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Original Paper

Ionic compositions play an important role on *in vitro* propagation of PLBs of spring-flowering *Calanthe*

Masao Yamamoto[1,2], Kazumitsu Miyoshi[3], Syoichi Ichihashi[4], Masahiro Mii[1,*]

[1]Graduate School of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan; [2]The Hiroshima Botanical Garden, Hiroshima, Hiroshima 731-5156, Japan; [3]Faculty of Bioresource Sciences, Akita Prefectural University, Akita 010-0195, Japan; [4]Department of Biology, Aichi University of Education, Kariya, Aichi 448-8542, Japan
*E-mail: miim@faculty.chiba-u.jp Tel: +81-47-308-8852 Fax: +81-47-308-8720

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Abstract The effect of ionic composition in medium on proliferation of shoot tip-derived PLBs of two *Calanthe* species, *C. aristulifera* and *C. striata*, was investigated by using the media with 12 different ionic compositions, which were systematically modified according to the method of Ichihashi (1992) based on the composition of modified Gamborg's B5 medium. These two species showed almost the same growth response to the ionic compositions and the growth of PLBs was inhibited on media with higher ratios of NH_4^+ and Ca^{2+}, but promoted with higher ratios of both K^+ and $H_2PO_4^-$. Based on the results of PLB growth, optimum cationic compositions calculated for *C. aristulifera* and *C. striata* were $NH_4^+ : K^+ : Ca^{2+} : Mg^{2+} = 10.4 : 70.0 : 9.6 : 10.0$ and $10.9 : 68.1 : 11.0 : 10.0$, respectively, whereas optimum anionic compositions were $NO_3^- : H_2PO_4^- : SO_4^{2-} = 42.1 : 43.7 : 14.2$ and $43.0 : 42.9 : 14.1$, respectively. The percentages of NO_3^- and $H_2PO_4^-$ in the optimum medium thus obtained were about half and 12-hold of the control B5 medium, respectively. Among the 100 micropropagated *C. striata* plants examined, no obvious variation in flower shape and color were observed except two plants which had larger and thicker flowers than original plants. Those two variants were confirmed to be chromosome doubled tetraploids by flow cytometric analysis.

Key words: *Calanthe*, FCM-analysis, medium, micropropagation, somaclonal variation.

The genus *Calanthe* consists of about 185 species, most of which are known to have high ornamental values because of their beautiful flowers (Kurzweil 2007). In Japan, 21 taxa of *Calanthe* are distributed throughout the country (Karasawa and Ishida 1998), and most of them are spring-flowering habit with difference in flower color, shape and fragrance. By utilizing these natural germplasms, numerous excellent cultivars with large variations in flower characters have already been bred through intra- and inter-specific hybridizations. Although most of these species are known to be recalcitrant in seed propagation, successful establishment of the methods for asymbiotic seed germination has contributed to solve the difficulty (Miyoshi and Mii 1988, 1995a, 1995b; Fukai et al. 1997; Park et al. 2000; Lee et al. 2007; Godo et al. 2010) and large scale production of seedlings has become relatively easy at present. In *Calanthe*, however, it takes more than 4 years to get flowers from seed sowing like as most of the other orchid species, which makes pure line breeding difficult and all of the cultivars are heterozygous. Consequently, the plants with outstanding horticultural values selected among the large seedling populations have been propagated vegetatively by conventional methods such as division of pseudo-bulbs.

In orchids, micropropagation has been employed successfully for the mass-propagation of desired genotypes, especially in various commercially important epiphytic orchids such as *Cymbidium*, *Dendrobium*, *Cattleya* and *Phalaenopsis*. However, micropropagation by tissue culture of terrestrial orchid genera such as *Paphiopedilum*, *Cypripedium* and *Calanthe* is still difficult and efficient method is not available commercially so far for most of these orchids. Although few trials for the *in vitro* culture of the genus *Calanthe* had been conducted till 1980s (Tahara 1977; Shimasaki and Uemoto 1987), first successful result on the micropropagation was reported in *C. sieboldii* by Yamamoto et al. in 1991 using shoot meristems as explants. In commercially micropropagated orchids such as *Cattleya*, *Cymbidium* and *Phalaenopsis*, somaclonal variations caused a serious problem for the production of true-to-type plants (George 1993) and differences in the frequencies of the somaclonal variation among the genotypes were reported (Tokuhara and Mii 1998). However, there have been no reports on the somaclonal variation in *Calanthe*.

