Establishment and application of endosperm culture systems to produce polyploid plants in Amaryllidaceae

（ヒガンバナ科植物における倍数体作出を目的とした胚乳培養系の開発と応用に関する研究）

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Abbreviations

BAP 6-benzylaminopuline

DAPI 4’,6-diamidino-2-phenylindole

PGRs Plant growth regulators

Picloram 4-amino-3,5,6-trichloropicolinic acid

MS Murashige and Skoog

NAA 1-naphthaleneacetic acid

WAF Weeks after flowering

Chapter 1: General introduction

1.1. Family Amaryllidaceae

In the Angiosperm Phylogeny Group III, the Amaryllidaceae family expanded with a subfamily Amaryllidoideae, the initial plant group, to the broader Amaryllidaceae (Amaryllidaceae *sensu lato*), and this new classification also includes the subfamily Agapanthoideae and Allioideae (Chase et al. 2009). The Amaryllidaceae are mainly perennial and bulbous flowering plants (Takos and Rook 2013). They have been widely used and cultivated for ornamental purposes because of their attractive flowers, such as those of *Narcissus*, *Lycoris*, and *Crinum* species (Torras-Claveria et al. 2017). In various bulbous plants, bulbs propagated from seeds undergo three developmental phases: juvenile vegetative, adult vegetative, and reproductive. The transition from the juvenile vegetative phase to the adult vegetative phase can take several years, before the transitioned bulbs become receptive to flower initiating signals (Leeggangers et al. 2013). Therefore, species of Amaryllidaceae are propagated vegetatively for commercial purposes to expediate production.

1.2. Polyploid plant production

Polyploid plants have more than two chromosome sets. They exhibit heterosis (hybrid vigor) by presenting with larger organs including flowers, leaves, and fruits, compared with diploid plants. Polyploid plants occur in nature, and it is known that two major pathways lead to polyploidy in plants: somatic doubling and formation of unreduced reproductive cells (Sattler et al. 2016).

Artificial polyploid plant production is a breeding strategy utilized in the propagation of ornamental plants. Generally, the production of polyploid plants with an even number of chromosome sets is performed via treatment with antimitotic agents (also known as spindle inhibitors) such as colchicine, oryzalin, and trifluralin to explants. Colchicine is commonly used

for chromosome doubling. In cells it binds to β-tubulin and prevents the formation of tubulin dimers, thereby preventing the formation of microtubules, preventing separation of replicated chromosomes, polar migration, and cell division. As a result, the treated cell doubles its chromosome number (Chaikam et al. 2019).

The most conventional method to produce polyploid plants with an odd number of chromosome sets is interploid sexual hybridization of diploid and tetraploid plants. Because spontaneous tetraploidy is extremely rare, it is necessary first to produce the tetraploid line by chromosome doubling (Wang et al. 2016). Issues arise in production of polyploid plants with an odd number of chromosome sets. One is that it requires a long time period because interploidy crossing needs to produce tetraploid plants with regulated flowering so that diploid and tetraploid plants bloom simultaneously. The other challenge for producing polyploid plants is the interploidy hybridization barrier: the failure of endosperm development in interploidy crosses, leading to abortion of seed production (Birchler 2014). One hypothesis to explain this phenomenon involves parental conflict regarding resource allocation to the progeny (Haig and Westoby 1989).

1.3. Endosperm culture

In diploid plants of most angiosperms, the endosperm is triploid tissue. This is a result of double fertilization, in which one of the male gametes fuses with the egg to form a zygote, and the other fuses with the central cell which contains two haploid nuclei to form a triploid endosperm. As compared to conventional methods, endosperm culture is a one-step and time-efficient protocol to produce triploid plants from diploid plants (Hoshino et a. 2011).

First attempts at endosperm culture took place in the 1930s (Lampe and Mills 1936). According to previous reviews of this process (Thomas and Chaturvedi 2008; Hoshino et al. 2011; Wang et al. 2016), endosperm culture has been attempted to produce triploid plants in nearly 70 species.

However, shoot initiation and triploid plant regeneration have been reported in only nearly 30 species including *Acacia* (Garg et al. 1996), *Azadirachta* (Chaturvedi et al. 2003), *Citrus* (Gmitter et al. 1990; Yang et al. 2000), *Gomortega* (Muñoz-Concha 2016), *Juglans* (Tulecke et al. 1988), and *Morus* (Thomas et al. 2000). Thus, plant regeneration by endosperm culture is often technically challenging (Wang et al. 2016).

1.4. Objectives of this study

The aims of this research were 1) to establish an endosperm culture system in Amaryllidaceae using endosperm culture in monocots and 2) to expand the availability of the endosperm culture systems to produce polyploid plants.

Chapter 2 describes how the endosperm culture was attempted in Amaryllidaceae members and the endosperm culture systems available to several genera were established. This suggests that the first aim was achieved. In addition, the stabilities of the frequencies of callus induction and shoot regeneration were investigated in *Haemanthus albiflos*, which exhibited the highest callus induction rate among the Amaryllidaceae members in the study, for use in further studies conducted and described in the subsequent chapters. Subsequently, as described in Chapter 3, hexaploid plant production was demonstrated through treatment with colchicine to the endosperm-derived triploid calli of *H. albiflos* as a novel example of utilizing endosperm culture. Chapter 4 describes the production of tetraploid and octoploid *H. albiflos* plants, demonstrating the utilization of the immature embryos obtained in the endosperm culture process described in Chapter 3. The second aim of the study was achieved as the production of plants with different ploidy levels, as described in Chapter 3 and Chapter 4. In Chapter 5, the utilization of endosperm culture systems for future polyploid breeding programs is discussed based on the present research findings.

Chapter 2: Establishment of endosperm culture systems in Amaryllidaceae

2.1. Introduction

2.1.1. Endosperm culture in monocots

Previous studies of callus induction from endosperm in monocots have been conducted mainly in the Poaceae and have been reported in *Cocos nucifera* (Kumar et al. 1985), *Hordeum vulgare* (Sun and Zhu 1981), and *Oryza sativa* (Bajaj et al. 1980; Nakano et al. 1975). Generally, the conditions of tissue culture in the relevant species are of priority for such research. To date, such research has been conducted on endosperm cultures in horticulturally important plants such as *Actinidia* (Kin et al. 1990; Góralski et al. 2005; Popielarska-Konieczna et al. 2006; Asakura and Hoshino 2017; Abdullah et al. 2021) and *Passiflora* (Mohamed et al. 1996; Antoniazzi et al. 2018; da Silva et al. 2020), and the specialized culture conditions for these plants are becoming clear. However, the biggest challenge for endosperm culture in most monocots is the lack of information on suitable tissue culture conditions. There are many plants of horticultural value in the Amaryllidaceae family, and the procedures of endosperm culture suitable for these plants are needed to improve their ornamental values. However, establishing suitable endosperm culture systems that induce plant regeneration from the endosperm is time-consuming and unique for each plant type. The present study attempted screening for endosperm culture using a medium supplemented with 4-amino-3,5,6-trichloropicolinic acid (picloram) and 6-benzylaminopuline (BAP), two compounds which have been utilized in tissue culture of Amaryllidaceae to elicit the conditions required for endosperm culture. In addition, the stability of callus induction and shoot regeneration in endosperm culture of *Haemanthus albiflos* was investigated. The high callus induction rate was utilized as a material for further study in following chapters.

2.2. Materials and methods

2.2.1. Plant materials

To determine the availability of endosperm culture in Amaryllidaceae, 27 species of 16 genera of Amaryllidaceae were investigated. Plants were purchased from seed companies or obtained from a local nursery in Japan. The detailed information of the plants is shown in Table 2.1. These plants were maintained in a greenhouse at Hokkaido University in Japan. Among them, ovaries with seed were obtained from open-pollination and used for endosperm culture in 17 species of 11 genera.

2.2.2. Preparation for endosperm culture

Ovaries were obtained from plants before the dehiscence occurred after endosperm cellularization. The period from flowering to ovary collection is described as weeks after flowering (WAF) in Table 2.1. In *Haemanthus albiflos*, ovaries were collected from four to ten WAF to investigate the effects of endosperm maturity on callus induction.

Ovaries were opened using a scalpel and a forceps, and ovules were removed from the ovaries. The integuments of ovules were removed using forceps and soaked distilled water during the process. The ovules were then surface sterilized by being soaked in sodium hypochlorite solution (1% active chlorine) for 5 minutes and then rinsed with sterilized distilled water.

2.2.3. Endosperm culture for callus induction

The endosperms of most plant materials were cultured on callus induction media in plastic petri dishes. Because ovules are difficult to remove as immature embryos, they were detached after sprouting during culture. The endosperms of *Haemanthus albiflos*, *H. pauculifolius*, and *Scadoxus multiflorus* were cut into four to eight segments, which were then cultured on a callus induction

medium. The cultures were incubated at 22°C under continuous light conditions (35 μmol $m^{-2} \cdot s^{-1}$). Subculturing was conducted every eight to ten weeks.

The callus induction medium was prepared as follows: a Murashige and Skoog (MS; 1962) medium supplemented with 5.0 mg L^{-1} picloram and 5.0 mg L^{-1} BAP, to which was added 30 g L^{-1} sucrose, solidified with 3 g L^{-1} gellan gum (WAKO Co. Ltd., Osaka, Japan). The pH of the medium was adjusted to pH 5.7 ± 0.1 before autoclaving at 121 °C for 15 min.

2.2.4. Effect of conditions on callus induction from endosperm in *Haemanthus albiflos*

To investigate the effect of conditions on callus induction from the endosperm, culturing of *H. albiflos* was conducted using different genotypes, concentrations of plant growth regulators (PGRs) in the medium, parts of the endosperm, and maturity of endosperm. The 105 segments cut from 18 endosperms from 16 ovaries open-pollinated in three plants were used as explants. The endosperm culture procedure is described in 2.2.3. After detachment of immature embryos, endosperm segments were cultured on callus induction medium separately so that endosperm segments derived from micropylar and chalazal endosperm be distinguished. Callus induction media supplemented with three different concentrations of picloram and BAP (2.5, 5.0 and 10 mg L^{-1}) were used. The frequency of callus induction was recorded 16 weeks after culture and one year after culture. To investigate the effect of maturation of endosperm, 80 and 64 ovules were collected four and six WAF and were cultured on MS medium supplemented with 5.0 mg L^{-1} picloram and 5.0 mg L^{-1} BAP.

2.2.5. Ploidy determination using flow cytometry

To investigate whether the ploidy level of endosperm was maintained in endosperm tissue culture, the ploidy levels of endosperm, callus, and plantlet were estimated using flow cytometry. Flow

cytometric analysis was conducted according to the modified procedure of Hoshino et al. (2019). The approximately 3-5 mm square segment of endosperm, callus, or leaf of plantlet was cut from a leaf segment of each mother plant using a razor blade in a plastic petri dish (3 cm diameter) containing 0.2 mL of ice-cold nuclei extraction buffer (Quantum Stain NA UV 2A; CytoTechs, Ibaraki, Japan). The leaves of each mother plant were used as the internal standard. After filtering through the 30 μm Nylon mesh (CellTrics®, CytoTechs), the nuclei sample was stained with 0.8 mL of DAPI (4',6-diamidino-2-phenylindole) solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) PVP K-30, 0.1% (w/v) Triton X-100, and 2 mg L^{-1} DAPI (pH 7.5) (Mishiba et al. 2000). After incubation for 5 min at room temperature, the nuclei fluorescent intensities of the samples were measured using a Ploidy analyzer (Partec PA; Partec). The ploidy levels of each sample were calculated from the peaks of fluorescent intensity in each sample compared to that in the internal standard.

2.2.6. Effects of conditions on shoot regeneration from endosperm-derived callus in *Haemanthus albiflos*

To investigate the effect of the medium components on shoot regeneration from endosperm-derived callus, three different media of 1/2MS medium without PGRs, MS medium without PGRs, and MS medium supplemented with 0.5 mg L^{-1} 1-Naphthaleneacetic acid (NAA) and 1.0 mg L^{-1} BAP were prepared. Fifty-five well-developed triploid calli derived from endosperm segments of 13 endosperm at 16 weeks after culture on callus induction medium were used. The calli except for B2-2 and C7-1 were cut into three segments and transferred to each medium. The single callus of B2-2 was cut into six segments, and the two calli of B7-1 were cut into a total of 27 segments. The number of regenerated shoots was recorded 31 and 37 weeks after transfer.

2.3. Results

2.3.1. Endosperm culture for callus induction in Amaryllidaceae

Calli were induced in endosperm culture of *Cyrtanthus mackenii*, *Haemanthus albiflos*, *H. pauculifolius*, *Hippeaskelia*, *Hippeastrum* 'Red Tiger', *Scadoxus multiflorus*, *Zephyranthes* 'Ajax', *Z. candida*, *Z. citrina*, and *Z. rosea* (Table 2.2).

