of explant is an essential factor for obtaining the cultures suitable for mass propagation. Small size of meristem explants has been used for elimination of viruses in many plant species. In *Cymbidium*, virus-free plant was obtained by culturing 0.1㎜ shoot meristem (Morel, 1960,1964).

As virus infection has been a serious problem for cultivated *Calanthe*, it is necessary to investigate whether virus free plants can be obtained by shoot meristem culture.

2) Effect of basal medium

Among the three basal media tested, B5 medium gave the highest survival rate of shoot meristem (56%), and those of 1/2 MS and MS were 15.7% and 4%, respectively (Table 2).

Table 2. Survival rate of shoot meristems of *Calanthe sieboldii* in different basal media

| Basal medium | Survival rate | | Plating season |
|---|---|---|---|
| MS [1] | 3/75 [3] | 4.0% | April 25 - May 16, 1988 |
| 1/2MS [2] | 8/51 | 15.7 | June 27 - July 4, 1988 |
| B 5 [1] | 43/77 | 56.0 | May 16 - May 25, 1988 |

[1] Concentration of each element for each medium was full strength. MS medium was supplemented with 3% sucrose, and B5 medium was supplemented with 2% sucrose.

[2] Concentrations of macro, micro and organic elements were reduced to the half of MS. 3% sucrose was added.

[3] Number of shoot meristems survived / (Number of shoot meristems plated – Number of meristems contaminated). Survived shoot meristems were defined as those not turning brown after one month of culture.

Table 3. Ionic concentration of MS, 1/2MS and B5 medium

| Medium | Composition of cation (%) | | | | | Composition of anion (%) | | | | Total ionic concentration of medium (me/ℓ) |
|---|---|---|---|---|---|---|---|---|---|---|
| | $NH_4^+$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | $Na^+$ | $NO_3^-$ | $H_2PO_4^-$ | $SO_4^{2-}$ | $Cl^-$ | |
| MS | 41.5 | 40.3 | 12.1 | 6.0 | | 79.4 | 2.4 | 6.0 | 12.1 | 49.6 |
| 1/2MS | 41.5 | 40.3 | 12.1 | 6.0 | | 79.4 | 2.4 | 6.0 | 12.1 | 24.8 |
| B5 | 6.4 | 77.4 | 6.4 | 6.4 | 3.4 | 77.4 | 3.4 | 12.7 | 6.4 | 31.9 |

In the previous reports of the shoot meristem culture of *Calanthe*, Tahara (1977) and Shimasaki and Uemoto (1987) used MS medium as the basal medium. Total ionic concentration of macro inorganic elements of B5 medium is lower than that of MS medium, but higher than that of 1/2MS medium (Table 3). Percentage of $NH_4^+$ in B5 medium is very low and that of $K^+$ is very high compared to other two media (Table 3). Therefore, it is possible that these differences of components might affect the survival of the meristem of *Calanthe*.

For shoot meristem culture of *Calanthe*, only agar medium has been used (Tahara, 1977; Shimasaki and Uemoto, 1987). In the present study, we used liquid medium with a rotary culture at 2 cycles/minute. In liquid culture, it is expected that nutrients can be continuously supplied to the meristem tissues. Moreover, both inhibition of shoot elongation and enhancement of shoot primordia formation occurred not only by application of appropriate growth regulators but also by changing the polarization to the direction of gravitation with continuous rotation. Hoppe and Hoppe (1987) used liquid medium by the same reason and succeeded in shoot meristem culture of an European terrestrial orchid, *Ophrys apifera*.

3) Effect of growth regulators

In MS and 1/2MS medium, the effect of supplemented growth regulators was not clean because plated shoot meristems almost died (Table 4,5). In contrast, they survived in all of the combinations of BA and NAA concentrations, when B5 medium was used. Consequently, the most effective combination of plant growth regulators was not clarified (Table 6).

In the previous reports, requirement of growth regulators differed according to the size of explants. Tahara (1977) reported that relatively big size(1.5-2.0㎜) of shoot-tips of *C. discolor* and *C. sieboldii* required the addition of NAA and BA to MS medium for the survival and the formation of PLBs and callus-like tissues. However, smaller size of shoot meristems of *C. discolor* survived on 1/8 MS medium without growth regulators and formed green tissues (Shimasaki and Uemoto, 1987).