The composition of the medium is an important factor for growth and development of plant tissues

Abbreviations: BA, N^6-benzyl adenine; PLBs, protocorm like bodies.
This article can be found at http://www.jspcmb.jp/

in *in vitro* culture, and successful plant tissue culture depends on the choice of nutrient medium (Gamborg et al. 1976). In orchids, various media have been applied for clonal micropropagation or propagation through seed germination for various plant materials (Arditti 2008). However, it is not easy to clarify the suitable medium composition for each plant material and most of the tissue culture studies have been conducted by selecting the better medium among the commonly used available ones. Under these circumstances, Ichihashi and Yamashita (1977) and Ichihashi (1978, 1979, 1980, 1992) has exceptionally conducted detailed studies on the suitable ionic compositions of the media for seed germination, seedling growth and PLBs proliferation of some orchid species by examining total ionic concentration, balance of NH_4^+ and NO_3^- and the ratio of major ionic elements among cations and anions. Through these studies, he clarified the importance of ionic composition for the growth of several orchids and proposed the optimum medium composition for each species (Ichihashi 1980).

The objectives of the present study are to evaluate the effects of media with different composition of ions on the multiplication of PLBs through shoot meristem tip culture and to clarify the somaclonal variation in *Calanthe*.

Materials and methods

Induction of PLBs from shoot meristem culture

Dormant shoot buds were collected from two spring-flowering *Calanthe* species, *C. aristulifera* Rchb. f. and *C. striata* R. Br. ex Lindl., which had been cultivated in the Hiroshima Botanical Garden, Japan, on February and March, respectively. Ten to twenty buds of both species were used for the induction of PLBs. After the removal of leaf sheaths, the shoot buds were sterilized successively with 0.1% benzalkonium chloride solution for 10 min, 1% sodium hypochlorite solution for 10 min, and 70% ethanol for 10 s, and then rinsed three times with sterilized distilled water. From apical and lateral buds in these shoot buds, shoot meristems each with one or two leaf primordia of ca. 0.5 mm width and length, were excised from the axillary buds of the shoot bud with surgical knife under a stereoscopic microscope. Then, each shoot meristem was placed in a test tube (30 mm×200 mm) containing 25 ml liquid medium on a vertical rotary culture equipment rotating at 2 cycles min^{-1}. Totally 35 and 76 shoot meristems were inoculated for *C. aristulifera* and *C. striata*, respectively.

In this study, modified B5 medium, previously employed for the shoot meristem culture of *Calanthe sieboldii* (Yamamoto et al. 1991), was used as the basal medium for both induction and subculture of PLBs. In this medium, original B5 medium (Gamborg et al. 1968) was modified as follows: most of the macro-elements ($NaH_2PO_4 \cdot 2H_2O$, KNO_3, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$) and organic components (nicotinic acid, thiamine·HCl, pyridoxine·HCl, myo-inositol) were reduced to the half strength, and $CaCl_2$ and the microelements (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) to the one-fourth strength, respectively. For the meristem culture of *Calanthe*, this modified B5 medium was used after supplementation with 2 mg l^{-1} N^6-benzyl adenine (BA) and 20 g l^{-1} sucrose. The medium pH was adjusted to 5.7 with 0.1 and 1.0 N NaOH prior to autoclaving at 120°C for 15 min. The cultures were kept at 23°C, under continuous illumination with cool white fluorescent light at photon flux density of 23 μmol m^{-2} s^{-1}.