2.3.2. Effect of culture conditions on callus induction from endosperm in *Haemanthus albiflos*

Total frequency of callus induction was 68 of 105 segments (64.8%) at 16 weeks after culture and 77 of 105 segments (73.3%) at the end of the study. Calli were induced from 15 of 18 different genotype endosperms. Among them, induction was observed in all segments of 11 different endosperms (Table 2.3). Frequencies of callus induction were 35 of 50 segments (70.0%) in micropylar endosperm and 39 of 51 segments (78.0%) in chalazal endosperm. The effect of the part of endosperm on callus induction was not observed. In endosperm culture with different concentrations of PGRs in callus induction media, the induction rates were 87.1% (2.5 mg L^{-1}), 90.5% (5.0 mg L^{-1}), and 37.5% (10 mg L^{-1}) at the end of the study (Table 2.4). The 2.5 and 5.0 mg L^{-1} PGRs were effective to cause callus induction from endosperm in *H. albiflos*.

In culture of ovules collected 4 and 6 WAF, most ovules were smaller than 3 mm and so fertilization could not be determined. Callus was induced in 13 of 65 ovules at 4 WAF and 3 of 41 ovules at 6 WAF (Table 2.5). The frequencies of callus induction were low in culture with ovules at 4 and 6 WAF compared to culture from endosperms at 8 to 10 WAF.

2.3.3. Ploidy analysis of callus induced through endosperm culture in Amaryllidaceae

The calli obtained from endosperm culture in *Cyrtanthus mackenii*, *Haemanthus pauculifolius*,

and *Scadoxus multiflorus* had 1.5 times the fluorescent intensity of the diploid mother plants (Figures 2.1 and 2.2). The plantlets developed from embryos derived from the same seed with endosperm used to callus induction were diploid in *H. pauculifolius* and *S. multiflorus*, suggesting these calli were derived from endosperm tissues.

Flow cytometric analysis of the endosperm culture of *H. albiflos* revealed that the calli from endosperms (except B2-1 and B4-1) had 1.5 times the fluorescent intensity of the diploid mother plant, indicating that these calli were derived from endosperm tissue and had maintained the ploidy level of that endosperm (Table 2.3). One of four calli induced from endosperm segments of B2-1 had three times fluorescent intensity and so was determined as hexaploid. Other calli from the segments of B2-1 were triploid. Four calli induced from segments of B4-1 resulted in the same fluorescent intensity of its diploid mother plant and so were determined as diploid; and 3 tetraploids were determined due to them having twice the fluorescent intensity of the mother plant.

In ovule culture collected at 4 and 6 WAF in *H. albiflos*, ploidy levels of all induced calli were determined (Table 2.5). Among 13 calli induced from ovules collected at 4 WAF ovules, 10 were diploid, 2 triploid, and 1 tetraploid. The calli induced from ovules collected at 6 WAF were all diploid.

2.3.4. Effect of conditions on shoot regeneration from endosperm-derived callus in *Haemanthus albiflos*

The total frequency of shoot regeneration in *H. albiflos* derived from endosperm was 97 of 191 callus segments (50.8%), and the average number of shoots per callus with shoot was 4.0 at 29 weeks after transfer. The shoot regeneration was observed in callus segments from all endosperms (Table 2.6). Among the endosperms, shoot regeneration rates varied from 16.7% of B2-1 and C5-

1 to 100% of B2-2 and B3-1. The number of shoots per callus segment was higher than the average (4.0) in endosperms of B2-2 (12.7), B3-1 (9.8), and S1-1 (7.0) (Table 2.7). As for the effect of part of endosperm on shoot regeneration, frequencies of shoot regeneration were 45 of 75 callus segments (60.0%) in micropylar endosperm and 40 of 83 callus segments (48.2%) in chalazal endosperm. There was no difference between micropylar endosperm and chalazal endosperm. In shoot regeneration with different medium components, frequencies of shoot regeneration were 32 of 64 callus segments (50.0%) in 1/2MS medium without PGRs, 38 of 64 callus segments (59.4%) in MS medium without PGRs, and 27 of 63 callus segments (42.9%) in MS medium supplemented with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP (Table 2.8). The numbers of shoot per callus segment on each medium were 5.0 in 1/2MS medium without PGRs, 5.1 in MS medium, and 3.4 in MS medium supplemented with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP. Both shoot regeneration rate and number of shoots per callus segment were high in MS medium without PGRs in *H. albiflos*.

2.4. Discussion

2.4.1. Endosperm culture in Amaryllidaceae

The present study screened endosperm culture with media supplemented with picloram and BAP in Amaryllidaceae. Callus induction by the endosperm culture was observed in the four species of three genera *Cyrtanthus mackenii*, *Haemanthus albiflos*, *H. pauculifolius*, and *Scadoxus multiflorus* among the 17 species of the 11 genera. Therefore, the present study provides new examples of the success of endosperm culture in monocots beside Poaceae. According to a phylogenetic hypothesis for Amaryllidaceae subfamily Amaryllidoideae (Rønsted et al. 2012), these genera which induced endosperm-derived calli were strongly supported as a sister clade, which is relevant to the results in the present study. The screening under the endosperm culture with the medium supplemented with picloram and BAP be able to induce endosperm-derived calli

in four species in three genera at once in the present study. However, this procedure is necessary to consider the risk of the failure to notice the plants with a low frequency of callus induction. Further study is needed with a different medium which does not induce calli, to understand the relations between conditions of endosperm culture and phylogenetic relationships.

2.4.2. Ploidy analysis of the calli induced from endosperm culture

The ploidy analysis revealed the calli obtained from endosperm culture were triploid in *Cyrtanthus mackenii*, *Haemanthus albiflos*, *H. pauculifolius*, and *Scadoxus multiflorus*. The presence of diploid embryo-developed plantlets from the same seed with endosperm that supported the triploid calli, were not derived from the triploid embryo fertilized with unreduced gamete and haploid gamete, but from the triploid endosperm tissue. In the endosperm culture of *H. albiflos*, diploid, tetraploid, and hexaploid calli were induced as well as triploid calli. In the B4-1 endosperm diploid and tetraploid calli, it is unlikely that contamination of the tissue occurred because the diploid embryo was well isolated and cultured. Many previous studies of endosperm culture reported diploid callus induction or diploid plant regeneration from endosperm-derived callus in *Azadirachta indica* (Chaturvedi et al. 2003), *Carica papaya* (Sun et al. 2011), and *Euonymus alatus* (Thammina et al. 2011). Sun et al. (2011) suggested that the recovery of diploid plants was attributed to incomplete fertilization, chromosome loss, or development from maternal tissues. In addition to these, it is possible that ploidy level of endosperm-derived calli decreased via chromosome elimination. Deumling and Clermont (1989) reported that DNA content and chromosome size of callus culture decreased in bulb tissue culture of triploid *Scilla siberica*. As for the tetraploid calli, they seemed to be derived by chromosome doubling on diploid callus during culture due to the presence of diploid callus. Increasing the number of subcultures and their duration enhances the rate of somaclonal variations (reviewed by Bairu et al. 2011). However,

it is not sure whether the diploid calli were induced from the non-fertilized diploid endosperm or the diploid maternal tissue. The hexaploid callus induced from the B2-1 endosperm segment seems to be derived by chromosome doubling in triploid calli due to other calli induced from the endosperm being triploid. Periodic measurement of ploidy level during culture and comparison of genotype between diploid calli, mother plant, and the embryo is necessary to reveal its origin.

2.5. Conclusion

The present study established the endosperm culture systems available for four species of several genera in Amaryllidaceae: *Cyrtanthus mackenii*, *Haemanthus albiflos*, *H. pauculifolius*, and *Scadoxus multiflorus*. Furthermore, the stability of callus induction and shoot regeneration was found by investigating the effect of culture conditions on endosperm culture in *H. albiflos*. Therefore, *H. albiflos* is considered a useful species for providing material to investigate the application of endosperm culture to produce polyploid plants.

Table 2.1 List of plant materials for this study.

	Plant material	Origin	Flowering	Seed set	Weeks after flowering
1	*Amaryllis belladonna*	Sakata Seed Co. Ltd.	○	○	4
2	*Cyrtanthus mackenii*	Takii Seed Co. Ltd.	○	○	7~8
3	*Clivia miniata*	N.D.	○	○	N.D.
4	*Crinum powelii pink*	Takii Seed Co. Ltd.	○	-	-
5	*Galanthus elwesii*	Takii Seed Co. Ltd.	○	-	-
6	*Eithea blumenavia*	N.D.	○	-	-
7	*Habranthus robustus*	N.D.	○	○	4
8	*Haemanthus albiflos*	Local plant grower (B, C) Botanic garden (S)	○	○	4, 6, 8, 10
9	*H. pauculifolius*	Local plant grower	○	○	12
10	*Hippeaskelia*	Takii Seed Co. Ltd.	○	○	6~8
11	*Hippeastrum* Red Tiger	Takii Seed Co. Ltd.	○	○	6~7
12	*H.* Sweet Orange	Takii Seed Co. Ltd.	○	-	-
13	*H.* Caramba	Takii Seed Co. Ltd.	○	-	-
14	*Hymenocallis festallis*	Takii Seed Co. Ltd.	○	-	-
15	*Leucojum aestivum*	Takii Seed Co. Ltd.	-	-	-
16	*Narcissus pseudonarcissus*	Takii Seed Co. Ltd.	○	-	-
17	*N. tazetta*	Takii Seed Co. Ltd.	○	-	-
18	*N.* Paper White	Takii Seed Co. Ltd.	○	○	7
19	*Nerine bowdenii*	Takii Seed Co. Ltd.	○	○	4
20	*Scadoxus multiflorus*	Sakata Seed Co. Ltd.	○	○	8
21	*Zephyranthes* Apricot Queen	Takii Seed Co. Ltd.	○	○	3~4
22	*Z.* Ajax	Takii Seed Co. Ltd.	○	○	3~4
23	*Z. candida*	Takii Seed Co. Ltd.	○	○	3~4
24	*Z. carinata*	Takii Seed Co. Ltd.	○	-	-
25	*Z. citrina*	Takii Seed Co. Ltd.	○	○	3~4
26	*Z.* Pride of Singapore	Sakata Seed Co. Ltd.	○	○	3
27	*Z. rosea*	Takii Seed Co. Ltd.	○	○	3~4

N.D. indicates no data.

The circles (○) and the hyphen (-) indicate whether flowering and seed set were observed or not.

Table 2.2. Callus induction in endosperm culture of Amaryllidaceae.

Plant material	PGRs of medium		No. of ovary	No. of endosperm	No. of calli	No. of endosperm-derived calli
	Picloram (mg L^{-1})	BAP (mg L^{-1})				
Amaryllis belladonna	5.0	5.0	3	5	0	-
Cyrtanthus mackenii	5.0	5.0	18	95	33	2
Clivia miniata	5.0	5.0	5	7	0	-
Habranthus robustus	5.0	5.0	15	411	0	-
	2.5	2.5	7	231	0	-
	10	10	5	132	0	-
Haemanthus albiflos	5.0	5.0	9	11 (42 segments)	11 (38 segments)	10 (34 segments)
	2.5	2.5	5	6 (31 segments)	6 (27 segments)	6 (27 segments)
	10	10	5	5 (32 segments)	3 (13 segments)	3 (13 segments)
H. pauculifolius	5.0	5.0	3	5 (17 segments)	4 (15 segments)	4 (15 segments)
	2.5	2.5	3	4 (16 segments)	4 (12 segments)	4 (12 segments)
Hippeaskelia	5.0	5.0	3	94	3	0
Hippeastrum Red Tiger	5.0	5.0	5	123	1	0
Narcissus Paper White	5.0	5.0	1	8	0	-
Nerine bowdenii	5.0	5.0	1	2	0	-
Scadoxus multiflorus	5.0	5.0	3	3 (17 segments)	1 (5 segments)	1 (5 segments)
Zephyranthes 'Ajax'	5.0	5.0	8	54	0	-
Z. 'Apricot Queen'	5.0	5.0	2	10	0	-
Z. candida	5.0	5.0	13	151	12	0
Z. citrina	5.0	5.0	8	198	0	-
Z. 'Pride of Singapore'	5.0	5.0	1	4	0	-
Z. rosea	5.0	5.0	14	48	6	0-

The number of segments in parentheses indicates the segments cut from the endosperms.

Table 2.3 The effects of accession and part of endosperm on callus induction from endosperm in *Haemanthus albiflos*.