On the shoot meristem culture of *Cymbidium* Sazanami var. "Haruno-umi", the optimum concentration of growth regulators was affected by the size of shoot meristem, namely number of leaf primordia. In the medium without growth regulators, organ formation rate in the culture of shoot apices with 4 and 6 leaf primordia (70-100%) was higher than that with 2 leaf primordia (30%). However, the rate of

Table 4. Results of the shoot meristem culture of *Calanthe sieboldii* in MS medium modified by the combination of NAA and BA

| NAA \ BA (mg/ℓ) | 0.0 | 0.02 | 0.2 | 2.0 | 4.0 |
|---|---|---|---|---|---|
| 0.0 | 1* 1/4 ** | 6 0/3 | 11 0/2 | 16 1/4 | 21 0/2 |
| 0.02 | 2 0/3 | 7 0/3 | 12 0/3 | 17 0/3 | 22 0/3 |
| 0.2 | 3 0/2 | 8 0/2 | 13 0/4 | 18 0/2 | 23 0/2 |
| 2.0 | 4 0/3 | 9 0/3 | 14 0/4 | 19 0/4 | 24 0/3 |
| 4.0 | 5 0/3 | 10 0/3 | 15 0/3 | 20 0/4 | 25 1/3 |

* Treatment No.

** Number of survived shoot meristems / (number of shoot meristems plated(4 shoot meristems) - number of shoot meristems contaminated). Survived shoot meristems were defined as those not turning brown after one month of culture.

Table 5. Results of the shoot meristem culture of *Calanthe sieboldii* in 1/2MS medium modified by the combination of NAA and BA

| NAA \ BA (mg/ℓ) | 0.0 | 0.02 | 0.2 | 2.0 | 4.0 |
|---|---|---|---|---|---|
| 0.0 | 1* 0/1 ** | 6 0/2 | 11 1/3 | 16 0/4 | 21 1/2 |
| 0.02 | 2 0/2 | 7 0/2 | 12 1/3 | 17 0/4 | 22 1/1 |
| 0.2 | 3 2/2 | 8 0/1 | 13 0/2 | 18 1/2 | 23 0/1 |
| 2.0 | 4 0/2 | 9 0/1 | 14 0/3 | 19 0/2 | 24 0/2 |
| 4.0 | 5 0/2 | 10 0/2 | 15 0/2 | 20 1/2 | 25 0/1 |

* Refer to table 4.

** Refer to table 4.

Survived shoot meristems were defined as those not turning brown after one month of culture.

Table 6. Results of the shoot meristem culture of *Calanthe sieboldii* in B5 medium modified by the combination of NAA and BA

| NAA \ BA (mg/ℓ) | 0.0 | 0.02 | 0.2 | 2.0 | 4.0 |
|---|---|---|---|---|---|
| 0.0 | 1* 2/4 ** | 6 0/3 | 11 2/3 | 16 2/2 | 21 1/3 |
| 0.02 | 2 3/4 | 7 3/4 | 12 2/4 | 17 3/3 | 22 0/4 |
| 0.2 | 3 2/3 | 8 1/3 | 13 1/3 | 18 2/3 | 23 2/2 |
| 2.0 | 4 2/3 | 9 3/4 | 14 0/3 | 19 1/2 | 24 1/1 |
| 4.0 | 5 1/4 | 10 2/3 | 15 1/3 | 20 2/2 | 25 3/4 |

* Refer to table 4.
** Refer to table 4.
Survived shoot meristems were defined as those not turning brown after one month of culture.

organ formation of the latter was increased to 80% by the addition of BA (Kim and Kako, 1982).

These results suggest the possibility that the shoot meristem culture of *Calanthe sieboldii* may also be affected by the growth regulators if the size of explants is changed.

4) Effect of each element of B5 medium

In each medium with 1/4 strength micro elements (medium No.7～9, No.16～18, No.25～ 27; see Table 7), survival rate of shoot meristems after one month of culture was higher than those containing 1/2 or full strength micro elements.

A similar result has been reported in shoot meristem culture of *C. discolor*, in which survival rate was the best at 1/8 strength of MS medium (Shimasaki and Uemoto, 1987). On the shoot meristem culture of *Ophrys apifera*, reducing the concentration of $Ca^{2+}$ to the level of micro elements resulted in high rate of survival (Hoppe and Hoppe, 1987). The addition of micro elements except Fe-EDTA caused marked inhibition of growth of seedlings of *Bletilla striata* (Ichihashi, 1979). From these results, therefore, it may be concluded that reduction of the concentrations of $Ca^{2+}$ and minor elements has beneficial effect on the survival of explants.