PLBs propagation medium

To clarify optimum composition of the medium for PLB growth, twelve media with different ionic compositions were prepared by changing the ratios of anions and cations systematically as indicated in Table 1 (Ichihashi 1992). Total ionic concentration of cations and anions were held at 20 me l^{-1} for all the media. In the cationic treatments (media Nos. 1–6), percentage of Mg^{2+} was held at 10% and that of each anionic component was kept constant to clarify the effect of cationic balances on the PLBs growth. Furthermore, in the media Nos. 1, 2 and 4, the balance of the cations, except NH_4^+ and K^+, was also kept constant to clarify the optimum K^+/NH_4^+ ratio. The difference in growth on theses three media was considered as the function of K^+/NH_4^+ ratio because no other factors were changed among these media. The difference in growth was approximated using a quadratic equation ($y=ax^2+bx+c$, y; fresh weight, x; K^+/NH_4^+ ratio). If there was a maximum value within the tested range, the optimum ratio of K^+/NH_4^+ could be fixed to one optimal ratio ($-b/2a$) (Ichihashi and Yamashita 1977; Ichihashi 1980). If there was no maximum value within the tested range, the ratio of ions, which showed the largest value of growth, was considered as the optimum ratio. In the same way, the optimums for K^+/Ca^{2+} and NH_4^+/Ca^{2+} were also determined from the data of media Nos. 4, 5 and 6, and Nos. 6, 3 and 1, respectively.

Next, optimum ratio of three cations, $NH_4^+ : K^+ : Ca^{2+}$ was fixed by using two optimum ratios calculated above as described previously (Ichihashi 1980). In the case of anionic treatments, favorable ratios of $NO_3^- : H_2PO_4^- : SO_4^{2-}$ were assumed by the same way.

The control medium was the same as the modified B5 medium as used for the induction of PLBs. For each treatment, 10 test tubes each containing 0.5 g PLBs were prepared, and increase in the fresh weight was measured after one month of culture. Culture condition was the same as that for induction of PLBs.

Flowering and variations in flower characters of micropropagated plants in C. striata

PLBs of *C. striata* were plated onto modified B5 medium supplemented with 0.02 mg l^{-1} NAA, 10 g l^{-1} sucrose and 2 g l^{-1} gellan gum for plant regeneration. The medium was adjusted to pH 5.7 prior to autoclaving at 120°C for 15 min. The cultures

Table 1. The media compositions tested with different ionic compositions arranged systematically

Medium No.	Percent of cations (%)					Percent of anions (%)			
	NH_4^+	K^+	Ca^{2+}	Mg^{2+}	Na^+	NO_3^-	$H_2PO_4^-$	SO_4^{2-}	Cl^-
1	70	10	10	10	—	70	10	20	—
2	40	40	10	10	—	70	10	20	—
3	40	10	40	10	—	70	10	20	—
4	10	70	10	10	—	70	10	20	—
5	10	40	40	10	—	70	10	20	—
6	10	10	70	10	—	70	10	20	—
7	40	30	20	10	—	80	10	10	—
8	40	30	20	10	—	60	30	10	—
9	40	30	20	10	—	60	10	30	—
10	40	30	20	10	—	40	50	10	—
11	40	30	20	10	—	40	30	30	—
12	40	30	20	10	—	40	10	50	—
Cont.	6.5	80.2	3.2	6.5	3.6	80.2	3.6	13	3.2

Each medium was supplemented with one fourth strength of micro elements, half strength of organic elements of B5 medium(Yamamoto et al. 1991), 2 mgl^{-1} BA and 20 gl^{-1} sucrose, and adjusted to pH 5.7. Total cationic and anionic concentrations of each medium were 20 mel^{-1}.

were grown at 23°C, with 16 h day^{-1} of cool white fluorescent light at a light intensity of 23 μmol m^{-2} s^{-1}.

Four to five months after plating on the medium, plantlets grown into 5–10 cm height with 2–3 leaves were potted with sphagnum moss after removing adhering gellan gum medium from roots, and acclimatized for 2–3 months in a styrofoam box covered with a sheet of vinyl film to keep humidity. After acclimatization, they were cultivated like seedlings in the greenhouse with a controlled minimum temperature of 5°C under natural light and humidity conditions for 4 years to flowering. Totally 100 plants which bore flowers were investigated for the variations in flower characters.