			16 weeks after culture			1 year after culture			
	No. of segments		No. of calli			No. of calli			
Endosperm	M	C	M	C	Callus induction rate	M	C	Callus induction rate	Ploidy level of calli
B1-1	2	1	1	0	33.3%	1	1	100%	Triploid
B2-1	2	2	2	0	50.0%	-	2	100%	Triploid, hexaploid
B2-2	1*	-	1	-	100%	-	-	100%	Triploid
B3-1	2	2	0	2	50.0%	0	-	50.0%	Triploid
B4-1	2	2	2	1	75.0%	-	1	100%	Diploid, tetraploid
B5-1	4	4	4	4	100%	-	-	100%	Triploid
B6-1	4	4	4	4	100%	-	-	100%	Triploid
B7-1	4	4	0	0	0%	1	2	37.5%	Triploid
B7-2	4	4	3	3	75.0%	0	1	87.5%	Triploid
B8-1	4	4	4	4	100%	-	-	100%	Triploid
S1-1	4	4	4	4	100%	-	-	100%	Triploid
C1-1	1*	-	0	-	0%	0	-	0%	Triploid
C2-1	4	4	2	2	50.0%	0	0	50.0%	Triploid
C3-1	3	4	0	0	0%	0	0	0%	Triploid
C4-1	4	4	0	0	0%	0	0	0%	Triploid
C5-1	4	4	4	4	100%	-	-	100%	Triploid
C6-1	3	4	4	4	100%	-	-	100%	Triploid
C7-1	2*	-	2	-	100%	-	-	100%	Triploid
Total	50 + 4*	51	33 + 3*	32	64.8%	2	7	73.3%	

M: micropylar endosperm. C: chalazal endosperm.

Hyphen (-) indicate there are no endosperm segment or already induced callus from the endosperm segments.

Asterisks indicate the endosperms could not be distinguished from the part of the endosperm.

Table 2.4. Effect of concentration of PGRs of callus induction medium on callus induction from endosperm in *Haemanthus albiflos*.

PGRs of medium		No. of segments	16 weeks after culture		1 year after culture	
Picloram (mg L^{-1})	BAP (mg L^{-1})		No. of calli	Callus induction rate	No. of calli	Callus induction rate
2.5	2.5	31	23	74.2%	27	87.1%
5.0	5.0	42	30	71.4%	38	90.5%
10.0	10.0	32	12	37.5%	12	37.5%

Table 2.5. Effect of maturation of endosperm on callus induction in *Haemanthus albiflos*.

WAF	No. of ovules	Contamination	Total	PGRs in medium		No. of calli	Ploidy of calli		
				Picloram (mg L^{-1})	BAP (mg L^{-1})		Diploid (2*x*)	Triploid (3*x*)	Tetraploid (4*x*)
4	80	15	65	5.0	5.0	13	10	2	1
6	64	23	41	5.0	5.0	3	3	0	0

Table 2.6. The effect of accession on shoot regeneration from endosperm-derived callus in *Haemanthus albiflos*.

Endosperm	No. of callus segments	No. of callus segments with shoot	Rate of shoot regeneration	No. of shoot	No. of shoot per callus segment with shoot
B1-1	3	1	33.3%	1	1.0
B2-1	6	1	16.7%	2	2.0
B2-2	3	3	100%	76	12.7
B3-1	6	6	100%	59	9.8
B5-1	24	17	70.8%	45	2.6
B6-1	24	7	29.2%	12	1.7
B7-2	17	8	47.1%	12	1.5
B8-1	24	9	37.5%	20	2.2
S1-1	24	23	95.8%	160	7.0
C2-1	6	4	66.7%	17	4.3
C5-1	12	2	16.7%	3	1.5
C6-1	12	7	58.3%	26	3.7
C7-1	27	6	22.2%	10	1.7
Total	191	97	50.8%	443	4.0

Table 2.7. Effect of part pf endosperm on shoot regeneration from endosperm-derived callus in *Haemanthus albiflos*.

Part of endosperm	No. of callus segments	No. of callus segments with shoot	Rate of shoot regeneration	No. of shoot	No. of shoot per callus segments with shoot
Micropylar side	75	45	60.0%	196	4.4
Chalazal side	83	40	48.2%	161	4.0

Data were excepted for B2-2 and C7-1 because these endosperms could not be distinguished from the parts of endosperm when they were cultured.
Data were recorded 37 weeks after transfer on shoot regeneration medium.

Table 2.8. Effect of medium composition on shoot regeneration from endosperm-derived callus in *Haemanthus albiflos*.

Medium composition	No. of callus segments	No. of callus segments with shoot	Rate of shoot regeneration	No. of shoot	No. of shoot per callus segment with shoot
1/2MS HF	64	32	50.0%	159	5.0
MS HF	64	38	59.4%	192	5.1
MS NAA+BAP	63	27	42.9%	92	3.4

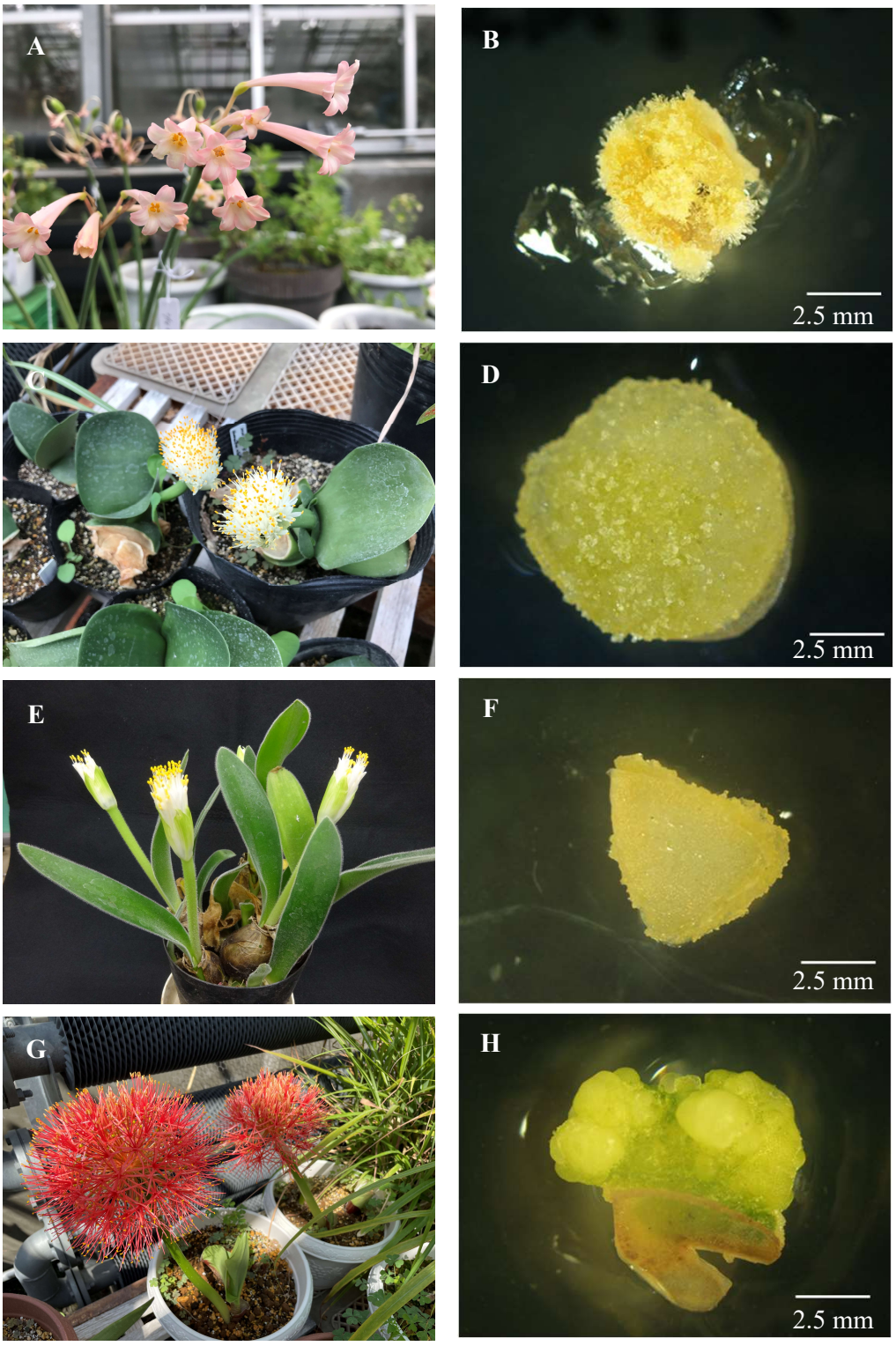

Figure 2.1. Images of flower and endosperm-derived callus in four species of three genera in Amaryllidaceae.

A and **B**. Flowers and endosperm-derived callus of *Cyrtanthus mackenii* Hook.f.

C and **D**. Flowers and endosperm-derived callus of *Haemanthus albiflos* Jacq.

E and **F**. Flowers and endosperm-derived callus of *Haemanthus pauculifolius* Snijman & A.E.van Wyk.

G and **H**. Flowers and endosperm-derived callus of *Scadoxus multiflorus* (Martyn) Raf.

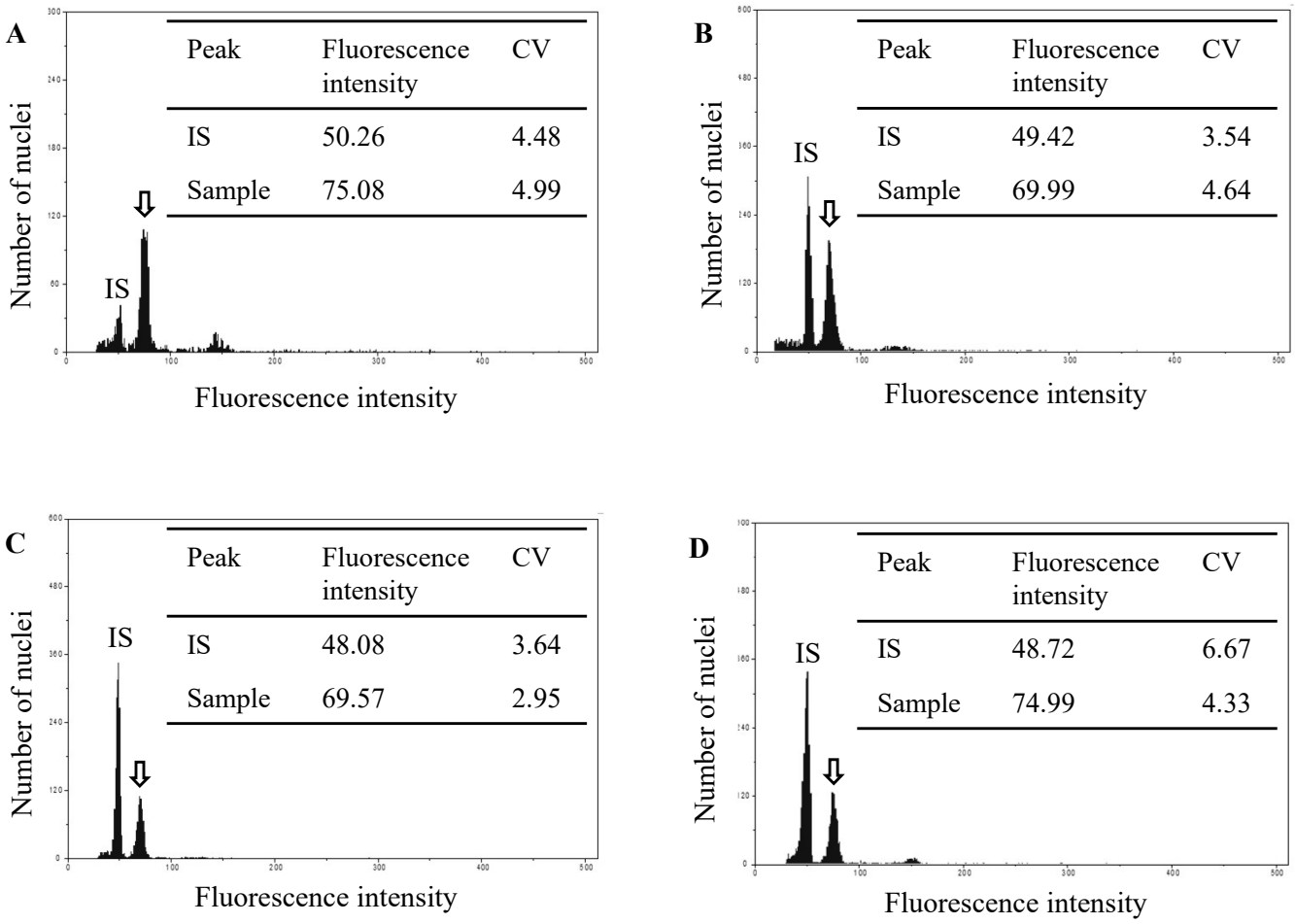

Figure 2.2. Histograms of relative fluorescence intensity of nuclei isolated from callus induced from endosperm culture of Amaryllidaceae using flow cytometry.

Peaks of fluorescence intensity of leaves from diploid plants were set 50 and used as internal standard (IS).

A. Endosperm-derived triploid callus of *Cyrtanthus mackenii.*

B. Endosperm-derived triploid callus of *Haemanthus albiflos.*

C. Endosperm-derived triploid callus *of Haemanthus pauculifolius.*

D. Endosperm-derived triploid callus of *Scadoxus multiflorus*

Chapter 3: Endosperm-derived triploid plant regeneration and hexaploid plant production from endosperm-derived callus treated with colchicine in diploid *Haemanthus albiflos*

This chapter is rewritten based on the publication in Plant Cell, Tissue and Organ Culture 144(3), 661-669 (2021) https://doi.org/10.1007/s11240-020-01974-4.