Three months after initiation of primary-culture, the cultures were classified into six types; (1) producing abnormal structure with growing leaf primordia, (2) slightly swell-

Table 7. Results of the shoot meristem culture of *Calanthe sieboldii* in different concentration and combination of macro, micro and organic elements of B5 medium

| Medium No. | Concentration and combination (ratio to the original medium) | | | Survival rate[1] | | Type of the cultures in B5-9 medium[3] | Type of the cultures in B5-16 medium[4] |
|---|---|---|---|---|---|---|---|
| | Macro elements | Micro elements | Organic elements | Number | % | | |
| 1 | 1 | 1 | 1 | 1/8 | 13 | —[2] | — |
| 2 | 1 | 1 | 1/2 | 0/8 | 0 | — | — |
| 3 | 1 | 1 | 1/4 | 2/8 | 25 | — | A1 |
| 4 | 1 | 1/2 | 1 | 3/7 | 43 | — | — |
| 5 | 1 | 1/2 | 1/2 | 5/7 | 71 | C1,SP1 | SP1 |
| 6 | 1 | 1/2 | 1/4 | 3/7 | 43 | — | — |
| 7 | 1 | 1/4 | 1 | 8/8 | 100 | C2,A1 | Ab1,A1 |
| 8 | 1 | 1/4 | 1/2 | 6/8 | 75 | C2,A1 | PLB1,Ab1 |
| 9 | 1 | 1/4 | 1/4 | 6/8 | 75 | SP1,Sh1 | A1,C1,PLB1 |
| 10 | 1/2 | 1 | 1 | 2/8 | 25 | — | — |
| 11 | 1/2 | 1 | 1/2 | 0/8 | 0 | — | — |
| 12 | 1/2 | 1 | 1/4 | 0/8 | 0 | — | — |
| 13 | 1/2 | 1/2 | 1 | 2/8 | 25 | — | Sh1 |
| 14 | 1/2 | 1/2 | 1/2 | 3/7 | 43 | — | Ab1 |
| 15 | 1/2 | 1/2 | 1/4 | 2/8 | 25 | — | A2,Sh1 |
| 16 | 1/2 | 1/4 | 1 | 6/7 | 86 | C1,A1 | A1,Ab3 |
| 17 | 1/2 | 1/4 | 1/2 | 7/8 | 88 | PLB1,Sh1 | C1,Ab1 |
| 18 | 1/2 | 1/4 | 1/4 | 4/8 | 50 | C1,Sh1 | C1,Ab1 |
| 19 | 1/4 | 1 | 1 | 0/8 | 0 | — | — |
| 20 | 1/4 | 1 | 1/2 | 1/8 | 13 | — | — |
| 21 | 1/4 | 1 | 1/4 | 0/8 | 0 | — | — |
| 22 | 1/4 | 1/2 | 1 | 1/8 | 13 | — | — |
| 23 | 1/4 | 1/2 | 1/2 | 1/8 | 13 | — | — |
| 24 | 1/4 | 1/2 | 1/4 | 1/8 | 13 | | |
| 25 | 1/4 | 1/4 | 1 | 5/8 | 63 | A2 | Sh1 |
| 26 | 1/4 | 1/4 | 1/2 | 6/8 | 75 | A2 | Sh1 |
| 27 | 1/4 | 1/4 | 1/4 | 5/8 | 63 | A1 | — |

Four shoot meristems were sampled in each medium.

1) Survival rate(%)= [Number of shoot meristems survived /(number of shoot meristems plated - number of shoot meristems contaminated)] × 100. Survival rate was investigated after one month of culture. The percentage in each medium (No.1-27) was calculated from the total explants cultured in B5-9 and B5-16 media.

2) Growth response after 3 months of culture. — : shoot meristems turned brown and died, A : shoot meristems which were slightly swollen but did not turn brown, C : callus-like tissues, SP : shoot primordia, Ab : Abnormal structure growing leaf primordia, PLB : protocorm like bodies and Sh : shoot.

3) B5-9=B5 medium supplemented with 0.02mg/ℓ BA and 2.0mg/ℓ NAA.

4) B5-16=B5 medium supplemented with 2.0mg/ℓ BA.