For histological observation, the petals and leaves of 2 propagated plants of *C. striata* which showed different flower characters were hand-sectioned, immersed with glycerin, covered with cover slip and observed under a light microscope.

Ploidy analysis of propagated plants

The ploidy level of variants was confirmed using flow cytometry according to the procedures described previously (Mishiba et al. 2000). About 1 cm^2 tissue segments were excised from young leaves of normal and variant plants of *C. striata*. These leaf segments were chopped into small pieces with surgical knives in 4,6-diamidino-2-phenylindole (DAPI) solution (10 mM Tris-HCl buffer at pH 7.5, 0.1% Triton X-100, 2 mM $MgCl_2$ and 2 mgl^{-1} DAPI) for releasing and staining the nuclei. The DAPI solutions containing free nuclei were filtered with 20 μm nylon mesh to remove the large cell debris, and used as samples for assessing DNA contents by flow cytometry (CA II, Partec Ltd. Munster, Germany). DNA contents of the variants were estimated by using *Bletilla striata* as an internal standard.

Statistical analysis

Statistical analysis was performed based on analysis of variance (ANOVA) followed by Student's *t*-test or Tukey's multiple range test at $P<0.05$. Data were expressed as means±standard deviation (SD).

Table 2. Effects of ionic compositions in medium on the growth of PLBs of *Calanthe aristulifera* and *C. striata*

Treatment	Medium no.	*C. aristulifera*	*C. striata*
		fresh weight (g)	fresh weight (g)
	Control	1.35±0.13 b	0.91±0.04 d
Cationic	1	0.83±0.07 cd	1.26±0.08 c
	2	1.21±0.10 bc	1.83±0.23 b
	3	1.21±0.33 bc	1.70±0.07 b
	4	2.28±0.32 a	2.29±0.15 a
	5	1.47±0.50 b	1.90±0.36 b
	6	0.67±0.14 d	1.27±0.10 c
	Control	1.35±0.13 ab	0.91±0.04 bc
Anionic	7	0.72±0.03 de	0.71±0.02 c
	8	1.04±0.15 c	1.42±0.19 a
	9	1.51±0.52 a	1.10±0.19 ab
	10	1.16±0.19 bc	1.20±0.32 ab
	11	0.74±0.06 d	0.75±0.08 c
	12	0.69±0.02 e	0.64±0.02 c

Initial fresh weight was 0.5 g. Fresh weight was measured after one month of culture. Each treatment consisted of 10 test tubes. Statistical analysis was performed separately between cationic and anionic treatments. Same letters in each of the cationic and anionic treatments for each species indicate insignificant difference by Tukey's multiple range test at $P<0.05$.

Results and discussion

Induction of PLBs

All of the shoot meristems of both species slightly became swollen two-three months after inoculation on medium containing 2 mgl^{-1} BA. However, most of the meristems turned brown and died thereafter and only three meristems survived in both species. Then PLBs were initiated to form at the base of the swollen shoot meristems, and they proliferated gradually as the mass of PLBs in the first year. Mass of PLBs was divided into

Table 3. Optimum medium compositions calculated for the culture of PLBs in *Calanthe striata* and *C. aristulifera*

Medium for:	% of cations					% of anions			
	NH_4^+	K^+	Ca^{2+}	Mg^{2+}	Na^+	NO_3^-	$H_2PO_4^-$	SO_4^{2-}	Cl^-
C. aristulifera	10.4	70.0	9.6	10.0	—	42.1	43.7	14.2	—
C. striata	10.9	68.1	11.0	10.0	—	43.0	42.9	14.1	—
Control (modified B5)	6.5	80.2	3.2	6.5	3.6	80.2	3.6	13.0	3.2

Total ionic concentration was adjusted to 20 $me l^{-1}$ for each medium. Other additional substances were shown in Table 1.

small mass and subcultured every 1–2 months for the next one year. In each species, one clone showing the most vigorous growth was selected for the experiments to determine the optimum ionic compositions.