3.1. Introduction

3.1.1 Production of triploid and hexaploid plants

Endosperm culture is a useful method to produce triploid plants directly from diploid plants. Generally, triploid plants are sterile and are utilized commercially to produce seedless fruits such as watermelon and banana. In breeding processes, it is often problematic that interploidy-crossed hybrid plants are not able to be propagated or used as parent plants due to the plant's sterility. Attempts to induce hexaploid plants from triploid plants requires use of antimitotic agents such as colchicine, oryzalin and trifluralin in order to restore fertility. Previous studies to produce hexaploid plants were reported in *Gossypium* hybrids (Chen et al. 2018), *Brassica* hybrids (Mei et al. 2015), *Lychnis senno* (Nonaka et al. 2011), *Drosera* hybrids (Tungkajiwangkoon et al. 2016), and *Pennisetum* hybrids (Campos et al. 2009). Investigation of the availability of endosperm culture to produce hexaploid plants is useful for polyploidy breeding. However, hexaploid production using endosperm culture in diploid plants has not been reported.

3.1.2. The genus *Haemanthus*

The previous chapter suggested that *Haemanthus albiflos* has stable regeneration ability in endosperm culture, and triploid calli and shoots were obtained. The genus *Haemanthus* belongs to the subfamily Amaryllidoideae of the family Amaryllidaceae. The genus includes 22 species

with a true bulb, distichous and fleshy leaves, and a diploid chromosome complement of 16 (Snijman 1984; Snijman and van Wyk 1993). The species is native to Southern Africa and Namibia. It was reported *Haemanthus pubescens* ssp. *pubescens* has a long lifecycle that includes a juvenile period of 9 years, a young reproductive period between 10 and 13 years, and a reproductive maturity peaking at 16 years. Plants older than 17 years showed a marked reduction in reproductive potential (Ruiters et al. 1993). Most species are deciduous, and only the three species *H. albiflos*, *H. deformis* and *H. pauculifolius* are evergreen (Snijman and van Wyk 1993). The species *H. albiflos* is the most commercially popular among those three species, with the common name brush lily for its brush-like umbel. The fruit of *H. albiflos* is a globose to ellipsoidal berry which is soft when ripe, and colored white, orange, or red (Snijman 1984).

The aims of the present study were to regenerate triploid plants from endosperm-derived calli and to investigate the availability of the endosperm culture to produce hexaploid plants in *H. albiflos*.

3.2. Materials and methods

3.2.1. Plant materials

The potted plants of diploid *Haemanthus albiflos* Jacq. ($2n = 2x = 16$) used in this study, were obtained from a local plant grower in Japan. The plants were propagated clonally and maintained in a greenhouse at Hokkaido University in Japan. Flowering was observed in early July 2019.

3.2.2. Callus induction from endosperm

Four ovaries were collected from one potted plant eight weeks after flowering. Each ovary contained one ovule, and four were used in this study. The ovules were surface sterilized in sodium hypochlorite (1% active chlorine) for five min and then rinsed in sterilized water. The ovules were

cut into approximately four to six segments and separated into endosperm tissue and immature embryos in a glass petri-dish. A total of 22 endosperm segments were obtained and used for callus induction. The segments were cultured on MS medium supplemented with 5.0 mg L^{-1} picloram and 5.0 mg L^{-1} BAP in plastic petri dishes.

To investigate the effect of light condition on callus induction from endosperm, ten of 22 segments were cultured under continuous illumination (35 μmol m^{-2} s^{-1}), and the remainder cultured under total darkness for eight weeks. The endosperm segments were observed to record frequency of callus induction at four and eight weeks after culture.

3.2.3. Colchicine treatment of endosperm-derived callus for chromosome doubling

To produce hexaploid plants, calli induced from endosperm were treated with colchicine. To prepare 2% (w/v) colchicine solution, 0.1 g colchicine powder was dissolved in 5 ml distilled water and filtered through a 0.22 μm filter (Merck Millipore, Darmstadt, Germany). The 2% (w/v) colchicine solution was diluted with sterilized distilled water to 0.2% just before treatment.

The endosperm-derived calli were cut into segments of 0.5 cm square, to ensure a sufficient number of explants for treatment, and then soaked in 5 ml of 0.2% (w/v) colchicine solution in plastic petri dishes (6 cm diameter). The plastic petri dishes were covered with aluminum foil and agitated on a shaker (EYELA Multi Shaker MMS-2000; Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at 100 rpm. The three different treatments of durations of 24, 48, and 72 hours were performed. After treatment, the calli were rinsed with sterilized water. Subsequently, the calli were used for differentiation. The calli not treated with colchicine served as a control. The survival rate of the calli was recorded at four weeks after treatment.

3.2.4. Plant regeneration from endosperm-derived callus

All endosperm-derived calli were transferred to 1/2MS medium without PGRs. The calli were transferred to MS medium supplemented with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP four weeks after culture on 1/2MS medium without PGRs. The calli were observed and the images were captured by iPhone7 once every week for the next four weeks. Somatic embryos and adventitious shoots that emerged from endosperm-derived calli were transferred to flesh 1/2MS medium without PGRs in plastic petri dishes. The cultures were grown under continuous illumination.

3.2.5. Histological observation of somatic embryos and adventitious shoots

To investigate the regeneration process in the calli, histological observation was conducted following a modified procedure of Hoshino et al. (2006). The calli-initiated regenerated shoots were fixed in sample tube bottles containing solution of acetic acid and ethanol in the ratio of 1:3 at 4°C overnight. The calli were transferred into 70% ethanol at 4°C overnight. The fixed calli were hydrolyzed with 1N sodium hydroxide at 60°C in a water bath for 10 min. After hydrolyzation, the sample tube bottle was transferred to ice-water and distilled water added immediately to stop the reaction. The hydrolyzed callus was put on a glass slide and had added to it a single drop of 50% (v/v) glycerin (distilled water: glycerin = 1:1). A cover slip was placed over the callus and gently pushed. The slide was observed using a dissecting microscope with a digital camera. The distinguishing of the somatic embryo and adventitious shoot was conducted based on the following characteristics: somatic embryos have bipolarity of apical and root meristems connected to the vascular bundle and are independent of the calli. Adventitious shoots are connected to and dependent on the calli.

3.2.6. Flow cytometric analysis to estimate ploidy level of calli and regenerated plantlets

To investigate whether the ploidy level of endosperm was maintained in endosperm culture, the fluorescent intensities of nuclei isolated from induced calli and leaves of plantlets regenerated from calli that were not treated with colchicine, were measured using a flow cytometer according to the procedure described in Chapter 2. In addition, to investigate whether hexaploid plantlets were produced from endosperm-derived calli treated and not treated with colchicine, the fluorescent intensities of nuclei isolated from the leaves of regenerated plantlets were measured using a flow cytometer. The 84 regenerated plantlets were randomly selected among regenerated plantlets. Leaves of the diploid mother plant were used as an internal standard.

3.2.7. Chromosome analysis of plantlets regenerated from endosperm-derived calli

Plantlets randomly selected among those which had their ploidy level determined by flow cytometry were used for chromosome counts. The following procedure was used: roots containing root tips were pretreated with 4 mL of 0.05% (w/v) colchicine solution in a sample tube bottle (AS ONE Co. Ltd., Osaka, Japan) at room temperature for four hours according to the procedure of Vosa and Marchi (1980). Subsequently, the pretreated roots were fixed with a solution of acetic acid and ethanol in the ratio of 1:3 at 4°C overnight. The fixed roots were stored in 70% ethanol at 4°C before use. The roots were stained with acetocarmine at room temperature for four hours. After staining, the sample tube bottles were heated to 70°C in a water bath for 50 min. The heavily stained root tip was separated from the root on a glass slide. A drop of 45% acetic acid was added to the root tip. A cover slip was placed over the root tip and pressed gently with a filter paper. The slides were observed using a standard light microscope, and the chromosome images were captured with a digital camera (DS-L1; Nikon, Tokyo, Japan).

3.2.8. Acclimatization to greenhouse environment

Plantlets developed from the adventitious shoots and the somatic embryos were transferred to 1/2MS medium without PGRs in a glass culture bottle (Sansyo Co. Ltd., Tokyo, Japan). Plantlets with several leaves and roots were then acclimatized to the greenhouse environment. The procedure was undertaken as follows: the culture medium was rinsed off carefully from the plantlets. The plantlets were then put into pots containing a mixture of potting soil, Akadama-soil, and Kanuma-soil in the ratio of 5:3:2. The pots were grown in a container covered with a plastic film to maintain high humidity. The plastic film was removed two days later.

3.2.9. Stomatal analyses of plants regenerated from endosperm-derived callus

The acclimatized plants were used for stomatal analyses to investigate the morphologies of each ploidy level. The leaf impressions of plants were obtained according to a modified protocol of Grant and Vatnick (2004). The abaxial leaf surfaces of plants were covered with a commercial clear nail polish. When dry, the nail polish was peeled off using a commercial clear adhesive tape and then pasted to a glass slide. The slide was observed using a standard light microscope with digital camera.

The length and width of 20 guard cells in nine triploid plants and two hexaploid plants were measured, and stomatal density was measured by counting the number of stomata per 1 mm^2 in more than three microscopic fields for each plant. Differences among regenerated plants were analyzed by one-way ANOVA, taking $p < 0.05$ as significant according to Tukey's multiple range test.

3.3. Results

3.3.1. Callus induction from endosperm

All 22 endosperm segments formed friable calli four weeks after culture regardless of the light conditions of either continuous illumination or total darkness. The colors of calli from the culture under continuous illumination were green and those from the culture under total darkness were white, turning green almost ten days after transfer to continual illumination. In this study, the only effect of light condition on callus induction from endosperm was the resulting color.

3.3.2. Plant regeneration from endosperm-derived callus and histological observation

Survival rates of calli treated with colchicine were 100% in control, 24- and 48- hour treatments, and 75% in 72-hour treatment (Table 3.1). Shoots were regenerated from the calli after transfer to 1/2MS medium without PGRs, and the regeneration was continued on MS medium supplemented with 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP. Histological observation of calli with shoots, to investigate the pathway of shoot regeneration, revealed that somatic embryos and adventitious shoots were regenerated from endosperm-derived calli. Therefore, the endosperm-derived calli retained two regeneration pathways of somatic embryogenesis and adventitious shoot organogenesis. The somatic embryos and adventitious shoots that were separated from the calli grew to plantlet stage four weeks after culture on 1/2MS medium without PGRs. More than 40 plants were acclimatized to greenhouse conditions however, most plants were dead.

3.3.3. Ploidy analyses of induced calli and regenerated plantlets

Flow cytometric analysis of 84 regenerated plantlets revealed that they comprised six diploid, 67 triploid, one tetraploid, and ten hexaploid plants among them (Table 3.1 and Figure 3.1). The diploid and tetraploid plants were regenerated from the two calli not treated with colchicine. One

of the calli was triploid, and the other one comprised both a diploid and triploid part. Chromosome counts of each plant determined the ploidy level by flow cytometry and clarified the chromosome numbers as $2n = 2x = 16$ in diploid, $2n = 3x = 24$ in triploid, and $2n = 6x = 48$ in hexaploid plants (data not shown). Chromosome counts could not be conducted for the tetraploid plant. Thus, hexaploid plants were produced successfully by colchicine treatment to endosperm-derived callus.

Hexaploid plants were produced from each duration of the colchicine treatment. The frequencies of hexaploid plants were two of 20 plantlets in 24 h, seven of 14 plantlets in 48 h, and one of nine plantlets in 72 h, indicating that the 48 h treatment was effective in this study.

3.3.4. Stomatal analyses of regenerated plants

The leaf impressions of triploid and hexaploid plants are showed in Figure 3.2. The mean length of guard cells was 49.7 ± 5.4 μm in triploid plants and 53.5 ± 6.3 μm in hexaploid plants. The difference between triploid and hexaploid plants was not observed (Figure 3.3A). The mean width of guard cells was 30.6 ± 5.7 μm in triploid plants and 41.0 ± 4.3 μm in hexaploid plants, those of hexaploid plants being significantly larger than those in triploid plants (Figure 3.3B). The mean number of stomata per 1 mm^2 was slightly lower in hexaploid plants (6.3 ± 1.4) than those in triploid plants (11.9 ± 4.2) (Figure 3.3C).

3.4. Discussion

3.4.1. Plant regeneration from endosperm-derived calli

Histological analysis revealed that the endosperm-derived calli of *Haemanthus albiflos* retained two regeneration pathways: somatic embryogenesis and adventitious shoot organogenesis. Several previous studies using histological observation of endosperm culture to determine regeneration pathways, reported somatic embryogenesis (Garg et al. 1996; Gmitter et al. 1990)

and adventitious shoot organogenesis (Antoniazzi et al. 2018). The regeneration pathway may be affecting the plant regeneration rate or the number of shoots from endosperm-derived callus as the difference reported in Chapter 2. Future investigations of these relationships in the future will be necessary.