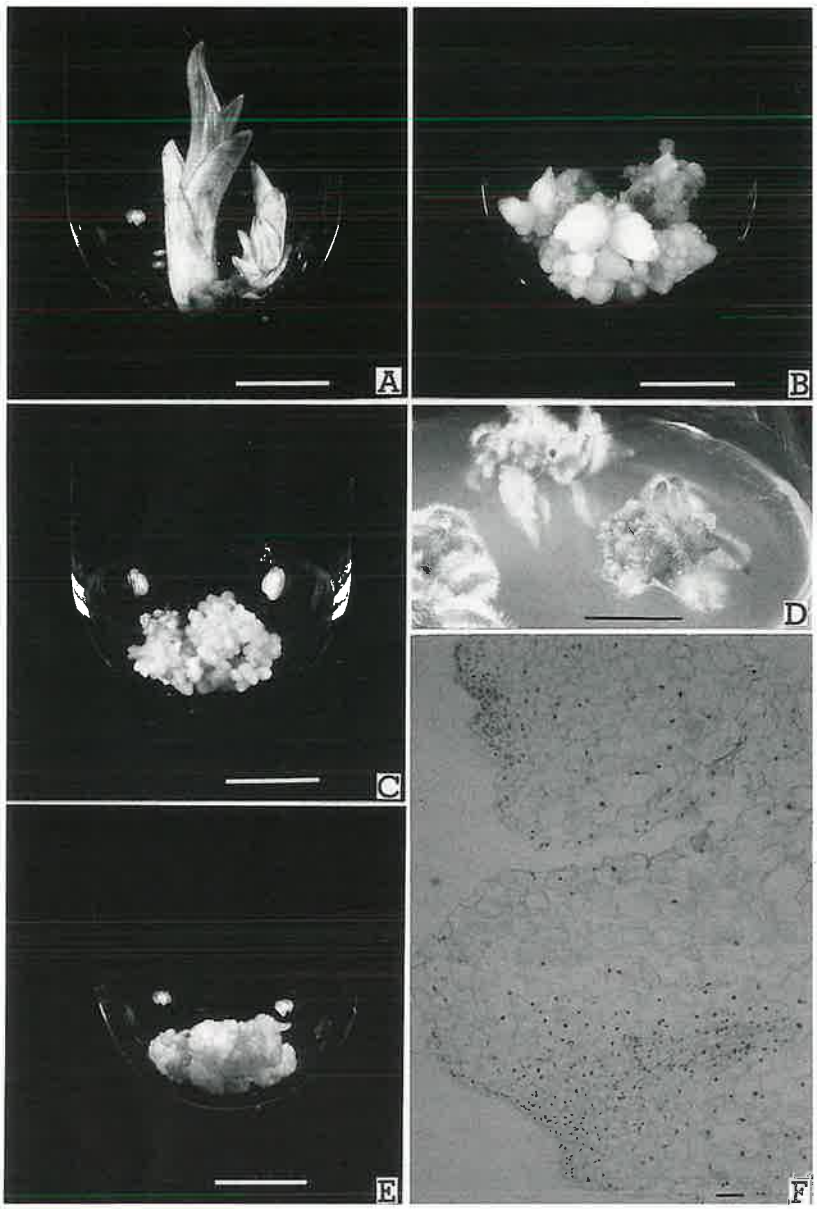


Fig.2. Various types of cultures induced from shoot meristems of *Calanthe sieboldii* in modified B5 medium and section of shoot primordia. A, Shoots (type 3) in B5-16 medium No.13. B, Abnormal PLBs (type 4) in B5-16 medium No.9. C, Callus-like tissues (type 5) in B5-9 medium No.19. D, Differentiated roots from callus-like tissues (type 5) after transplanting on agar medium. E, Shoot primordia (type 6) in B5-16 medium No.5. F, Section of shoot primordia induced in B5-16 medium No.5. A-E ; Scale bar=1cm, F; scale bar=0.2mm.

ing but not turning brown in color, (3) producing one or two shoots in most cases (Fig.2-A), and multiple shoots (five to ten shoots) in few cases, (4) becoming protocorm-like bodies (PLBs) which consisted of the green normal type and the white swollen abnormal type (Fig.2-B), (5) producing friable callus-like tissues with yellowish white-color (Fig.2-C) which turned into shaggy structure with differentiating roots after transfer onto agar medium (Fig.2-D), (6) producing compact shoot primordia with milk-white-colored, conglomerate structure with many small round nodules (Fig.2-E), which obviously differing from callus-like tissues and PLBs.

According to the results on morphological and anatomical analyses for the shoot meristem culture of *Spiranthes sinensis* (Sato *et al.*, 1987), some of the PLBs are considered as precocious branches but most of them were considered as developing somatic embryos (Taniguchi and Tanaka, 1990).