Optimum medium composition for PLBs propagation

The growth of PLBs was greatly affected by the ionic composition of medium (Table 2). Especially, composition of cationic ions affected more than that of anions, and medium No. 4 in cationic treatments gave the highest fresh weight (ca. 4.6 times of initial PLBs weight of 0.5 g) among all the treatments in both *C. aristulifera* and *C. striata* after one month of culture. In contrast, on media Nos. 1, 6, 7, 11 and 12 in *C. aristulifera* and Nos. 7, 11 and 12 in *C. striata*, PLBs turned brown and showed low multiplication rates (less than twice). The results on the cationic treatments clearly indicate that PLB growth was inhibited by higher NH_4^+ ratio (medium No. 1) and higher Ca^{2+} ratio (No. 6), but promoted by higher K^+ ratio (medium No. 4). In the medium where growth rate was high, i.e. No. 4, the composition of cationic ion is similar to that of the control medium (modified B5 medium). Although anionic treatment also affected PLB growth, the preferable medium composition was different in each species, medium No. 9 for *C. aristulifera* and No. 8 for *C. striata*, respectively. Based on the results in Table 2, optimum medium compositions for *C. aristulifera* and *C. striata* were obtained by calculating with the method of systematic variations (Table 3). Compared to the control medium, the optimum medium thus obtained had similar composition of cationic ions but greatly different composition of anionic ions, in which % of NO_3^- in the optimum medium was about half of the control medium and 12-hold in % of $H_2PO_4^-$ in comparison with control medium. Since the control medium is based on the medium used for shoot meristem tip culture reported by Yamamoto et al. (1991), it might be that the requirements of anionic ions for PLBs propagation are different from that for shoot meristem culture in *Calanthe*.

For orchid tissue culture and seed germination, some media such as Murashige and Skoog medium (1962), Knudson's B medium (1922), Vacin and Went medium (1949) have been used predominantly without detailed comparative studies with other media despite of the large difference in ionic compositions. It is highly possible that mineral requirements are different from species to species and that the composition of medium for tissue culture should be optimized according to the mineral requirements of the target species. In this respect, Ichihashi (1980) examined the optimum ionic compositions for the growth of seedlings of three species (*Dendrobium tosaense, Calanthe furcata, Phaius minor*) by using the triangle method developed by Hamner (1940) and Takano and Kawazoe (1973). The results indicated that optimum cationic percent compositions for *D. tosaense*, *C. furcata* and *P. minor* were $NH_4^+ : K^+ : Ca^{2+} : Mg^{2+} = 60 : 15 : 15 : 10$, $25 : 55 : 10 : 10$, and $40 : 30 : 20 : 10$, and optimum anionic compositions for these species were $NO_3^- : H_2PO_4^- : SO_4^{2-} = 70 : 20 : 10$, $70 : 20 : 10$, and $70 : 10 : 20$, respectively (Ichihashi 1980). Optimum cationic and anionic compositions of medium for proliferation of PLBs induced from young flower stalks of *Phalaenopsis* were also revealed by the same method as $NH_4^+ : K^+ : Ca^{2+} : Mg^{2+} = 25 : 38 : 27 : 10$ and $NO_3^- : H_2PO_4^- : SO_4^{2-} = 60 : 17 : 23$, respectively (Ichihashi 1992). The optimum medium obtained in the present study for both *C. aristulifera* and *C. striata* showed similar cationic balance, but different anionic balance from these previously reported media. Since high ratio of $H_2PO_4^-$ is a characteristic feature of the medium suitable for *Calanthe*, it will be necessary to clarify the specific role of $H_2PO_4^-$ for the propagation of *Calanthe*.