3.4.2. Endosperm culture to produce hexaploid plants from crossing diploid plants

To date, the production of hexaploid plants from the hexaploid endosperm of seed-crossed tetraploid plants have been reported in *Actinidia arguta* (Abdullah et al. 2021) and *Lonicera caerulea* var. *emphyllocalyx* (Miyashita et al. 2009). However, the production of hexaploid plants through the utilization of the triploid endosperm of seed-crossed diploid plants was uncertain. Therefore, this study is the first report that demonstrated the combination of endosperm culture and colchicine treatment to produce hexaploid plants, expanding the availability of endosperm culture to produce polyploid plants. Compared with the conventional methodology for hexaploid plant production by diploid plant and tetraploid plant interploidy hybridization and chromosome doubling, the endosperm culture combined with colchicine treatment is efficient, time-saving, and convenient.

3.4.3. Stomatal analyses of triploid and hexaploid plants regenerated from endosperm culture

Regenerated hexaploid plants had slightly larger guard cells and a smaller number of stomata compared to triploid plants. The same trend has been reported as a significant difference in previous studies which compared triploid and induced hexaploid plants (Gonzalez and Hanna 1984; James et al. 1987; Tungkajiwangkoon et al. 2016). Due to there being a lack of significant differences in the results of previous studies, stomatal analyses in this study were performed in

small plants with leaves less than 10 mm long. *Haemanthus albiflos* produces two to six leaves as large as 90 – 400 mm long (Snijman 1984), but the measurement of fully grown leaves was difficult due to the species' slow growth. Therefore, future investigations into the effect of ploidy level on stomatal morphology need to measure stomata on fully grown leaves.

3.5. Conclusion

The present study showed a new example of plant regeneration from endosperm tissue through somatic embryogenesis and adventitious shoot organogenesis in *H. albiflos*. Furthermore, hexaploid plant production was demonstrated by the combination of endosperm culture and colchicine treatment. Thus, this study provides a method for the simultaneous production of triploid and hexaploid plants from the endosperm crossed from diploid plants, which can be utilized for polyploid breeding.

Table 3.1. Survival rate of endosperm-derived calli treated with colchicine and ploidy level of regenerated plantlets in *Haemanthus albiflos*.

Colchicine treatment							Ploidy level of regenerated plantlets			
Concentration (%)	Duration (h)	Replication	No. of calli	Survival rate (%) [a]	No. of calli with shoot	No. of plantlets analyzed	Diploid (2*x*)	Triploid (3*x*)	Tetraploid (4*x*)	Hexaploid (6*x*)
0.0	0	1	10	100	4	15	2	12	1	0
		2	10	100	1	5	4	1	0	0
		3	10	100	4	21	0	21	0	0
0.2	24	1	10	100	7	17	0	16	0	1
		2	10	100	7	3	0	2	0	1
		3	10	0*	-	-	-	-	-	-
0.2	48	1	10	100	8	13	0	7	0	6
		2	10	0*	-	-	-	-	-	-
		3	10	100	1	1	0	0	0	1
0.2	72	1	10	60	1	0	-	-	-	-
		2	10	90	6	9	0	8	0	1
		3	10	0*	-	-	-	-	-	-
Total			120		39	84	6	67	1	10

a Survival rate was recorded at four weeks after culture on shoot induction medium.

Asterisks indicate the ten calli were lost by contamination.

Hyphens indicate there were no calli or no regenerated plantlets.

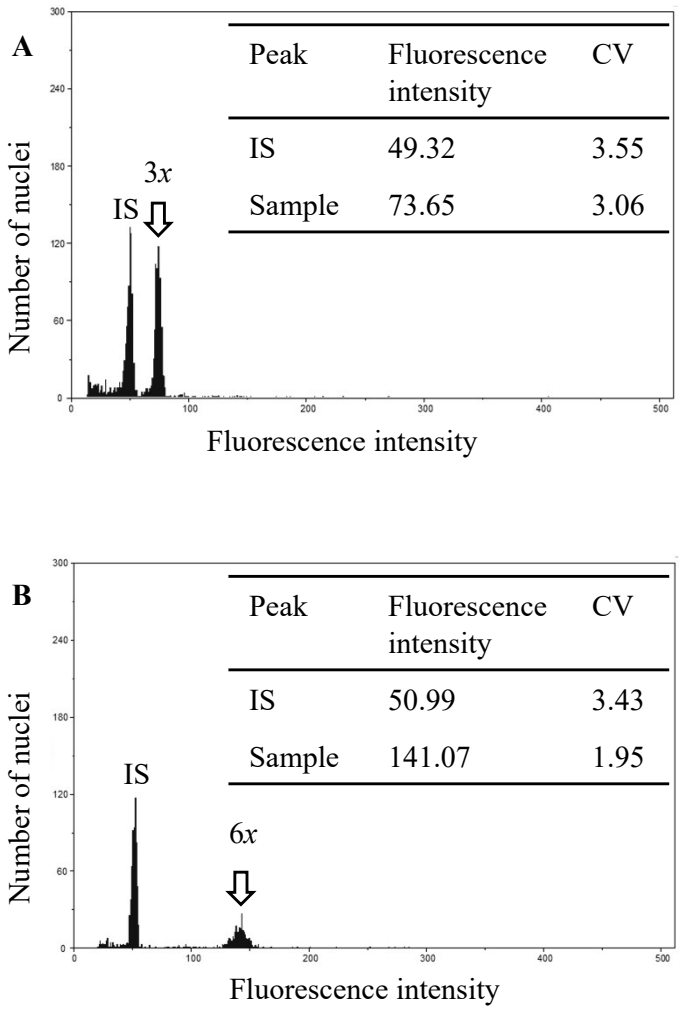

Figure 3.1. Histograms of the relative fluorescence intensity of nuclei isolated from regenerated plantlets with an internal standard by flow cytometry in *Haemanthus albiflos*.

A. A regenerated triploid (3*x*) plantlet.

B. A hexaploid (6*x*) plantlet regenerated from the callus treated with colchicine. IS means internal standard.

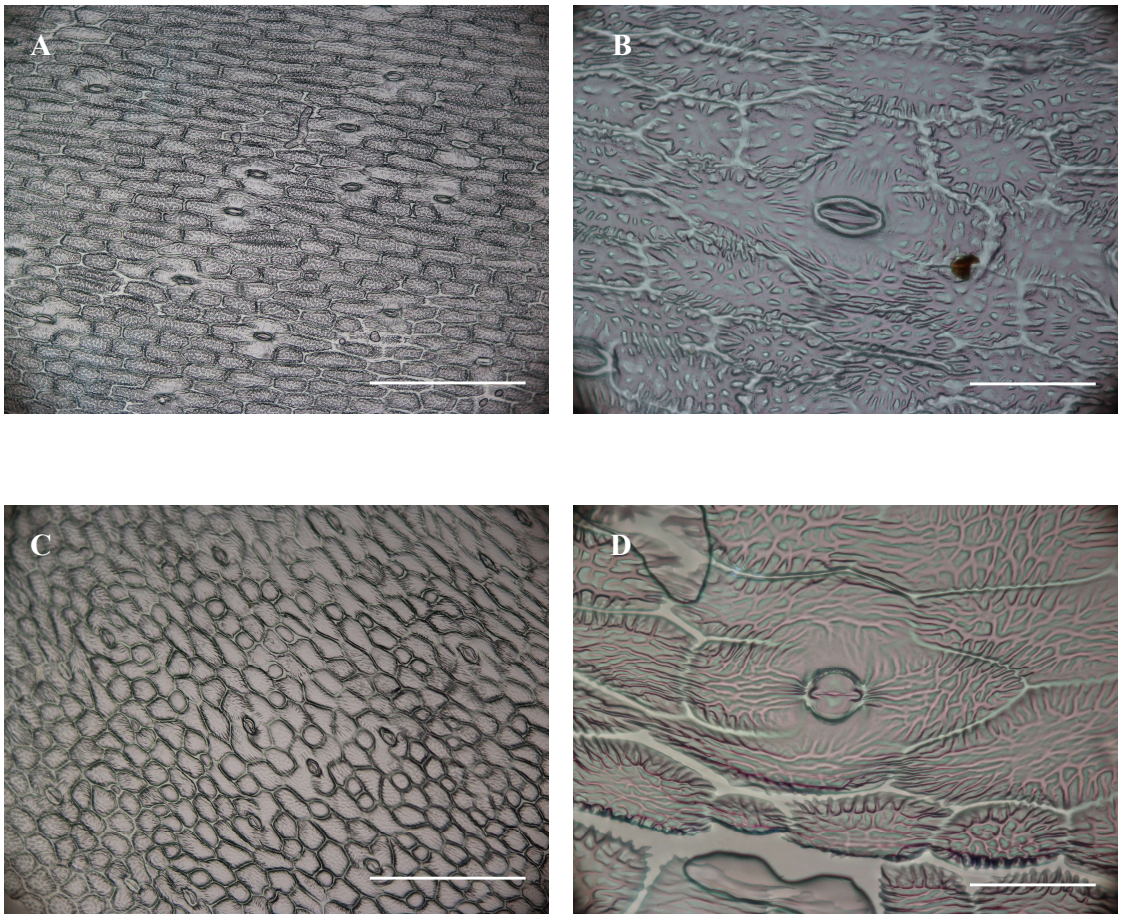

Figure 3.2. Images of leaf impressions from triploid and hexaploid plants of *Haemanthus albiflos*.

A and **B**. Images of stomata in triploid plants.

C and **D**. Images of stomata in hexaploid plants.

Scale Bar: 0.5 mm (A and C), 0.1 mm (B and D).

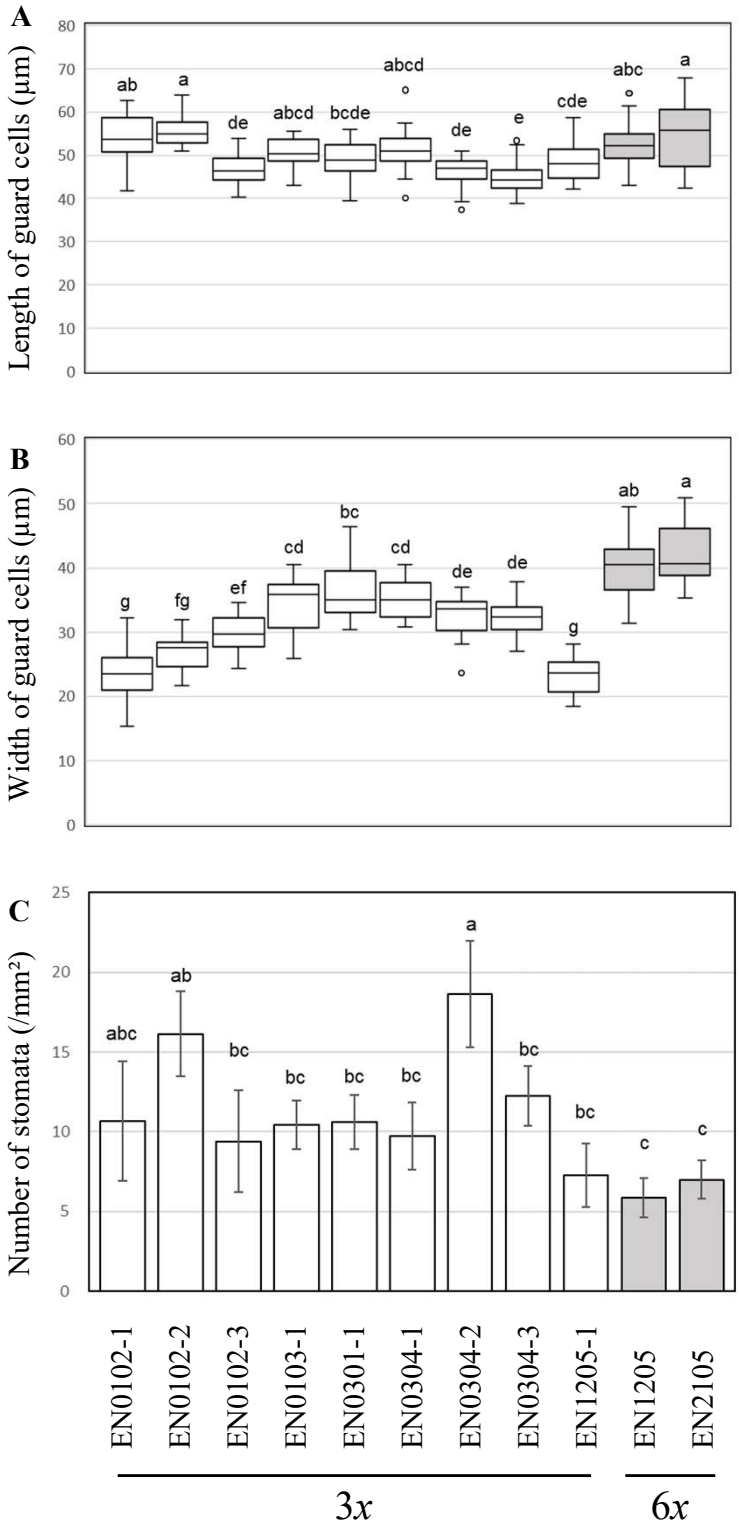

Figure 3.3. Stomatal analyses of triploid and hexaploid plants in *Haemanthus albiflos*.