Shoot primordia of *Calanthe sieboldii* induced in the present experiment were conglomerate with small round nodules about 0.5mm in diameter, and without leaf primordia which are common in PLB. By the anatomical observation of shoot primordia, meristem tissues were shown in epidermal layer as well as inner region (Fig.2-F). Therefore, this mass of shoot primordia seemed like a mixture type of a somatic embryo (PLBs) and a shoot primodium (Sato *et al.*,1987).

5) Induction of PLBs

Shoot primordia induced in B5-9 medium No.5 (see table 7) were transplanted onto No.5 agar medium supplemented with 0.02mg/ℓ BA and 0.02mg/ℓ NAA. After three months of culture, many PLBs were induced on the surface of the shoot primordia (Fig.3-A).

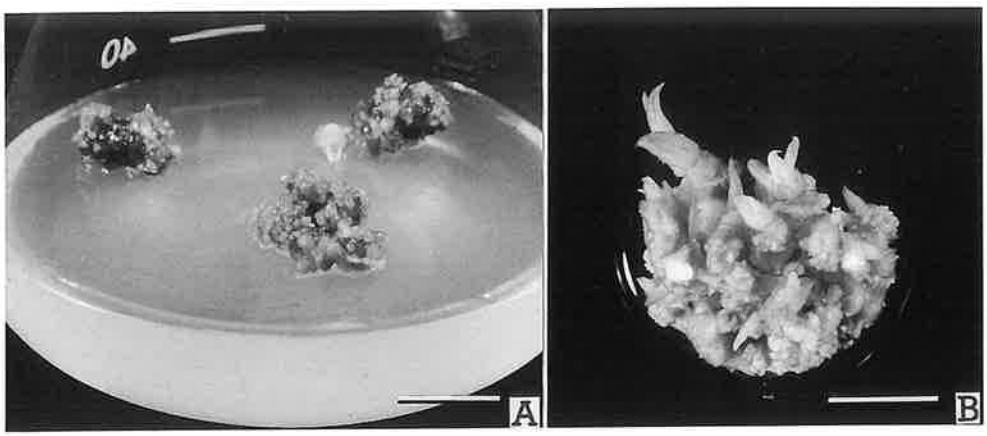


Fig.3-A, PLBs induced from shoot primordia on medium No.5 supplemented with 0.02mg/ℓ BA and 0.02mg/ℓ NAA. B, Proliferated PLBs. Scale bar=1cm.

6) Growth rate of PLBs

Since PLBs did not grow on agar medium 5 months after transfer, they were transplanted into B5 liquid medium (B5-16 medium No.5, see table 7). Then PLBs started to grow again on this medium and formed numerous small PLBs on the base of each PLB. They proliferated vigorously and rapidly (Fig.3-B) and number of PLBs increased about 11 times, and fresh weight about ten times respectively after two months of subculture (Table 8). The growth rate of PLBs of *Calanthe* has not been reported previously because of the difficulty in its induction from shoot meristem. In *Cymbidium* Sazanami var. "Haruno-umi" which had been considered to have high multiplication rate, 7.9 times increase in fresh weight was obtained 8 weeks after inoculation of 0.4g mass of PLBs on optimal ionic composition medium for *Cymbidium* (Ichihashi, 1989). Therefore, the growth rate of *C. sieboldii* was better than that of *Cymbidium* Sazanami var. "Haruno-umi" although the culture medium and condition used were different between these two species.

Thus, practical clonal mass-propagation of *C. sieboldii* seems to be possible by employing our culture method.

7) Shoot induction

PLBs plated onto No.5 agar medium (see table 7) grew vigorously irrespective of the concentrations of BA, NAA and sucrose tested and regenerated roots and shoots. Particularly PLBs plated on medium with 0.02mg / ℓ NAA and 10g/ ℓ sucrose showed best growth. Twenty days after transplating to this medium, PLBs differentiated into young plantlets with buds, rhizoids and roots, and leaves were produced on the plantlets by the 60th days (Fig.4-A,B,C). Plantlets were, then, potted with sphagnum moss after removing agar from roots and acclimatized for 2-3 months in a small space covered with vinyl. These plantlets grew well like seedlings in green house after acclimatization (Fig.5).