Variation of micropropagated plants

Among the 100 micropropagated plants of *C. striata*, no obvious variations in flower shape and color were observed (Figure 1) except for two plants, which possessed apparently larger and thicker flowers than the original plant (Figure 2). Flow cytometric analysis revealed that these two plants contained twice the amount of nuclear DNA compared to the original one, suggesting that they are tetraploid (Figure 3). The tetraploid had significantly larger values than the diploid in all the flower characters examined, and showed 8.4% increase in flower width, 20.1% in flower height, 41.3% in width of petal, and 22.7% in width of dorsal sepal, respectively, compared to the diploid (Table 4). The tetraploid plants also had thicker petals and leaf than the diploid; petal thickness was 380 μm and 510 μm in diploid and tetraploid, and leaf thickness was 250 μm and

300 μm in diploid and tetraploid, respectively (Figure 2).

It is well known that tissue culture sometimes causes somaclonal variations in orchids (Vajrabhaya 1977; Tokuhara and Mii 1998). However, there have been no reports on the variation of micropropagated *Calanthe* species because of the difficulties of propagation by tissue culture. Tetraploid plants are well known of their larger flowers and longer flower longevity than diploid plants in many orchids such as *Calanthe* (Tahara 1987) and *Phalaenopsis* (Chen et al. 2009). Since the same characteristics were confirmed in the tetraploid *C. striata* in the present study, tetraploids could be utilized beneficially as more valued horticultural crops in the genus *Calanthe*. Recently in *Phalaenopsis*, chromosome doubled plants were efficiently produced by inducing secondary PLBs from lower half of PLBs, which was rich in endoreduplicated cells (Chen et al. 2009). Therefore, it is possible that the spontaneous production of tetraploid in the present study might have caused by the same mechanism. Detailed study will be needed to confirm such possibility to produce tetraploids efficiently. Since the two *Calanthe* species showed almost the same optimum ionic compositions for PLB growth in the present study, the optimum media

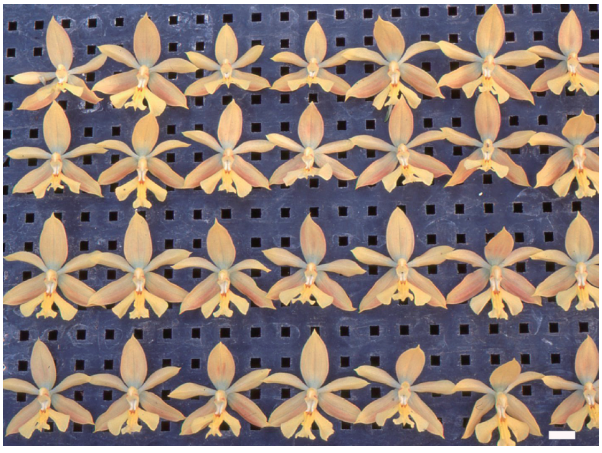

Figure 1. Variation in flower morphology of micropropagated plants of *Calanthe striata*. Each flower was collected from different individuals. Bar=10 mm.

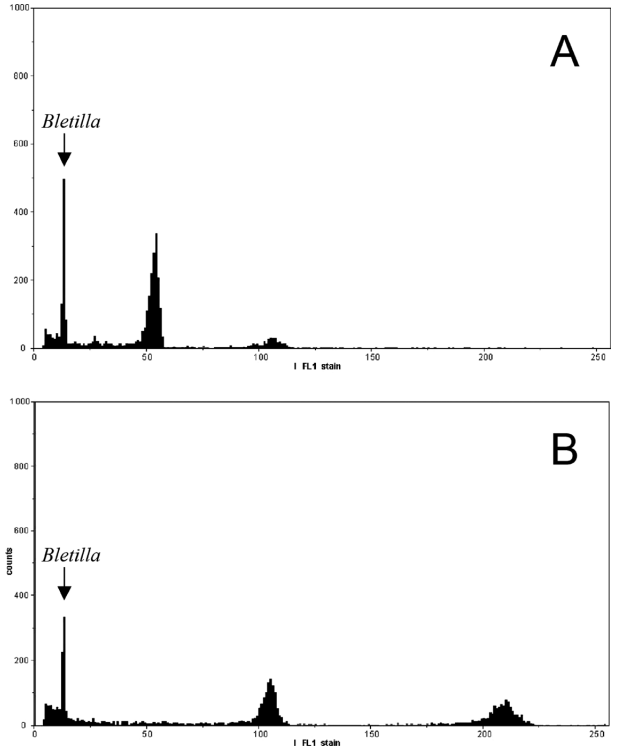

Figure 3. Flow cytometric analysis of nuclei isolated from young leaves of 2 types, 2×(A) and 4×(B), of micropropagated plants of *Calanthe striata*. DNA contents of the variants were estimated by using *Blettila striata* as an internal standard.