A. Boxplot of length of guard cells.

B. Boxplot of width of guard cells.

C. Bar graph of stomatal density per 1 mm².

White color indicates triploid plantlets, and gray color indicates hexaploid plantlets. Different letters above boxplots and bar graph indicate significant differences between plantlets (Tukey's HSD test; $p < 0.05$).

Chapter 4: Production of tetraploid and octoploid plants from immature embryo-derived callus treated with colchicine in *Haemanthus albiflos*

This chapter is rewritten based on the manuscript that has been submitted to Plant Cell, Tissue and Organ Culture.

4.1. Introduction

4.1.1. Production of tetraploid and octoploid plants

In the ornamental plant industry, variations in the structure, color, and shelf life of plants are of commercial importance. Polyploid plants have increased productivity, both morphologically, and biochemically (Eng and Ho 2019). The production of tetraploid and octoploid plants has been attempted to accomplish morphological change and so improve plant value. The response of polyploidization varies in plants, and the increase of the size of flower or plant was reported in *Rosa* (Kermani et al. 2003), *Anemone* (Zahumenická et al. 2018), *Arabidopsis* (Robinson et al. 2018), and *Hemerocallis* (Podwyszyńska et al. 2015). Conversely, a decrease in size was reported in *Tulipa* (Podwyszyńska et al. 2018), *Petunia* (Regalado et al. 2017), and *Rhododendron* (Mo et al. 2020). Production of tetraploid plants has been attempted in Amaryllidaceae. Previous studies performed polyploidization by treating immature embryos of *Clivia miniata* with colchicine (Wang and Lei 2012), bulblets of *Nerine bowdenii* with oryzalin (van Tuyl et al. 1992), and bulblets of *Rhodophiala montana* with colchicine (Muñoz et al. 2006). As for *Haemanthus albiflos*, there is no reported protocol for producing tetraploid plants. Therefore, the present study aimed to investigate the availability of immature embryos obtained from endosperm culture to produce tetraploid *H. albiflos* plants.

4.2. Materials and methods

4.2.1. Plant materials

Ovaries derived from open pollination of diploid *Haemanthus albiflos* plants ($2n = 2x = 16$) were used in this study. Ovules excised from the ovaries were surface-sterilized and the endosperm tissue and immature embryo were separated according to the protocol described in Chapter 3. The immature embryos were used for callus induction.

4.2.2. In vitro culture of immature embryo for callus induction

Four immature embryos were cultured for callus induction on MS medium supplemented with 5.0 mg L^{-1} picloram and 5.0 mg L^{-1} BAP. The medium contained 30 g L^{-1} sucrose and 3 g L^{-1} gellan gum. The pH of the medium was adjusted to pH 5.7 ± 0.1. The conditions of continuous light and temperature during culture were used as described in Chapter 2.

4.2.3. Colchicine treatment to immature embryo-derived callus for chromosome doubling

The calli induced from all four immature embryos were used for colchicine treatment to produce polyploid plants. To ensure sufficient material for colchicine treatment, the four calli were cut into 120 segments 16 weeks after culture on callus induction medium. Colchicine treatment was performed according to the protocol described in Chapter 3. Ten callus segments were soaked in 0.2% (w/v) colchicine solution for three different durations of 24, 48, and 72 hours. The treated calli were rinsed with sterilized water and then used for shoot regeneration. Non-treated calli were used as a control, and all treatments were replicated three times. The survival rate of calli were recorded four weeks after treatment.

4.2.4. Induction of somatic embryogenesis in immature embryo-derived calli

The immature embryo-derived calli were transferred to 1/2MS medium without PGRs. The images of calli were captured using iPhone7 once every week for the next four weeks. Somatic embryos regenerated from calli were separated from the calli and transferred to flesh 1/2MS medium without PGRs in plastic petri dishes. The images of early regenerated somatic embryos were captured using a dissecting microscope fitted with a digital camera once every week for the next four weeks. Plantlets grown from the somatic embryos were transferred to 1/2MS medium without PGRs in glass culture bottles. Subsequently, plantlets with several leaves and roots were greenhouse acclimatized, as described in Chapter 3.

4.2.5. Histological analysis of regenerated shoots

Regenerated shoots, comprising cup-like tissue, cultured from immature embryo-derived callus not treated with colchicine were used for histological analysis to investigate the regeneration pathway. Histological analysis was conducted according to the modified protocol of Chapter 3.

4.2.6. Flow cytometric analysis to estimate ploidy level of plantlets regenerated from immature embryo-derived calli

Ploidy levels of plantlets regenerated from the calli both treated and non-treated with colchicine were investigated using a flow cytometer according to the protocol described in Chapter 2. The 187 plantlets randomly selected among regenerated plantlets were used as samples. Leaves of the sample and diploid mother plant were measured together, and the diploid mother plant was used as an internal standard. Ploidy level was determined by comparing the peaks of relative fluorescence intensities between the sample and internal standard. The means of frequency of polyploid plants were calculated and compared using one-sided Student's *t*-test after an *F* test.

Statistical significance was considered at $p < 0.05$.

4.2.7. Chromosome counting in plants regenerated from immature embryo-derived calli

Roots of regenerated plantlets randomly selected from among each one determined the ploidy level by flow cytometry and were used for chromosome counts. The procedures of pretreatment, fixation, and chromosome observation are described in Chapter 3.

4.2.8. Stomatal analysis of plants regenerated from immature embryo-derived calli

Stomatal analyses of regenerated plants were conducted to investigate the effects of ploidy level on morphological traits. The procedure for the production of leaf impressions, measurement of length and width of guard calls and stomatal density were described in Chapter 3. In this study, the stomata of five diploid, six tetraploid, and one octoploid plants were investigated. The length and width of 20 guard cells and number of stomata per 1 mm^2 in five microscopic fields were measured for each plant. Differences among regenerated plants were analyzed according to the procedure described in Chapter 3.

4.3. Results

4.3.1. Embryogenic callus formation in immature embryo and histological analysis

Calli were formed from all four immature embryos that were cultured. Shoot regeneration was observed four weeks after culture on 1/2MS medium without PGRs. Histological observation revealed that cup-like shoots had apical and root meristems connected by vascular bundles, indicating that the shoots were regenerated by somatic embryogenesis. The somatic embryos grew into plantlets and subsequently were acclimatized to greenhouse conditions.

4.3.2. Determination of ploidy levels of regenerated plantlets

All 120 callus segments survived four weeks after treatment regardless of the duration of treatment. Thereafter shoots were regenerated from the calli and grew to plantlet stage. Flow cytometric analysis of 187 regenerated plantlets revealed that there were 141 diploid, 38 tetraploid, seven octoploid, and one mixoploid ($4x + 8x$) plants among them (Table 4.1 and Figure 4.1). The mixoploid plant had equal number of nuclei with fluorescent intensities of tetraploid and octoploid plants. Chromosome counts clarified that the chromosome numbers were $2n = 2x = 16$ in diploid, $2n = 4x = 32$ in tetraploid, and $2n = 8x = 64$ in octoploid plants (data not shown).

Tetraploid plants were regenerated from the calli regardless of the duration of treatment with colchicine, and octoploid plants were regenerated from the calli exposed to colchicine for 48 and 72 h. The frequency of production of tetraploid plants was significantly higher for the 48-hour treatment compared to that in the control. Therefore, tetraploid, octoploid, and mixoploid plants were regenerated successfully by treating immature diploid embryo-derived embryogenic calli with colchicine in *H. albiflos*.

4.3.3. Stomatal analyses

The leaf impressions of diploid, tetraploid, and octoploid plants are showed in Figure 4.2. The mean length of guard cells was 46.7 ± 3.8 µm in diploid plants, 59.9 ± 4.2 µm in tetraploid plants, and 64.9 ± 5.8 µm in octoploid plant (Figure 4.3A). Stomata of polyploid plants were significantly longer compared to those of diploid plants. The mean width of guard cells was 27.2 ± 4.1 µm in diploid plants, 41.8 ± 5.2 µm in tetraploid plants, and 51.5 ± 4.1 µm in octoploid plant (Figure 4.3B). Stomata of polyploid plants were also significantly larger than those in diploid plants. Stomatal density was varied among each ploidy, and the mean number of stomata in octoploid plants (6.6 ± 1.4) was significantly lower than in diploid (29.9 ± 6.7) and tetraploid (18.3 ± 3.7)

plants (Figure 4.3C).

4.4. Discussion

4.4.1. Production of polyploid plants from immature embryo-derived calli treated with colchicine

It was demonstrated in this study that embryogenic calli were induced from immature embryos obtained in endosperm culture of *Haemanthus albiflos* on a medium with the same components as endosperm culture. Furthermore, the production of tetraploid and octoploid plants was able to be induced from the calli treated with colchicine. There are few previous studies of production of octoploid plants in Amaryllidaceae. Octoploid plants were regenerated from calli treated with colchicine for 48 and 72 h, suggesting that the second cell cycle occurred within 24 to 48 h in diploid calli of *H. albiflos*. Consequently, treatment with 0.2% (w/v) colchicine for 48 h could produce both tetraploid and octoploid plants.

In the present study, suppression in the appearance of mixoploid plants was observed, suggesting an efficient protocol to produce polyploid plants. Low frequency of mixoploid plants was also reported in *Citrus* cultivars (Wu and Mooney 2002) and *Vitis vinifera* (Acanda et al. 2015). A common feature of these studies is the use of somatic embryogenic calli as an explant of colchicine treatment. It has been described that somatic embryos developed from embryogenic cultures originate from single cells, therefore the chimera frequency is very low (Button et al. 1974; Ji et al. 2011). Thus, embryogenic calli can be used for highly efficient polyploid production. In member of the Amaryllidaceae, chromosome doubling has been performed with different tissues and antimitotic agents to produce polyploid plants (Muñoz et al. 2006; van Tuyl et al. 1992; Wang and Lei 2012). The present study indicates that embryogenic calli can serve as a useful tissue for polyploid production in Amaryllidaceae.

4.4.2. Stomatal analyses in diploid, tetraploid, and octoploid plants

The comparison of stomatal morphology of diploid, tetraploid, and octoploid plants indicated that polyploid plants have large guard cells and a smaller number of stomata than diploid plants. In addition to these differences, previous studies reported a morphological change of plant size in polyploid plants (Mo et al. 2020; Regalado et al. 2017). The characteristics of the induced polyploid plants need to be evaluated in further research.

4.5. Conclusion

The present study showed the immature embryo obtained from endosperm culture in Chapter 3 is suitable to produce tetraploid and octoploid plants. Thus, various polyploid plants, including those that are triploid (3*x*), tetraploid (4*x*), hexaploid (6*x*), and octoploid (8*x*) are able to be produced from the seed of crossed diploid (2*x*) plants in *Haemanthus albiflos*.

Table 4.1. Survival rate of immature embryo-derived calli treated with colchicine and ploidy level of regenerated plantlets in *Haemanthus albiflos*.

Colchicine treatment						Ploidy level of regenerated plantlets			
Concentration (%)	Duration (h)	Replication	No. of calli	Survival rate of calli (%)	No. of plantlets analyzed	Diploid (2*x*)	Tetraploid (4*x*)	Octoploid (8*x*)	Mixoploid (4*x* + 8*x*)
0	0	1	10	100	0	-	-	-	-
		2	10	100	10	10	0	0	0
		3	10	100	15	15	0	0	0
0.2	24	1	10	100	15	12	3	0	0
		2	10	100	10	10	0	0	0
		3	10	100	25	21	4	0	0
0.2	48	1	10	100	10	8	2	0	0
		2	10	100	26	16	7	2	1
		3	10	100	13	2	8	3	0
0.2	72	1	10	100	35	30	5	0	0
		2	10	100	28	17	9	2	0
		3	10	100	- *	-	-	-	-
Total			120		187	141	38	7	1

Asterisk and hyphens indicate the 10 calli were lost by contamination and no plantlet analyzed the ploidy level.