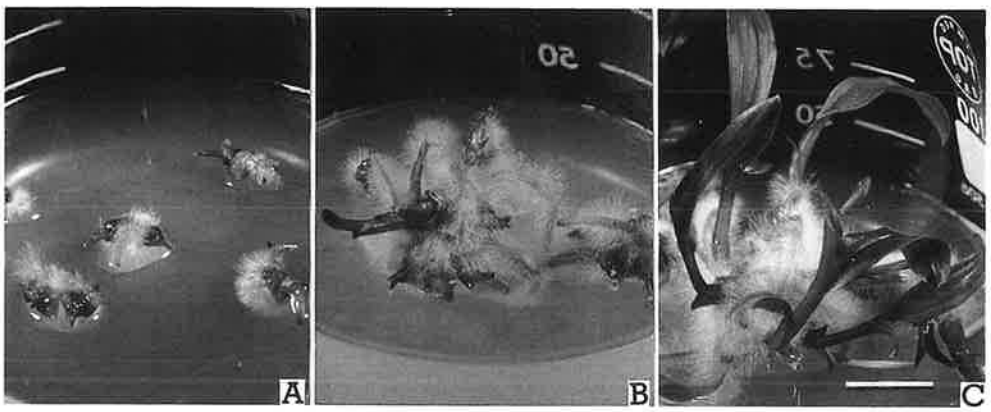


Fig.4. Plantlets fromation from PLBs, 20(A), 60(B) and 120days(C) after transplantaion onto the agar plate. Scale bar=1cm.

Table 8. Propagation rate of PLBs of *Calanthe sieboldii*

| Test tube No. | Initial | | Growth | | | |
|---|---|---|---|---|---|---|
| | | | After one month of culture | | After two months of culture | |
| | Number of PLBs | Fresh weight | Number of PLBs[1] | Fresh weight | Number of PLBs | Fresh weight |
| 1 | 3 | 0.11g | 26 [8.7][2] | 0.56g [5.1][3] | 45 [15.0][2] | 1.86g [16.9][3] |
| 2 | 3 | 0.12 | 5 [1.7] | 0.43 [3.6] | 23 [ 7.7] | 0.70 [ 5.8] |
| 3 | 3 | 0.14 | 12 [4.0] | 0.53 [3.8] | 35 [11.7] | 0.97 [ 6.9] |
| Average | 3 | 0.12 | 14.3 [4.8] | 0.51g [4.3] | 34.3 [11.4] | 1.18g [9.8] |

Three PLBs originated from same bud were plated in each test tube.
1) Number of PLBs over 1.5mm diameter was counted.
2) Number of PLBs after culture / initial number of PLBs.
3) Fresh weight after culture / initial fresh weight.
Medium; B5-16 medium No.5 (see Table 7).

## Concluding remarks

The method described above has a potential to be applied for clonal mass-propagation of other species and hybrids of *Calanthe*. More experiments on media, culture conditions and other factors will be required to apply the method for broad range of genotypes of *Calanthe*. For *C. sieboldii*, somaclonal variation must be investigated using acclimatized plantlets to realize the commercial scale of production by utilizing the propagation method developed in the present study.

Fig.5. Plantlets after acclimatization. Scale bar=5cm.

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## 摘　　要

キエビネは日本に自生する地生ランであり、花が美しいため人気の高いランのひとつである。しかし、繁殖率が低く、また組織培養による大量増殖も困難であるため、苗条原基法（田中・池田1983）を適用し、大量増殖を試みた。

新芽の殺菌方法を改善することにより、11～5月に摘出した茎頂の汚染率は0～6％と低くなった。培地は液体培地とし、直径0.3～0.5㎜の茎頂を植え付けた。生存率は、B5培地においてMSならびに1/2MS培地より高かった。B5培地の成分を無機多量要素、無機微量要素と$CaCl_2$ならびにFe-EDTA、有機微量要素の3要素に分け、それぞれ1、1/2、1/4倍に組み合わせた培地で培養した結果、無機微量要素と$CaCl_2$ならびにFe-EDTAが減少するほど茎頂の生存率は高くなった。無機多量要素を1倍、無機微量要素と$CaCl_2$ならびにFe-EDTAを1/2倍、有機微量要素を1/2倍とした修正B5培地にBAを2.0mg/ℓ添加した培地において、苗条原基集塊が形成された。この苗条原基集塊を寒天培地に置床することによりPLBが誘導された。これらのPLBは苗条原基集塊が形成された液体培地において再び回転培養することにより、急速に増殖した。PLBの増殖率は2か月間の培養においてPLB数は約11倍、生重量は約10倍となった。増殖したPLBは寒天培地に置床することにより容易に幼植物体となり、その後馴化できた。

以上のようにキエビネのクローン大量増殖が可能となり、今後エビネ属の他の種についてもクローン大量増殖の可能性が示唆された。

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