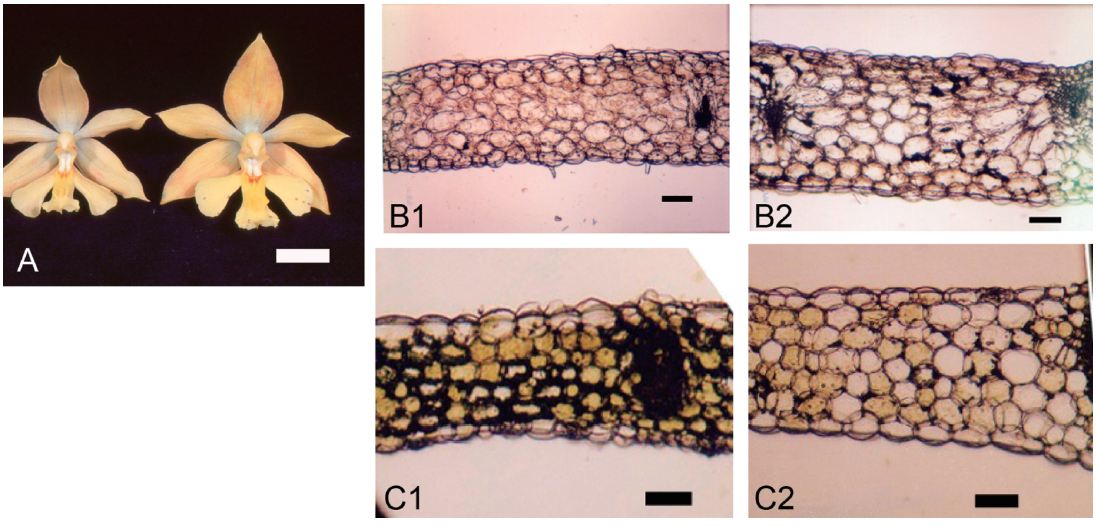

Figure 2. Flowers and transverse sections of lateral petals and leaves of micropropagated *Calanthe striata*. A: flowers, left diploid, right: tetraploid. Bar=10 mm. B: transverse sections of lateral petals of diploid (B1) and tetraploid (B2). C: transverse sections of leaf of diploid (C1) and tetraploid (C2). Bar=100 μm.

Table 4. Characteristics of diploid and tetraploid of micropropagated *Calanthe striata*

Ploidy level	Width of flowers (mm)	Height of flowers (mm)	Width of petal (mm)	Width of dorsal sepal (mm)
Diploid	39.5±3.5 a	35.8±3.0 a	6.3±0.4 a	9.7±0.4 a
Tetraploid	42.8±2.1 b	43.0±2.3 b	8.9±0.2 b	11.9±0.6 b
Rate of increase[1)]	8.4%	20.1%	41.3%	22.7%

Number of investigated plants; width and height of flowers, diploid=100 flowers from 100 plants, tetraploid=10 flowers from 2 plants. Width of lateral petals and dorsal sepals, diploid=10 flowers from 10 plants, tetraploid=10 flowers from 2 plants.[1)] (Data of tetraploid−data of diploid)/data of diploid×100. Data expressed mean±SD. Values followed by different letters within a column indicate significant differences at $P<0.05$ by Student's *t*-test.

developed are expected to be efficiently used not only for the propagation of true-to-type elite clones but also for inducing chromosome-doubled plants in wide range of *Calanthe* species.

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