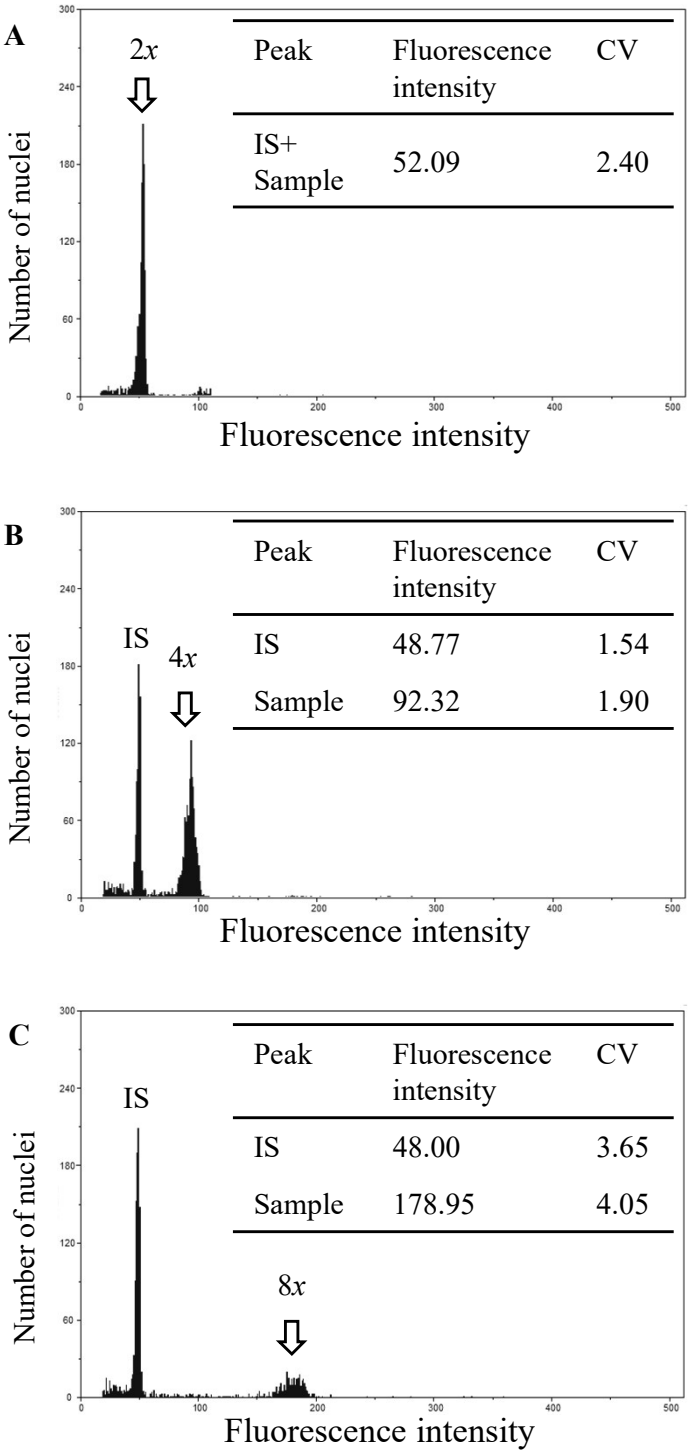

Figure 4.1. Histograms of the relative fluorescence intensity of nuclei isolated from regenerated plantlets of *Haemanthus albiflos* with an internal standard by flow cytometry.

A. Diploid (2*x*) regenerated plantlet.

B. Tetraploid (4*x*) plantlet regenerated from callus treated with colchicine.

C. Octoploid (8*x*) plantlet.

IS indicates an internal standard. White allows indicate sample peaks.

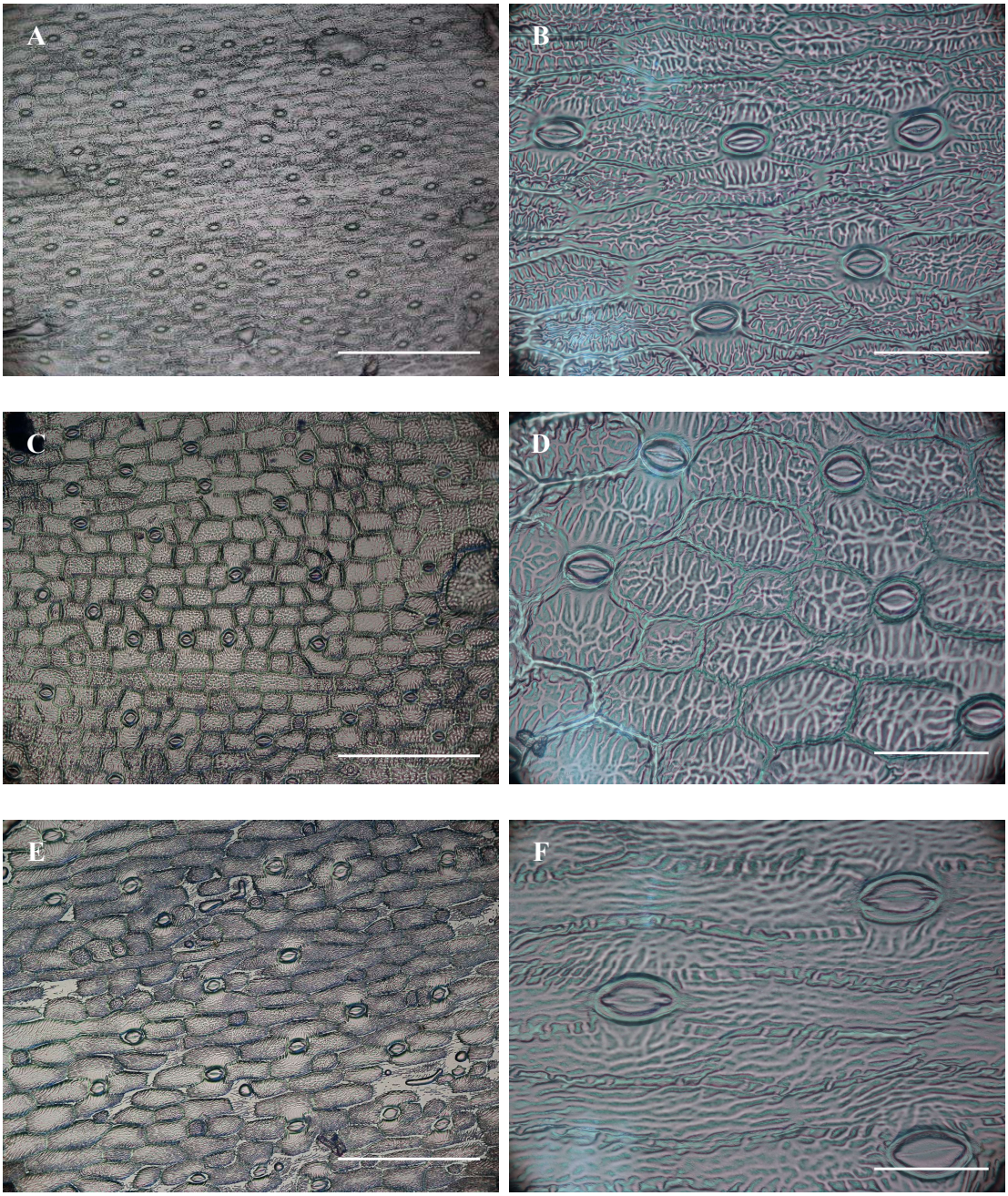

Figure 4.2. Images of stomata of plants regenerated from immature embryo-derived callus treated colchicine in *Haemanthus albiflos*.

A. and **B**. Images of stomata in diploid plants (A and B: EM3104-1).

C. and **D**. Images o stomata in tetraploid plants (C: EM3107-1, D: EM3201-3).

E and **F**. Images of stomata in octoploid plants (E and F: EM2201-1).

Scale Bar: 0.5 mm (A, C, and E), 0.1 mm (B, D, and F).

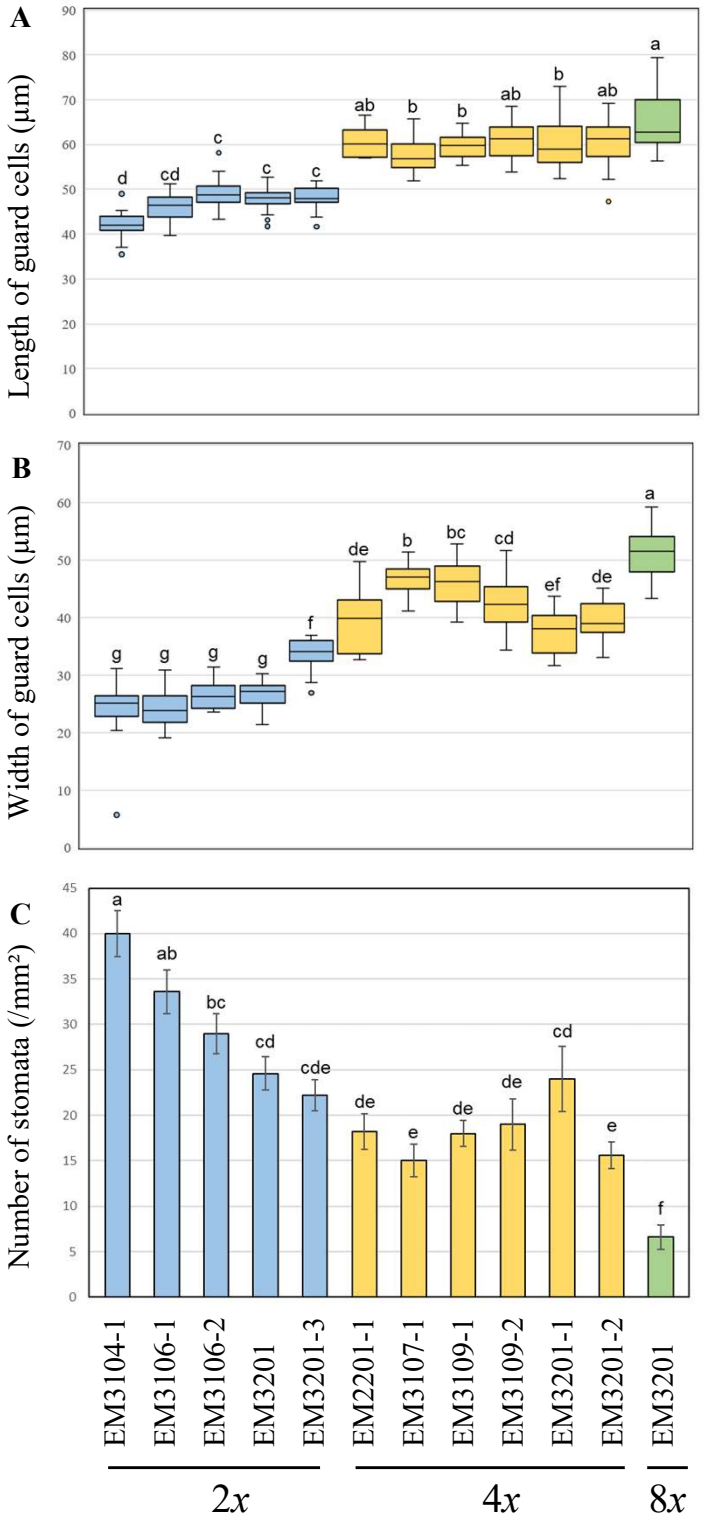

Figure 4.3. Stomatal analyses of plants regenerated from immature embryo-derived callus treated with colchicine in *Haemanthus albiflos*.

A. Boxplot of length of guard cells.

B. Boxplot of width of guard cells.

C. Bar graph of stomatal density per 1 mm².

Blue color indicates diploid plants, yellow color indicates tetraploid plants, and green color indicates octoploid plants. Different letters above boxplots and bar graph indicate significant differences between plants (Tukey's HSD test; $p < 0.05$).

Chapter 5: General discussion

5.1. Establishment of endosperm culture systems in Amaryllidaceae members

The culturing of endosperm is considered to be a technical challenge. Understanding the molecular mechanisms of plant regeneration is important because it tackles many fundamental questions in cell and developmental biology. The mechanisms of plant regeneration from tissues including leaf, hypocotyl, and root, have been investigated in *Arabidopsis thaliana* (reviewed by Ikeuchi et al. 2019). Although the endosperm is tissue derived from fertilization, as is the same as the embryo, it is different in its timing of programmed cell death. The present study provided novel examples of endosperm culture in monocots. This study should facilitate the research of endosperm culture in monocots such as Liliaceae. The accumulated data of endosperm culture contributes to the understanding of developmental biology, ideally such that future endosperm culturing will not be challenging.

5.2. Expanding the availability of endosperm culture to produce polyploid plants

In previous studies, Inoue et al. (2004) conducted protoplast culture in *Iris fulva* and reported the simultaneous regeneration of diploid, triploid, tetraploid, and hexaploid plants. The present study is a novel example to produce various polyploid plants in the Amaryllidaceae, and the most valuable result found is the demonstration of production of polyploid plants including diploid, triploid, tetraploid, hexaploid, and octoploid, from seeds crossed between diploid plants. The interploid hybridization barrier on polyploid plant production was overcome. Moreover, because *Haemanthus albiflos* has a long lifecycle, the procedure described here is a time-saving method of propagation.

The procedure could be utilized also to produce a polyploid plant series with the same genome. In angiosperms, a polygonum type embryo sac is common: a haploid egg cell, two haploid

synergid cells, three haploid antipodal cells, and a diploid central cell (Davis 1966). These cells are genetically homogenous because of the embryo sac being monosporic. Hybridized male gamete cells are also genetically homogenous because they originate from the same generative cell. Therefore, the endosperm and embryo have different parental genome balances but the same genome.

From the present study, it could not be concluded that the polyploid plants were genomic homogenous, because it has been reported there are several developmental types of embryo sac in Amaryllidaceae, such as Allium type, Polygonum type, and Endymion type (Ekici and Dane 2008). Allium and Endymion type resemble Polygonum type, however the genotypes of central cell and egg cell in these embryo sacs are different. The reason is the developing pattern termed bisporic megasporogenesis in which cytokinesis after the second meiotic division of the megasporocyte nucleus is skipped, and one of the binucleate cells developing into embryo sac contains two haploid nuclei that contribute to the female gametophyte (Simpson 2019). Therefore, confirmation of the genotype when investigating the effect of genome dosage by chromosome doubling is necessary.

Finally, the establishment and application of endosperm culture systems in Amaryllidaceae demonstrated in the present study, provide valuable information for production of polyploid plants and will contribute significantly to plant breeding programs.

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Summary

There are many species of perennial bulbous plants, including *Narcissus*, *Lycoris*, and *Crinum*, that have attractive flowers. Therefore, these plants are widely cultivated and used for ornamental purposes. However, flowering requires the full development of the bulb, and the process of development from seed to flowering takes several years or more. For this reason, they are generally propagated vegetatively.

Polyploid breeding is one of the breeding strategies. Polyploid plants with three or more sets of chromosomes exhibit more vigorous growth and larger organs than diploid plants. Since their characteristics are useful horticulturally, the production of polyploid plants has been attempted in many plant species. In general, chromosome doubling by mitotic inhibitors such as colchicine and oryzalin is used to artificially produce polyploid plants with even number sets of chromosomes such as tetraploid and octoploid. In addition, interploid hybridization with a cross between diploid and tetraploid is required to produce polyploid plants with odd number sets of chromosomes such as triploid. However, there are two problems with interploid hybridization. First, it is necessary to produce tetraploid plants in advance and to regulate the flowering of diploid and tetraploid plants simultaneously. The second is the interploid hybridization barrier, which causes seed abortion due to abnormalities in endosperm development. Because of these issues, the production of polyploid plants with odd number sets of chromosomes requires a longer period and more labor than the production of polyploid plants with even number sets of chromosomes.

As a method of polyploid plant production, endosperm culture has been attracting attention. The endosperm of diploid plants in angiosperms is generally a triploid tissue formed through the fusion of a binucleate central cell and a haploid sperm cell. Therefore, it is possible to produce triploid plants from the seeds crossed from diploid plants by endosperm culture, which leads to a

significant reduction in the breeding period. However, almost 30 species of triploid plants have been produced from nearly 70 species of plants so far, and it is considered technically difficult to produce triploid plants using endosperm culture.

The objectives of this study were 1) to establish endosperm culture systems in Amaryllidaceae and 2) to expand the availability of endosperm culture. As described above, Amaryllidaceae members have long-term life cycles, which makes it very effective to shorten the period of polyploid plant production through endosperm culture. Previous studies on endosperm culture in monocots have been limited to Poaceae, and the establishment of endosperm culture systems in Amaryllidaceae was also expected to be a useful point for application to other monocotyledonous plants such as Liliaceae. In addition, I tried to produce not only triploid but also other polyploid plants by applying the established endosperm culture systems. I hope that the present study will improve the availability of endosperm culture, promote research, and lead to the investigation of factors that make endosperm culture technically difficult in the future.

As described in Chapter 2, we first attempted the callus induction from endosperm in 27 plant species of 16 genera in the subfamily Amaryllidoiceae (the family Amaryllidaceae). The culture medium was used Murashige Skoog (MS) medium supplemented with picloram as auxin and 6-benzylaminopuline (BAP) as cytokinin. As a result of endosperm culture, calli were induced in 10 species of six genera. To investigate the origin of the calli from endosperm tissue, the relative nuclear DNA content was measured using flow cytometry with diploid plants as an internal standard to confirm whether the ploidy of callus was the same as that of endosperm tissue. Flow cytometric analysis revealed that the calli obtained from *Cyrtanthus mackenii*, *Haemanthus albiflos*, *H. pauculifolius*, and *Scadoxus multiflorus* were triploid and considered to be derived from the endosperm, indicating that endosperm culture can be used for Amaryllidaceae members. Next, I evaluated the stability of endosperm culture in *H. albiflos,* which showed the highest callus

induction rate, for the application of the endosperm culture system. The callus induction and shoot regeneration rates were examined in terms of seed, endosperm maturity, parts of endosperm, and medium composition. As a result, stable callus induction and shoot regeneration were observed, although some variation was observed depending on the origin of the seeds.

As described in Chapter 3, to expand the availability of endosperm culture, I attempted to produce hexaploid plants by the established endosperm culture system of *H. albiflos*. The production of hexaploid plants was performed with an aim to restore the fertility of triploid plants which show sterility due to unbalanced chromosome pairing. I treated triploid endosperm-derived calli with colchicine, a mitotic inhibitor, and cultured them on the shoot regeneration medium. Then, the shoot regeneration pathway was also investigated through histological observation of the calli regenerating shoots. The shoots regenerated from the treated calli grew into plantlets. Ploidy analyses of flow cytometry and chromosome counts revealed the presence of hexaploid plants among the regenerated plantlets, indicating that hexaploid plants could be produced from the endosperm crossed from diploid plants by a combination of endosperm culture and colchicine treatment. Histological observations revealed that the endosperm-derived calli retained two regeneration pathways of somatic embryogenesis and adventitious shoot organogenesis.

As described in Chapter 4, I attempted to produce tetraploid and octoploid plants from diploid immature embryos obtained in the process of the endosperm culture described in Chapter 3. The immature embryos were cultured on MS medium supplemented with picloram and BAP for callus induction from the endosperm. After colchicine treatment to the immature embryo-derived calli, shoot regeneration was induced on 1/2MS medium without plant growth regulators. The regeneration pathway from the calli was investigated through histological observation of regenerated shoots. Ploidy analyses of regenerated plantlets revealed that tetraploid and octoploid plants regenerated from the treated calli. Previous studies have reported the decrease in the

efficiency of polyploid plant production by the appearance of mixoploid. In this study, only one mixoploid was used, and the efficiency was high. It was thought to be related to the regeneration pathway by somatic embryogenesis.

The present study demonstrated that diploid, triploid, tetraploid, hexaploid, and octoploid plants could be produced from seeds crossed from diploid plants through the establishment of endosperm culture system and their application. This is expected to contribute to polyploid breeding because it can overcome the long-term production and the interploid hybridization barrier, which are major challenges in polyploid breeding. In addition, the established endosperm culture system of *H. albiflos* in this study can be employed for stable callus induction and plant regeneration. It is expected to contribute to the elucidation of plant regeneration mechanisms in endosperm tissue through its use in molecular genetic research.

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要約 (Summary in Japanese)

主に多年生の球根植物であるヒガンバナ科植物には，スイセンやリコリス，クリナムといった魅力的な花を咲かせる種が多く属している．そのため，これらの植物は観賞用として広く栽培および利用されている．しかし，開花には球根の充実が必要であり，種子から開花までに数年以上の長期間を要するため，一般的には球根の分球による栄養繁殖で増殖されている．

育種の手法として倍数性育種がある．染色体セットを三組以上もつ倍数性植物は，二倍性植物に比べ，旺盛な生育や器官の大型化といった特徴を示す．このような性質は，園芸的に有用であるため多くの植物種で倍数体化が試みられている．一般的に，四倍体や八倍体のような偶数の倍数体の人為的な作出には，コルヒチンやオリザリンのような有糸分裂阻害剤による染色体倍加が利用されている．また，三倍体のような奇数の倍数体の作出には，二倍体と四倍体との交雑のような異なる倍数体間の交雑（倍数体間交雑）が利用されている．しかし，倍数体間交雑には次の二つの課題がある．ひとつは，事前の四倍体作出および二倍体と四倍体が同時に開花するように調節する必要があることである．二つ目は，胚乳発生の異常（胚乳崩壊）に起因する胚の発生不全が生じる受精後障壁である．このような課題から，奇数の倍数体作出は偶数の倍数体作出よりも長い期間と労力が必要である．

倍数体作出の方法として，胚乳培養が注目されている．多くの被子植物において二倍性植物の胚乳は，二核性の中央細胞と半数性の精細胞との融合により形成される三倍性組織である．そのため，胚乳培養により二倍体同士の交雑種子から三倍体を作出することが可能であり，作出期間の大幅な短縮につながる．しかし，これまでに 70 種近くの植物で試みられてきたが，実際に三倍体作出に至った事例は 30 種程度に留まっており，胚乳培養による倍数体作出は技術的に困難であると考えられている．

本研究の目的は，1）ヒガンバナ科植物における胚乳培養系の開発および 2）胚乳培養の利用可能性の拡大である．ヒガンバナ科植物は上述のように長い生活環をもつため，胚乳培養による倍数体作出期間の短縮は非常に効果的と考えられた．また，単子葉植物における胚乳培養に関する先行研究はイネ科植物に限られており，ヒガンバナ科植物における胚乳培養系の開発はユリ科植物など他の単子葉植物への応用の起点となることが期待される．さらに，開発した胚乳培養系を応用し，三倍体のみならず他の倍数体作出を試みた．これにより胚乳培養の利用価値の向上および研究を促し，将来的に胚乳培養が技術的に困難とされる要因の追究へつながることを期待している．

本研究では始めに，ヒガンバナ科の主要なグループであるヒガンバナ亜科に属する 16 属 27 種の植物を対象に胚乳培養によるカルス誘導を試みた．培養にはオーキシンとしてピクロラム，サイトカイニンとしてベンジルアミノプリンを添加したムラシゲ・スクーグ（MS）培地を用いた．胚乳培養の結果，6 属 10 種においてカルスが誘導された．

カルスが胚乳組織に由来することを調査するため, 二倍性植物を内部標準とした相対的核 DNA 含量をフローサイトメーターにより測定しカルスの倍数性が胚乳組織と同一であるか確認した．その結果，*Cyrtanthus mackenii*，*Haemanthus albiflos*，*H. pauculifolius*, *Scadoxus multiflorus* において得られたカルスは胚乳に由来であると考えられ，ヒガンバナ科植物における胚乳培養の利用可能性を明らかにした．次に，最も高いカルス誘導率を示した *H. albiflos* において，胚乳培養系の応用に利用するため，胚乳培養の安定性を評価した．由来する種子，胚乳の成熟度，部位，および培地組成におけるカルス誘導率およびシュート再生率を調査した結果，由来する種子による変動がみられたものの，安定的なカルス誘導およびシュート再生が観察された．

胚乳培養の利用可能性の検証として，開発した *H. albiflos* の胚乳培養系を利用し，六倍体の作出を試みた．六倍体作出は，染色体不対合による不稔性を示す三倍体の稔性回復を目的に行われている．胚乳培養により誘導した三倍性カルスに，有糸分裂阻害剤であるコルヒチンを処理し，シュート再生培地で培養を行った．同時に，シュートを再生しつつあるカルスの組織学的観察によりシュート再生経路の調査も行った. 処理を施したカルスから再生したシュートは，小植物体へ成長した．フローサイトメーターおよび染色体数の調査による倍数性判定の結果, 再生した小植物体に六倍体の存在が確認され, 胚乳培養とコルヒチン処理の組み合わせにより, 二倍体同士の交雑に由来する種子の胚乳を材料に六倍体が作出可能であることを明らかにした．また，組織学的観察の結果，

カルスは不定胚再生および不定芽再生の二種類の経路を保持していることが明らかになった.

次に，胚乳培養の過程で得られる未熟胚の活用方法として，四倍体および八倍体の作出を試みた．上述の *H. albiflos* の胚乳培養の過程で得られた未熟胚を，胚乳培養と同様にピクロラムとベンジルアミノプリンを添加した MS 培地で培養し，二倍性カルスを誘導した．さらに，得られたカルスに対してコルヒチン処理を行ったのち，植物成長調節物質を含まない 1/2MS 培地でシュート再生を試みた．同時に，再生したシュートを用いた組織学的観察による再生経路の調査も行った. 小植物体における倍数性調査の結果, コルヒチン処理を施したカルスから四倍体および八倍体が再生していることが明らかになった．先行研究では，しばしば異なる倍数性の細胞が混在する mixoploid による倍数体作出効率の低下が報告されている．本研究では，mixoploid は 1 個体であり，高い倍数体作出効率を示した. これは, 不定胚による再生経路が関与していると考えられた.

本研究は，ヒガンバナ科植物における胚乳培養系を開発するとともに，胚乳培養の利用可能性を拡大することで二倍体間の交雑由来の種子から，二倍体，三倍体，四倍体，六倍体，および八倍体を作出可能であることを実証した．これは倍数性育種の課題である長期間の作出期間や倍数体間交雑における障壁を克服しうるものであり, 今後の倍数性育種に資することが期待される．また，本研究で開発された *H. albiflos* の胚乳培養系は安定したカルス誘導および植物体再生が可能であることから，今後，分子遺伝学的研

究への利用により胚乳組織における植物体再生機構の解明への貢献が期待される.